UNIVERSITY OF TURKU

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## ABSTRACT

Ischemic heart disease is the leading cause of death globally, even though the prognosis of the disease has improved with advancing treatments. One particular phenomenon which could be treated more effectively is ischemia-reperfusion injury. While reperfusion, the return of blood flow to the heart after infarction, is necessary for the functionality of the heart, it is a stress itself and contributes to the myocardial damage. Ischemia-reperfusion injury could be prevented pharmacologically, and novel targets for this are needed.

Heart failure is a life-threatening disorder with high death rates within years from its diagnosis. In addition to acquired causes, there is a strong heritable component to its risk. Only a few of genetic variants attributing to the risk are well characterized.

This thesis aimed to identify novel therapeutic targets for ischemic heart disease, especially ischemia-reperfusion injury, and genetic markers associated with risk of heart failure. Large-scale screenings on cell and tissue samples, as well as genome-wide screening on human genetic data from the FINRISK study cohort, were carried out. First, the family of receptor tyrosine kinases (RTK), a family of cell surface receptors involved in cellular survival and proliferation, was assessed in the context of ischemia-reperfusion injury. A member of the family, ROR1, was detected as a potential candidate for therapeutic targeting. In the second study, an unbiased screen exploiting a combination of drug and shRNA libraries identified another member of RTK family, EGFR, as a putative therapeutic target for ischemia-reperfusion. Gefitinib, an EGFR tyrosine kinase inhibitor clinically used as a cancer drug, was identified as a candidate for drug repurposing. The genetic association study on the FINRISK population identified an E140K variant of the *TRIM55* gene to be associated with heart failure. *TRIM55* E140K was also shown to negatively affect cardiomyocyte-specific functions *in vitro* and *in vivo*.

Altogether, the findings demonstrate that novel candidates for therapeutic targets and genetic markers for cardiac disorders can be effectively identified by preclinical and genetic screening methods. ROR1 and EGFR were identified as potential targets for treatment of ischemia-reperfusion injury, and *TRIM55* E140K as a genetic marker potentially modifying the risk for heart failure.

KEYWORDS: genetics, heart failure, high-throughput screening, ischemia-reperfusion injury, myocardial infarction

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## TIIVISTELMÄ

Iskeeminen sydänsairaus on maailmanlaajuisesti yleisin kuolinsyy. Vaikka taudin hoidossa on edistytty, siihen liittyy yhä merkittävää sairastavuutta ja kuolleisuutta. Iskemia-reperfuusiovaurio on tautiin liittyvä alihoidettu ilmiö. Reperfuusio eli verenkierron palautuminen sydämeen infarktin jälkeen on tarpeellista sydämen toimintakyvyn palautumiselle, mutta se on itsessään stressitekijä, joka tekee lisänsä sydänlihasvaurioon. Vauriota voidaan estää lääkehoitojen avulla, ja uusien lääkevaikutuskohteiden tunnistaminen tähän tarkoitukseen on tarpeen.

Sydämen vajaatoiminta on hengenvaarallinen sairaus, joka johtaa usein kuolemaan jo muutamassa vuodessa. Hankinnaisten syiden lisäksi perinnölliset tekijät vaikuttavat vahvasti sen riskiin. Kuitenkin vain kourallinen riskiä lisääviä geneettisiä variantteja on kuvattu tarkasti.

Tämän väitöskirjan tavoitteena oli tunnistaa uusia lääkevaikutuskohteita iskeemisen sydänsairauden, etenkin iskemia-reperfuusiovaurion, hoitoon, ja sydämen vajaatoiminnan riskiin liittyviä geneettisiä tekijöitä. Työssä hyödynnettiin seulontoja solu- ja kudosnäytteillä ja toteutettiin genominlaajuinen seulonta FINRISKI-tutkimuksen geenidatasta. Seuloimme iskemia-reperfuusiovaurioon liittyen reseptorityrosiinikinaasien (RTK) perhettä. RTK-perhe koostuu solunpinnan reseptoreista, jotka säätelevät solujen selviytymistä ja jakautumista. Perheen jäsen ROR1 tunnistettiin lupaavana vaurion hoitokohteena. Toisessa osatyössä tunnistettiin lääkeaine- ja shRNA-kirjastoja hyödyntävän seulonnan avulla toinen RTK-perheen jäsen, EGFR, mahdollisena lääkehoitokohteena. EGFR:ää estävä syöpälääke, tyrosiinikinaasi-inhibiittori gefitinibi, tunnistettiin potentiaalisena lääkkeenä, joka voidaan uudelleenkohdistaa iskemia-reperfuusiovaurion hoitoon. FINRISKI-aineiston geneettisessä assosiaatiotutkimuksessa havaittiin *TRIM55*-geenin E140K-variantin liittyvän sydämen vajaatoiminnan riskiin. Variantin osoitettiin vaikuttavan negatiivisesti sydänsolujen toimintaan *in vivo* ja *in vitro*.

Uusia lääkehoitokohteita ja geenitekijöitä voidaan tehokkaasti löytää prekliinisten ja geneettisten seulontametodien avulla. ROR1 ja EGFR tunnistettiin lupaavina iskemia-reperfuusiovaurion lääkehoitokohteina, ja *TRIM55* E140K sydämen vajaatoiminnan riskiin vaikuttavana geneettisenä tekijänä.

AVAINSANAT: genetiikka, iskemia-reperfuusiovaurio, suurten aineistojen seulonta, sydämen vajaatoiminta, sydäninfarkti

# Table of Contents

# Abbreviations

| | |
|---|---|
| ADAM | a disintegrin and metalloproteinase |
| ACE | angiotensin-converting enzyme |
| Akt | v-akt murine thymoma viral oncogene homolog |
| ANP | atrial natriuretic peptide |
| ARB | angiotensin receptor blocker |
| cGMP | cyclic guanosine monophosphate |
| DAG | diacylglycerol |
| DCM | dilated cardiomyopathy |
| ECD | extracellular domain |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| ErbB | v-erb-b avian erythroblastic leukemia viral oncogene homolog |
| ERK | extracellular signal-regulated kinase |
| FGFR | fibroblast growth factor receptor |
| GRB2 | growth factor receptor-bound protein 2 |
| GWAS | genome-wide association study |
| HB-EGF | heparin-binding EGF-like growth factor |
| HCM | hypertrophic cardiomyopathy |
| HFpEF | heart failure with preserved ejection fraction |
| HFmrEF | heart failure with mid-range ejection fraction |
| HFrEF | heart failure with reduced ejection fraction |
| ICD | intracellular domain |
| IGF | insulin-like growth factor |
| IP3 | inositol trisphosphate |
| JAK | Janus kinase |
| JM | juxtamembrane |
| MAPK | mitogen activated protein kinase |
| MPTP | mitochondrial permeability transition pore |
| MRA | mineralocorticoid receptor antagonist |
| MURF | muscle ring finger |
| NRG | neuregulin |

| | |
|---|---|
| NO | nitric oxide |
| (N)STEMI | (non-)ST-segment elevation myocardial infarction |
| PDGFR | platelet-derived growth factor receptor |
| PI3K | phosphoinositol-3 kinase |
| PIP2 | phosphatidylinositol 4,5-bisphosphate |
| PIP3 | phosphatidylinositol 3,4,5-trisphosphate |
| PKC | protein kinase C |
| PKG | protein kinase G |
| PLC | phospholipase C |
| PTB | phosphotyrosine-binding |
| RBCC | RING-B-Box-coiled-coil |
| RIP | regulated intramembrane proteolysis |
| RTK | receptor tyrosine kinase |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| TRIM | tripartite motif |
| RISK | reperfusion injury salvage kinase |
| ROR | receptor tyrosine kinase-like orphan receptor |
| SAFE | survivor activating factor enhancement |
| SGLT2 | sodium-glucose cotransporter 2 |
| SH2 | Src-homology 2 domain |
| SOS | son of sevenless |
| STAT | signal transduced and activator of transcription |
| TACE | tumor-necrosis factor-α converting enzyme |
| TKI | tyrosine kinase inhibitor |
| TM | transmembrane domain |
| TNF-α | tumor necrosis factor alpha |
| VEGFR | vascular endothelial growth factor receptor |

# List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Heliste J, Jokilammi A, Paatero I, Chakroborty D, Stark C, Savunen T, Laaksonen M, Elenius K. Receptor tyrosine kinase profiling of ischemic heart identifies ROR1 as a potential therapeutic target. *BMC Cardiovascular Disorders*, 2018; 18:196.

II Heliste J, Paatero I, Jokilammi A, Vaparanta K, Elenius K. Combined genetic and chemical screens indicate protective potential for EGFR inhibition in cardiomyocytes under hypoxia. Manuscript.

III Heliste J, Chheda H, Paatero I, Salminen TA, Akimov Y, Paavola J, Elenius K, Aittokallio T. Genetic and functional implications of an exonic *TRIM55* variant in heart failure. *Journal of Molecular and Cellular Cardiology*, 2020; 138:222-233.

# 1 Introduction

Ischemic heart disease, or coronary artery disease, and heart failure cause significant disease burden globally. Diagnostic methods, treatment and prognosis have improved stably over years but the diseases remain leading killers worldwide. Part of this is related to poorly characterized genetics and intracellular signaling events related to the diseases. Better characterization of these factors allows the design of novel treatments, as well as more efficient ways to diagnose and monitor patients with the disorders.

Cardiomyocytes are mostly regarded as cells which do not divide after their maturation. This leads to scarring in the case of myocardial infarction as the lost cardiomyocytes cannot be efficiently replaced. Therefore, during and after infarction and the resulting ischemia-reperfusion injury, the cardiomyocyte death should be maximally prevented. There is considerable, underutilized potential to limit the ischemia-reperfusion injury and to improve the outcome of the infarction. This thesis aimed to detect novel treatment targets which could be utilized in this setting.

Genetic factors substantially contribute to the risk of heart failure but the the underlying variants and their effects on cardiomyocyte signaling are poorly characterized. Better characterization of these variants improves the knowledge of intracellular events which lead to heart failure. Therefore, as a part of this thesis work, a genetic association study of heart failure was performed. A likely functional variant in *TRIM55* was characterized in detail.

# 2 Review of the Literature

## 2.1 Ischemic heart disease

### 2.1.1 Ischemic heart disease is a major cause of death

Ischemic heart disease (coronary artery disease, coronary heart disease) is caused by reduced blood flow to the heart. This is due to accumulation of atherosclerotic plaque in the coronary arteries (Libby & Theroux 2005). It is the leading cause of death and disability globally (World Health Organization 2018). Its significance has been increasing during the last decades also in less developed countries, emphasizing its global importance even more (GBD 2017 Causes of Death Collaborators 2018; World Health Organization 2018). The disease risk is modified by lifestyle factors such as smoking and diet, concomitant diseases such as diabetes and hypertension, as well as genetics. A recent genome-wide association study (GWAS) attributed 21.2% of the heritability of the disease to 304 variants (Nelson et al. 2017) in genes related e.g. to vascular development, lipid metabolism and inflammation (Nelson et al. 2017; Nikpay et al. 2015).

### 2.1.2 Acute myocardial infarction and ischemia-reperfusion injury

Acute myocardial infarction is defined as ischemia-related myocardial injury with necrosis, evidenced by significant elevation of cardiac troponin values in the plasma, together with symptoms consistent with myocardial ischemia (Ibanez et al. 2018; Thygesen et al. 2019). Most often the infarction is caused by an atherothrombotic event which means that an atherosclerotic plaque in a coronary artery ruptures and the artery gets occluded. Infarction can, however, happen without atherothrombosis, e.g. due to simple narrowing of the coronary arteries due to atherosclerosis, vasospasm, coronary dissection or imbalance of oxygen supply and demand alone (Thygesen et al. 2019). Clinically, different types of infarction are divided to subcategories of ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (NSTEMI), according to electrocardiography findings. ST-segment is a specific region in an

electrocardiogram, denoting the timeframe between the end of the so called QRS complex, which represents the depolarization of the ventricles, and the start of the T wave, which represents the repolarization of the ventricles. Changes in the level of the ST segment signal are observed during ischemia.

## Cellular events mediating ischemia-reperfusion injury

Ischemia, resulting in lack of oxygen and nutrients in the myocardium, causes myocyte damage in many ways. Oxidative phosphorylation and production of ATP ceases which stops the contractile function, raises lactate level due to switching to anaerobic glycolysis, reduces intracellular pH and eventually raises intracellular calcium levels (Hausenloy & Yellon 2013). Both cellular necrosis and the programmed cell death, i.e. apoptosis, have been shown to contribute to the ischemic myocyte loss (Hausenloy & Yellon 2004; Kajstura et al. 1996).

Reperfusion means the return of blood flow to the heart after infarction. It is an inevitable goal in order to maintain the functionality of the heart. Reperfusion is, however, a stress itself and it exacerbates the injury initiated by ischemia (Hausenloy & Yellon 2013; Piper et al. 1998; Yellon & Hausenloy 2007). The consequential total effect is called ischemia-reperfusion injury. Reversible forms of ischemia-reperfusion injury are arrhythmias (Hausenloy & Yellon 2013) and myocardial stunning, a reversible contractile dysfunction (Kloner et al. 1998). Irreversible events in ischemia-reperfusion injury are microvascular obstruction and lethal myocardial reperfusion injury which means the reperfusion-induced death of cardiomyocytes viable at the end of ischemia (Hausenloy & Yellon 2013).

Lethal myocardial reperfusion injury is due to multiple factors. Oxidative stress by the restoration of oxygen supply adds to the injury caused by ischemia alone (Hearse et al. 1973). During ischemia, intracellular calcium concentration increases abruptly. Upon the return of oxygen and subsequent re-energization of the cardiomyocytes, myofibrillar structures start contracting. Due to the concomitant cytosolic calcium overload, this leads to hypercontracture and mechanical rupture of the cellular structures (Piper et al. 1998). This damage is potentially enhanced by rapid restoration of cellular pH which activates the myofibrillar ATPase (Lemasters et al. 1996; Piper et al. 1998). Additionally, restoration of pH leads to functional activation of proteases and phospholipases which is elicited by ischemia (Lemasters et al. 1996).

One key factor in the lethal events is the mitochondrial permeability transition pore (MPTP). It is a non-selective pore protein in the inner membrane of mitochondria which remains closed during ischemia but opens upon reperfusion due to mitochondrial calcium overload, oxidative stress and ATP depletion (Griffiths & Halestrap 1995). Restoration of pH also contributes to MPTP opening (Lemasters et

al. 1996). This leads to mitochondrial membrane depolarization, mitochondrial swelling, ATP depletion and release of proapoptotic factors to the cytosol (Kim et al. 2006). Additional contributing factors to cardiomyocyte damage include inflammation (Hausenloy & Yellon 2013) and restoration of tissue osmolality which leads to cellular swelling due to the gradient between intra- and extracellular osmolality (Inserte et al. 1997; Piper et al. 1998).

A major regulator of cellular responses to hypoxia is the hypoxia-inducible factor 1α (HIF-1α) which is normally quickly degraded and thus present in low quantities in normoxic cells (Kaelin & Ratcliffe 2008). However, in hypoxia HIF-1α levels are quickly increased, and as a transcription factor, it increases the expression of genes crucial for survival in and adaptation to hypoxia, such as *EPO* (increasing red blood cell production), *VEGF* and *ANGPT1* (promoting angiogenesis and vascular remodeling) (Semenza 2014). HIF-1α has been shown to have protective effects against cardiac ischemia and pressure-overload heart failure (Semenza 2014).

### RISK and SAFE signaling pathways in ischemia-reperfusion injury

Among various intracellular signaling pathways activated upon ischemia-reperfusion injury, two central cascades have been named as the reperfusion injury salvage kinase (RISK) pathway and the survivor activating factor enhancement (SAFE) pathway. RISK pathway includes the phosphatidylinositol-3-OH kinase (PI3K)-Akt pathway and the extracellular signal-regulated kinases (ERK1/2) pathways (Hausenloy & Yellon 2004). Both of these anti-apoptotic signaling cascades are activated upon reperfusion and their activation confers cardioprotection during ischemia-reperfusion injury in preclinical models (Fujio et al. 2000; Matsui et al. 1999; Shimizu et al. 1998; Yue et al. 2000). Activation of the RISK pathway leads to prevention of MPTP opening, inhibition of proapoptotic proteins including BAD, BAX and BIM and activation of signaling proteins such as protein kinase C (PKC), endothelial nitric oxide synthase (eNOS) and several transcription factors (Hausenloy & Yellon 2004).

The SAFE pathway involves the Janus Kinases (JAK) and their downstream signal mediator signal transducer and activator of transcription 3 (STAT3) which are activated by e.g. tumor necrosis factor alpha (TNFα) through TNF receptor 2 (Lecour 2009). Cardioprotection in ischemia-reperfusion by the SAFE pathway is independent of the RISK pathway (Lecour et al. 2005).

### 2.1.3 Current treatment of ischemic heart disease and myocardial infarction

As ischemic heart disease is mostly caused by lifestyle factors, treatment of chronic or stable ischemic heart disease includes lifestyle adjustments, such as cessation of smoking, appropriate diet and exercise. Additionally, symptoms, the course of the disease, and prognosis can be adjusted by pharmacological therapies, including beta-blockers, calcium channel blockers, short- and long-acting nitrates, antiplatelet and antithrombotic medications (e.g. acetylsalicylic acid and clopidogrel), statins and other lipid lowering drugs, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB), mineralocorticoid receptor antagonists (MRA), neprilysin antagonists and diuretics (Knuuti et al. 2020).

Treatment of acute myocardial infarction aims for timely reperfusion of the heart, either through percutaneous coronary intervention (PCI) or pharmacological fibrinolysis (Ibanez et al. 2018). Additionally, pharmacological therapies used during and directly after the ischemia and reperfusion include antiplatelet therapies (e.g. acetylsalicylic acid, clopidogrel, prasugrel, ticagrelor, abciximab, eptifibatide and tirofiban), anticoagulant therapies, ACE inhibitors or ARBs, nitrates and beta-blockers (Ibanez et al. 2018). Pharmacological therapies are thus mainly the same ones as used for the treatment of the stable disease. The target of the therapy is the reperfusion, inhibiting coronary restenosis by platelet inhibition and reducing the workload of the heart. No therapies which are directly cardioprotective or, even better, regenerative at the level of cardiomyocytes are currently in use. Noteworthy however, antiplatelet therapeutics clopidogrel and cangrelor have shown direct cardioprotective efficacy in a rabbit infarction model (Yang et al. 2013).

### 2.1.4 Treatments under development for ischemia-reperfusion injury

Multiple promising strategies for preventing ischemia-reperfusion injury have been assessed preclinically and clinically. These therapies target cardiomyocytes or other involved cell types such as endothelial and other vascular cells, platelets, fibroblasts or inflammatory cells. In addition to cardioprotection by inhibiting cell death during ischemia-reperfusion injury, regeneration of cardiomyocytes e.g. through forcing the cells to re-enter cell cycle and divide is another highly studied area (Cahill et al. 2017). Here, the focus is on the strategies aimed at the salvage of cardiomyocytes per se and which have proceeded to clinical trials.

A mechanical way to prevent ischemia-reperfusion injury is called ischemic conditioning in which short periods of induced ischemia and reperfusion are applied. This can be done with an angioplasty balloon within the coronary artery before ischemia (in elective settings) or directly after the reperfusion (Davidson et al. 2019).

The procedure is called ischemic pre- or post-conditioning, respectively (Davidson et al. 2019; Heusch 2015). Another way is to cause ischemia-reperfusion remotely to another organ before, during or after the reperfusion, e.g. with a blood pressure cuff on a limb (Hausenloy et al. 2019). This is called remote ischemic conditioning. The phenomenon of ischemic preconditioning was first described by Murry et al. who demonstrated reduction of infarct size in dogs by four cycles of five-minute occlusion of circumflex coronary artery prior to a 40-minute occlusion (Murry et al. 1986). Cardioprotective mechanisms of ischemic conditioning are not totally clear but they can be mediated by humoral or neuronal mechanisms or locally (Heusch 2015). Intracellular signaling mechanisms activated by ischemic conditioning include, among many others, the RISK pathway (Hausenloy et al. 2005; Tong et al. 2000), the SAFE pathway (Lecour et al. 2005), and nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) pathway (Penna et al. 2007), as shown in isolated perfused rat hearts. Ischemic conditioning has proceeded to phase III clinical trials. After successful phase II trials, a large randomized clinical trial (DANAMI-3–iPOST) addressing the efficacy of ischemic post-conditioning failed to reduce mortality and hospitalization due to heart failure (Engstrøm et al. 2017). Similarly, in a large clinical trial called CONDI-2/ERIC-PPCI remote ischemic conditioning did not produce significant differences in cardiac death or hospitalization for heart failure (Hausenloy et al. 2019).

Among the multitude of pharmacological strategies for reduction of ischemia-reperfusion injury, a few have proceeded to clinical trials. Insulin has been shown to reduce infarct size in a perfused rat heart model (Jonassen et al. 2001). The IMMEDIATE trial assessed the efficacy of glucose-insulin-potassium infusion given to infarction patients early in the ischemia but no significant differences in the clinical outcomes were observed in the whole cohort (Selker et al. 2014). In the subset of patients with STEMI, however, a significant decrease in mortality, cardiac arrest and hospitalization for heart failure after one year of follow-up (Selker et al. 2014). Exenatide, a glucagon-like peptide-1 analog used for treatment of type 2 diabetes, has also been reported to decrease infarct size in clinical trials including STEMI patients (Lønborg et al. 2012; Woo et al. 2013), while no differences in cardiac death, infarction, stent thrombosis or stroke were observed at 30-day follow-up (Lønborg et al. 2012). Atrial natriuretic peptide (ANP) has also been shown to reduce infarct size and improve left ventricular ejection fraction at 6–12 months when given to patients with acute myocardial infarction (Kitakaze et al. 2007).

Nitric oxide has been studied in two trials with intravenous or intracoronary administration. In a trial with intracoronary administration of sodium nitrite, reduction in infarct size was not observed while significant reduction in major adverse cardiac events at one year in the nitrite group was reported, though with few cases only (Jones et al. 2015). In a trial with intravenous administration of sodium

nitrite no significant differences in infarct size were detected (Siddiqi et al. 2014). In the NOMI trial where inhaled NO was used, reduction of infarcted area was demonstrated in inhaled NO-treated patients who had not had intra-arterial or intracoronary nitroglycerine (i.e. nitroglycerine-naïve patients) but no significant differences in clinical outcomes (death, recurrent ischemia, stroke, or rehospitalizations) were observed (Janssens et al. 2018).

Cyclosporin A, an inhibitor of MPTP opening (Griffiths & Halestrap 1995), has also been considered a promising candidate for ischemic cardioprotection. However, in two large clinical trials, no significant differences in infarct size or in clinical outcome were observed (Cung et al. 2015; Ottani et al. 2016). Moreover, several clinical trials with intracoronary or intravenous adenosine have been performed, and a meta-analysis indicated that intracoronary adenosine protected from heart failure while the individual trials did not demonstrate significant efficacy (Bulluck et al. 2016). Larger trials with intracoronary adenosine are thus warranted. Finally, metoprolol, a beta-blocker, has been considered to have direct cardioprotective efficacy (Hausenloy et al. 2017a). In the METOCARD-CNIC trial, intravenous metoprolol prior to PCI reduced both infarct size and hospitalization for heart failure (Ibanez et al. 2013; Pizarro et al. 2014). On the other hand, in the EARLY BAMI trial no differences in infarct size were detected although there was a reduction in malignant arrhythmias (Roolvink et al. 2016). These clinical trials and their outcomes are summarized in Table 1.

Suggested reasons for the failure of translation of ischemia-reperfusion therapies to clinic include differences between preclinical models and human, duration and setting of ischemia, comorbidities and concurrent medical therapies, and the capability of the therapeutics to reach the ischemic myocardium, affected by the lack of circulation in the heart during ischemia (Davidson et al. 2019). One noteworthy reason is also age. Animal models used in the preclinical trials are mostly healthy and young, and the ischemia is artificially produced by coronary artery ligation (Hausenloy et al. 2017a). Mostly preclinical models lack the effects of slow deterioration of the heart and vessels and prolonged ischemic stress typical for ischemic heart disease in human. There are, however, animal models which mimic the effects of e.g. hypertension, hyperlipidemia and atherosclerosis on the heart (Ferdinandy et al. 2014). Additionally, comedication often seen in patients with ischemic heart disease may affect cardioprotection and these effects of certain drugs, such as antidiabetics and statins, have been studied in animal models (Ferdinandy et al. 2014). In designing the future clinical trials, patient selection, drug dosing and timing should be carefully considered (Hausenloy et al. 2017a). As many of the cardioprotective strategies act via inhibiting redundant mechanisms of cell death, approaches with single modalities may not be sufficient, and synergistic strategies targeting multiple pathways may be necessary (Davidson et al. 2019; Hausenloy et

al. 2017a). Another important aspect in cardioprotection is the timing of the intervention, i.e. before or after the reperfusion (Davidson et al. 2019). A novel way to offer cardioprotection would be to administer a pharmacological agent before the ischemia, i.e. as prophylaxis before the onset of an acute ischemic event. This could be used as secondary prevention in patients with known ischemic heart disease and possibly a prior infarction.

**Table 1.** Summary of clinical trials of ischemia-reperfusion therapies. Result abbreviations: 0, neutral outcome; +, significant result for a subset of patients or for a surrogate marker (e.g. reduction in infarct size); ++, significant result for a clinical outcome (e.g. cardiac death or hospitalization for heart failure). Phase information was retrieved from ClinicalTrials.gov or from the study article, when available. N/A, not available. References for each study are stated in the preceding paragraph.

| THERAPEUTIC APPROACH | TRIAL NAME(S) | TRIAL PHASE | RESULT |
|---|---|---|---|
| Ischemic post-conditioning | DANAMI-3-iPOST | III | 0 |
| Remote ischemic conditioning | CONDI-2/ERIC-PPCI | N/A | 0 |
| Glucose-insulin-potassium | IMMEDIATE | III | + |
| Exenatide | N/A, EMPIRE | I, IV | + |
| ANP | J-WIND-ANP, J-WIND-KATP | N/A | + |
| Intracoronary sodium nitrite | NITRITE-AMI | II | ++ |
| Intravenous sodium nitrite | NIAMI | II | 0 |
| Inhaled nitric oxide | NOMI | II | + |
| Cyclosporin A | CYCLE, CIRCUS | II, III | 0 |
| Adenosine | meta-analysis | N/A | ++ |
| Metoprolol | METOCARD-CNIC, EARLY BAMI | IV, N/A | ++, 0 |

## 2.2 Heart failure

### 2.2.1 Pathophysiology of heart failure

Heart failure is a clinical syndrome defined by characteristic signs and symptoms, such as fatigue, shortness of breath, peripheral edema and pulmonary crackles, which result in reduced cardiac output and/or elevated intracardial pressures (Ponikowski et al. 2016). One important diagnostic factor is ejection fraction (EF) which means the amount of blood pumped from the left ventricle, measured in percentage. In heart failure with reduced ejection fraction (HFrEF), EF is < 40% and in heart failure with

preserved ejection fraction (HFpEF), EF is ≥ 50% (Ponikowski et al. 2016; Yoon & Eom 2019). In HFpEF, EF is thus of normal range (defined as ≥ 50%), but the patients exhibit typical heart failure symptoms. Left ventricular wall thickness is often increased and there are potentially problems with diastolic filling of the ventricle, but the term diastolic dysfunction should not be used interchangeably with HFpEF (Ponikowski et al. 2016). Additionally, heart failure patients with EF between 40 and 49% are currently defined as having heart failure with mid-range EF (HFmrEF) (Ponikowski et al. 2016). Classification of heart failure types according to EF is important therapy-wise: traditional pharmacological treatments are effective for HFrEF while for HFpEF there are currently no well-defined treatment strategies (Yoon & Eom 2019).

There are multiple causes underlying heart failure and the problem can affect the myocardium itself, endo- or pericardium, cardiac valves, rhythm and conduction (Ponikowski et al. 2016). The major risk factors for heart failure are ischemic heart disease, hypertension, diabetes and obesity (Dunlay et al. 2009). In addition to acquired causes, there is fairly strong inherited predisposition to heart failure (Lee et al. 2006; Lindgren et al. 2018) as discussed below.

### 2.2.2 Genetics of heart failure

Estimates of heritability of sporadic heart failure range from 18 to 26% (Lee et al. 2006; Lindgren et al. 2018). A distinct entity among heart failure subtypes are the familial cardiomyopathies which eventually cause heart failure, such as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), arrhythmogenic cardiomyopathy (AC) and restrictive cardiomyopathy (RCM) (Cahill et al. 2013). These syndromes are often caused by a monogenic, dominant mutation and are often early onset. HCM is the most common inherited heart disease and currently approximately 60% of the cases are explained by characterized mutations in sarcomeric genes, most frequently in *MYH7* (myosin heavy chain 7, cardiac muscle, beta) and *MYBPC3* (myosin-binding protein C, cardiac) (Cahill et al. 2013). DCM can be caused by acquired causes such as ischemia, infection and toxins, as well as due to a genetic cause. The genetic background of DCM is diverse, and in the familial forms of DCM causative mutations are identified in 30-35% of the cases (Cahill et al. 2013), while estimates of up to 65% of inherited background of DCM have been published (Petretta et al. 2011). The most frequently mutated genes in DCM include *TTN* (titin), *LMNA* (lamin A/C), *MYH7* and *TNNT2* (cardiac troponin T) (Cahill et al. 2013).

Sporadic heart failure is a polygenic disorder, and there are numerous potential minor genetic variants contributing to its pathogenesis (Guo et al. 2016; Li & Zhang 2017). Among noteworthy examples are the members of the renin-angiotensin-

aldosterone pathway, such as *ACE*, *AT1R* (angiotensin II type 1 receptor), and *AGT* (angiotensinogen) (Guo et al. 2016). Association of an insertion-deletion (I/D) polymorphism in *ACE* to heart failure incidence has been studied widely with contradictory results, and a meta-analysis found no significant association (Bai et al. 2012). *ACE* D allele has been, however, shown to be associated with improved outcome when patients are treated with high-dose ACE inhibitor and beta-blockers, although with low-dose ACE inhibitor treatment there were more adverse events in carriers of the D allele (McNamara et al. 2004). A recent meta-analysis has also found an association between the D allele and development of left ventricular hypertrophy (Fajar et al. 2019).

Another set of genes closely related to the disease pathogenesis are the genes encoding adrenergic receptors such as *ADRB1* (adrenoceptor beta 1), *ADRB2* (adrenoceptor beta 2), and *ADRA2C* (adrenoceptor alpha 2C) (Guo et al. 2016). These genes have several variants which have been studied according to heart failure prognosis, susceptibility, and treatment outcomes. *ADRB1* Arg389Gly variant has been shown to be associated with reduced treatment efficacy of beta-blockers (Liu et al. 2012; Parikh et al. 2018). Reports about the *ADRB2* Arg16Gly variant have shown inconsistent associations with heart failure (Guo et al. 2016), while a recent study demonstrated worse prognosis and better response to beta-blockers in Gly16 allele carriers (Huang et al. 2018). An interesting modulator of therapeutic response to the beta-blocker metoprolol is the hepatic enzyme CYP2D6 accounting for metabolism of metoprolol. Genetic variants cause decreased or increased enzymatic activity of CYP2D6. In patients with decreased enzymatic activity of CYP2D6, i.e. poor and intermediate metabolizers, plasma concentrations and efficacy of metoprolol are increased (Rau et al. 2009). A pharmacogenetic dosing guideline suggests dose reductions in poor and intermediate CYP2D6 metabolizers and, on the other hand, dose increase in ultrarapid CYP2D6 metabolizers (Swen et al. 2011).

Only a few large-scale all-cause heart failure GWAS have been published and they have identified a handful of variants significantly associated with heart failure (Li & Zhang 2017). Smith et al. identified two heart failure incidence-associated loci with a *P*-value $< 5 \times 10^{-7}$ (Smith et al. 2010). The closest genes to these identified loci are *USP3* (ubiquitin specific peptidase 3) and *LRIG3* (leucine rich repeats and immunoglobulin like domains 3) (Smith et al. 2010). Another study from the same cohorts identified one intronic variant in *CMTM7* (CKLF-like MARVEL transmembrane domain containing 7) to be associated with heart failure mortality (Morrison et al. 2010). Cappola et al. identified two heart failure-associated intronic variants with a *P*-value $< 6 \times 10^{-5}$ (Cappola et al. 2010), the other one of which at *HSPB7* was later shown to be linked to a likely functional variant in *CLCNKA* (ClC-$K_a$, (renal) chloride voltage-gated channel $K_a$) (Cappola et al. 2011).

A more recent GWAS identified several novel loci associated with heart failure, from which a few mediated their effect through risk factor associations, such as ischemic heart disease, atrial fibrillation, or obesity (Shah et al. 2020). Altogether, the number of identified individual variants is low compared to other cardiovascular disorders, and most of the heritability of heart failure may be more attributable to subphenotypes of the disease than to a common final pathway (Shah et al. 2020). As biobanks currently allow for even larger GWAS cohorts, and also a possibility to define detailed subclasses of disease phenotypes, the heart failure GWAS are finally gaining the needed power and accuracy in the mixed landscape of multiple etiologies of the disease. Aragam et al. performed GWAS both on all-cause heart failure and on a defined subclass of cases: individuals with left ventricular dysfunction without ischemic heart disease (non-ischemic cardiomyopathy, NICM) (Aragam et al. 2019). They identified a strong association with NICM for a variant at *BAG3* (BCL2-associated athanogene 3), earlier associated with DCM (Esslinger et al. 2017). The same variant was later found to be associated with sporadic heart failure (Shah et al. 2020). Additionally, association with NICM was also detected for a variant at *CLCNKA* (Aragam et al. 2019).

A few loci have thus been eventually confirmed by multiple GWAS analyses and the landscape of central genes and signaling pathways is starting to shape up. Many of these loci still need characterization of the functional mechanisms underlying the pathogenesis as well as of prognostic and therapeutic significance.

### 2.2.3 Current treatment of heart failure

Current pharmacological therapies for chronic HFrEF include ACE inhibitors or ARBs, beta-blockers, MRAs, neprilysin inhibitors, ivabradine, diuretics, and digoxin (Ponikowski et al. 2016). Treatments aim at reducing the congestion and workload of the heart, thus relieving symptoms. Additionally, some treatments, such as ACE inhibitors, beta-blockers, MRAs and a neprilysin inhibitor combined with an ARB (sacubitril and valsartan) reduce mortality and morbidity (Ponikowski et al. 2016). For HFmrEF and HFpEF, there are currently no treatment strategies with sufficient evidence for improved survival and merely diuretics and treatment of comorbidities are recommended (Ponikowski et al. 2016). Additionally, none of the treatments so far have been aimed at regeneration of the heart.

### 2.2.4 Treatments under development for heart failure

Clinical trials assessing SGLT2 (sodium-glucose cotransporter 2) inhibitors, originally used for type 2 diabetes treatment, in the treatment of heart failure have been performed. In the DAPA-HF trial on HFrEF patients, dapagliflozin reduced

heart failure worsening and cardiovascular deaths in diabetic as well as non-diabetic patients (McMurray et al. 2019). Interestingly, another SGLT2 inhibitor empagliflozin has been shown to reduce left ventricular wall mass and improve diastolic function in a rat model of HFpEF (Connelly et al. 2019), and clinical trials are underway to specifically address the efficacy of SGLT2 inhibitors in HFpEF patients. Another therapeutic approach for HFpEF which has proceeded to clinical trials is the combination of neprilysin inhibitor sacubitril with valsartan (an ARB). The latest phase III trial with this combination against valsartan alone in HFpEF patients was neutral in cardiovascular deaths and heart failure hospitalizations overall, although in female patients the reduction was significant with the combination of sacubitril and valsartan (Solomon et al. 2019). NO-cGMP-PKG signaling is considered a promising therapeutic target for HFpEF, which is also the pathway the neprilysin inhibitors act upon, and novel therapies with e.g. nitrites and phosphodiesterase-5a inhibitors targeting the pathway are being assessed (Yoon & Eom 2019; Zakeri & Cowie 2018).

Regeneration of cardiomyocytes to prevent or treat heart failure is an intriguing option, although difficult as cardiomyocytes mostly stop dividing and exit cell cycle after birth. Several approaches to regenerate heart muscle have been proposed and tested in preclinical models, e.g. with transplantation of induced pluripotent stem cell (iPSC)-derived cardiomyocytes or embryonic stem cells and activation of cardiomyocyte proliferation with exogenous ligands such as neuregulin-1 (NRG-1), fibroblast growth factor 1 (FGF-1), or insulin-like growth factor 1 (IGF-1) (Cahill et al. 2017). NRG-1 has been tested in clinical trials on stable heart failure patients and a relatively recent phase I trial using cimaglermin, full-length recombinant NRG-1β3, improved ejection fraction over three-month follow-up (Lenihan et al. 2016).

## 2.3 Receptor tyrosine kinases

### 2.3.1 Structure and activation of receptor tyrosine kinases

Protein tyrosine kinases are enzymes which phosphorylate other proteins on tyrosine residues as a means of signal transduction and regulation within and between cells. Among over 90 tyrosine kinases, 55 receptor tyrosine kinases (RTK) form a subfamily of cell surface-located, transmembrane proteins which bind extracellular ligands and mediate their signal inside of the cell (Blume-Jensen & Hunter 2001; Wheeler & Yarden 2015). RTKs regulate central cellular processes such as proliferation, migration, survival and metabolism, and are involved in disease pathogenesis, especially in cancer (Lemmon & Schlessinger 2010) as well as in cardiac diseases and development of the heart (Cahill et al. 2017).

The structure of RTKs is roughly similar among family members: extracellular domain contains the ligand binding site; transmembrane domain consists of a single hydrophobic α helix; and intracellular domain (ICD) contains the tyrosine kinase domain with its regulatory element, the activation loop (Lemmon & Schlessinger 2010). When not phosphorylated, the activation loop inhibits the kinase domain (Hubbard & Till 2000). A common event in the initiation of RTK signal transduction upon ligand binding is receptor dimerization followed by phosphorylation of the activation loop in *trans* by the dimer partner or in *cis* by autophosphorylation (Hubbard & Till 2000). This leads to subsequent phosphorylation of tyrosine residues in the c-terminal tail of the RTK and activation of intracellular signaling pathways.

### 2.3.2 Signaling pathways activated by RTKs

Activation of intracellular signaling pathways involves the binding of signaling and adaptor proteins to the phosphorylated docking sites at the intracellular domain of an RTK via the Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains of the downstream signaling proteins (Blume-Jensen & Hunter 2001). Among the many pathways activated by RTKs, central examples include the mitogen-activated protein kinase (MAPK) pathway, the PI3K-Akt pathway, the phospholipase C gamma (PLC-γ) pathway, and the JAK-STAT pathway. The pathways are illustrated in Figure 1.

RTKs activate the MAPK pathway by recruiting the growth factor receptor-bound protein 2 (GRB2) – son of sevenless (SOS) complex, which in turn activates RAS (Lemmon & Schlessinger 2010). Subequently, RAS activates the Raf-MEK-ERK pathway. Phosphorylated ERK translocates to the nucleus and regulates through transcription e.g. cell cycle progression, cell death or proliferation (Rose et al. 2010). In the heart, ERK activity can promote hypertrophy or cardioprotection during ischemia (Rose et al. 2010). Another important kinase among the MAPKs is the stress-activated p38 which is activated by a large variety of extracellular stress stimuli leading to activation of downstream kinases such as MEKK1-4 or TAK1 and subsequently MKK3, -6 and -4 (Rose et al. 2010). Different subunits of p38 are activated and necessary in ischemic conditioning (Heusch 2015) but, on the other hand, the inhibition of p38, most likely the α subunit, has been shown to be cardioprotective in preclinical models (Rose et al. 2010). Additionally, p38α inhibition has been shown to enable proliferation of adult rat cardiomyocytes (Engel et al. 2005).

RTKs activate the PI3K pathway by binding of PI3K through its SH2 domains which leads to conversion of phosphatidylinositol (4,5)-bisphosphate (or phosphoinositol-2-phosphate, PIP2) into phosphatidylinositol (3,4,5)-trisphosphate

(phosphoinositol-3-phosphate, PIP3) (Blume-Jensen & Hunter 2001). Additionally, Ras kinase can activate PI3K via directly binding to it (Blume-Jensen & Hunter 2001). PIP3 recruits Akt and its activator kinase PDK1 to the cell membrane which leads to Akt phosphorylation and activation (Blume-Jensen & Hunter 2001). Akt itself has multiple targets within the cell and its activity leads to e.g. proliferation and inhibition of apoptosis (Manning & Cantley 2007). Together, the activated PI3K-Akt and MAPK/ERK pathways in the heart are called the RISK pathway which is crucial for cardioprotection in ischemia-reperfusion injury.

Another RTK-activated pathway using phosphoinositol as a signal mediator is the PLC-γ pathway. RTKs phosphorylate PLC-γ, after which the activated PLC-γ hydrolyzes PIP2 to inositol (1,4,5)-trisphosphate (inositol trisphosphate, IP3) and diacylglycerol (DAG) (Lemmon & Schlessinger 2010; Rhee 2001). DAG activates PKC and IP3, in turn, increases intracellular calcium levels (Rhee 2001). Subsequent signaling activities regulate e.g. cellular migration and survival (Suh et al. 2008). Activity of PKC is involved in ischemic preconditioning through e.g. translocation to sarcoplasmic reticulum and subsequent reduction of calcium content (Heusch 2015).

RTKs can activate STAT signaling directly or through phosphorylation of JAKs (Blume-Jensen & Hunter 2001). STATs are encoded by seven genes in human (Yu et al. 2009). They are activated through phosphorylation which leads to their dimerization and nuclear translocation, and in the nucleus they act as transcription factors, regulating the expression of genes related to e.g. survival and proliferation (Yu et al. 2009). Especially STAT3 has been shown to have cardioprotective effects in multiple pathological conditions, such as ischemia-reperfusion, hypertrophy and heart failure in murine models *in vivo* (Harhous et al. 2019). STAT3 is central member of the cardioprotective SAFE pathway.

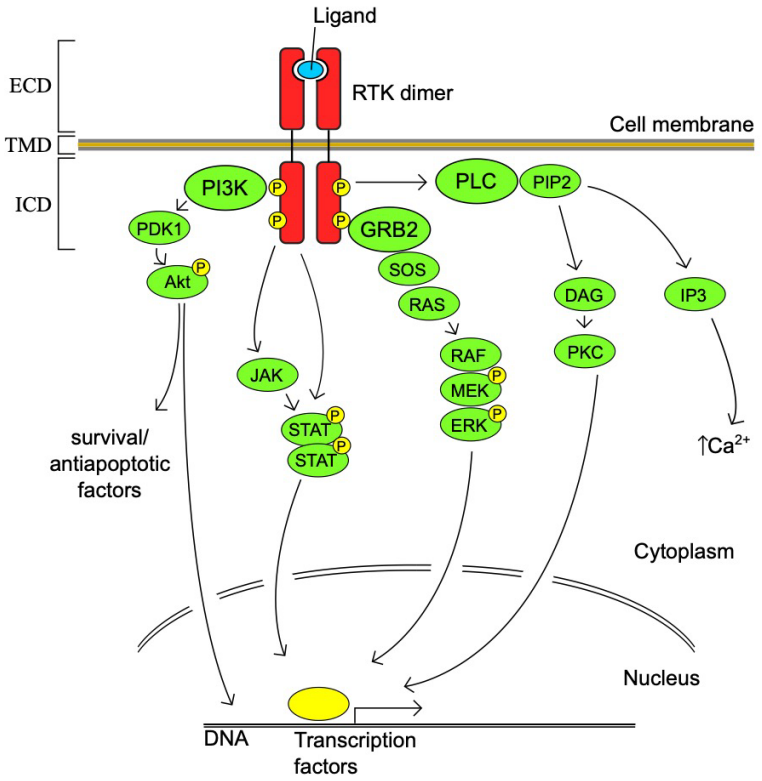


**Figure 1.** Schematic presentation of common signaling pathways activated by RTKs upon ligand binding and receptor dimerization. Abbreviations: ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; P, phosphorylated tyrosine residue.

### 2.3.3 Regulated intramembrane proteolysis

In addition to the classical signal trandsuction by ligand binding, dimerization, autophosphorylation and activation of downstream signaling proteins, a subset of RTKs can undergo a process called regulated intramembrane proteolysis (RIP) (Merilahti et al. 2017). RIP is initiated, often after ligand binding, by cleavage of the extracellular domain of an RTK by a protease (also known as sheddase) belonging e.g. to the families of disintegrin and metalloproteases (ADAM) or matrix metalloproteases (MMP) (Beel & Sanders 2008). This exposes a cleavage site in the transmembrane domain which is cleaved by another protease presented in the form of γ-secretase complex (Beel & Sanders 2008) (Figure 2). Released ICD can translocate to the nucleus and act there as a transcriptional regulator (Merilahti & Elenius 2019). Altogether 27 RTKs have been shown to undergo RIP (Merilahti et al. 2017). Additionally, an RTK can translocate into the nucleus as an intact

molecule, as has been shown e.g. for epidermal growth factor receptor (EGFR, ErbB1) (Marti et al. 1991).

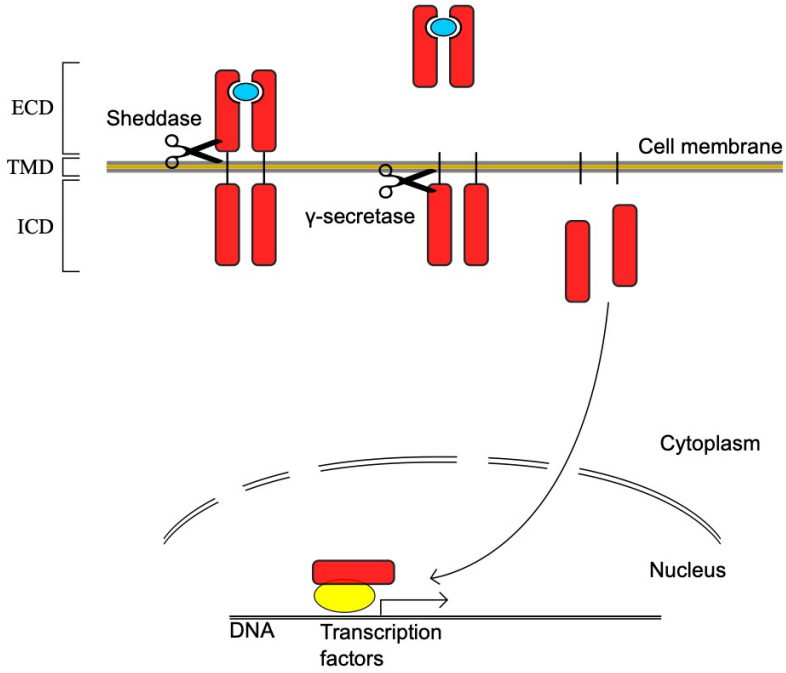


**Figure 2.** Schematic illustration of regulated intramembrane proteolysis (RIP) of an RTK. RIP starts with the cleavage of the ECD by a sheddase which is followed by γ-secretase-mediated release of the ICD. Abbreviations: ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain.

### 2.3.4 ErbB receptor family

ErbB receptor family (named originally as v-erb-a erythroblastic leukemia viral oncogene homolog, also known as EGFR or HER family) consists of four members in mammals, EGFR or ErbB1 (Ullrich et al. 1984), ErbB2 (HER2) (Stern et al. 1986), ErbB3 (HER3) (Plowman et al. 1990) and ErbB4 (HER4) (Plowman et al. 1993). They have at least 11 ligands (Stein & Staros 2006). The binding specificities of the ligands are presented in Table 2 (Linggi & Carpenter 2006). Notably, ErbB2 has no high-affinity ligand detected so far.

ErbB receptors are strongly expressed in the heart, skeletal muscle, central nervous system, mammary gland, kidney, lung, and their knockout produces problems in development of these organs (Chan et al. 2002; Chen et al. 2000; Elenius et al. 1997; Erickson et al. 1997; Gassmann et al. 1995; Lee et al. 1995; Sibilia et al. 2007). The role of ErbBs in cardiac development, as well as in cardiovascular

diseases, is discussed in detail below. ErbB receptors have also multiple implications in cancer pathogenesis, especially breast, lung, brain, skin, ovarian and head and neck cancers (Sibilia et al. 2007).

ErbB receptors are mainly activated in the manner common for RTKs, including ligand binding, homo- or heterodimerization and phosphorylation of the kinase domain (Linggi & Carpenter 2006). However, ErbB receptor activation does not require phosphorylation of the autoinhibitory activation loop but instead an allosteric mechanism allows activation of the kinase domains in the dimer which results in phosphorylation and activation (Lemmon & Schlessinger 2010). Several mechanisms to inhibit or modify ErbB-mediated signals have been identified, notably dephosphorylation by phosphatases (Lemmon & Schlessinger 2010), ubiquitination (see chapter 2.3.1 for detailed definition) and subsequent internalization and degradation of the receptor (Carraway 2010), or SUMOylation followed by nuclear localization and either repression (Sundvall et al. 2012) or enhancement (Knittle et al. 2017) of signaling activities.

Among the ErbB family, ErbB4 is an exception in its ability to undergo RIP. Two alternatively spliced isoforms at the juxtamembrane (JM) region of ErbB4 exist: JM-a exclusively carrying exon 16 and JM-b exclusively exon 15 (Elenius et al. 1997). JM-a is the only cleavable isoform (Elenius et al. 1997). Extracellular domain of JM-a is cleaved by the sheddase TNFα converting enzyme (TACE) / ADAM17 which generates an 80-kilodalton (kDa) membrane-bound fragment. This is subsequently cleaved by γ-secretase, leading to the release of an ICD which can translocate into the nucleus (Ni et al. 2001), and regulate transcription and differentiation (Knittle et al. 2017; Merilahti et al. 2017; Muraoka-Cook et al. 2006; Paatero et al. 2012). In addition to JM isoforms of ErbB4, its cytoplasmic part varies by inclusion (CYT-1 isoform) or exclusion (CYT-2 isoform) of exon 26. CYT-1 can directly bind and activate PI3K (Elenius et al. 1999). Additionally, it can bind the ubiquitin ligase Itch which leads to ubiquitination and endocytosis of CYT-1 (Sundvall et al. 2008).

**Table 2**. Ligands of ErbB family receptors (modified from Linggi and Carpenter 2006). Abbreviations: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; TGF-α, transforming growth factor alpha.

| LIGAND | EGFR | ErbB2 | ErbB3 | ErbB4 |
|---|---|---|---|---|
| Amphiregulin | x | | | |
| EGF | x | | | |
| Epigen | x | | | |
| TGF-α | x | | | |
| Betacellulin | x | | | x |
| Epiregulin | x | | | x |
| HB-EGF | x | | | x |
| Neuregulin-1 | | | x | x |
| Neuregulin-2 | | | x | x |
| Neuregulin-3 | | | | x |
| Neuregulin-4 | | | | x |

### 2.3.5 ROR receptors

ROR (receptor tyrosine kinase-like orphan receptor) family of RTKs consists of two members, ROR1 and ROR2 (Masiakowski & Carroll 1992). The name of the family stems from the notion that there would be no specific ligands for the receptors but it was later discovered that WNT5A can bind and activate both RORs (Fukuda et al. 2008; Liu et al. 2008; Oishi et al. 2003; Yu et al. 2016). RORs heterodimerize with each other (Yu et al. 2016). Additionally, homodimerization of ROR2 (Liu et al. 2008) and ROR1 (Hojjat-Farsangi et al. 2013) has been demonstrated. Activation of RORs causes phosphorylation, binding of signaling proteins to the phosphorylated docking sites, and activation of pathways common for RTKs, such as the PI3K, JAK-STAT, and MAPK pathway (Hojjat-farsangi et al. 2014). Additionally, ROR receptors can act as coreceptors for the GPCR Frizzled (Park et al. 2015) and dimerize with other RTKs, such as EGFR (Yamaguchi et al. 2012). WNT5A-mediated activation of ROR1/ROR2-Frizzled complex leads to activation of relatively less studied RTK signaling pathways, such as planar cell polarization pathway through Rho-Rac-Cdc42 proteins, as well as to inhibition of the canonical WNT pathway (Kamizaki et al. 2020).

RORs are expressed in many tissues during embryonic development, especially in neural tissues, heart, muscle, bones, limb, and gut (Al-Shawi et al. 2001; Matsuda et al. 2001; Oishi et al. 1999). Knockout of *Ror2* in mice causes dwarfism, cyanosis,

dysplastic lungs, short limbs and tails, and ventricular septal defects (VSD) (Oishi et al. 2003; Takeuchi et al. 2000). A similar phenotype have been reported for *Wnt5a* deficient mice (Oishi et al. 2003). *Ror1* knockout alone in mice does not cause cardiac or skeletal abnormalities but the mice die postnatally due to respiratory problems and unexpanded alveoli in the lungs (Nomi et al. 2001). Defects in cardiac development due to knockout of ROR receptors are discussed later in detail.

Expression of RORs is decreased in normal adult tissues compared to the levels in embryo. In mouse, Ror2 expression is virtually absent in adult tissues (Oishi et al. 1999). Similarly, Ror1 expression is decreased by age and in the adult mouse, expression is mainly detected in the heart (Oishi et al. 1999). Strong expression of ROR1 in adult human tissues has been shown in pancreatic, parathyroid and gastrointestinal tissues (Balakrishnan et al. 2017). ROR1 expression in adult heart was not detected by Balakrishnan et al. (Balakrishnan et al. 2017) Dave et al. identified a truncated 50-kDa isoform and only very low expression of the full length receptor in pediatric heart samples, and additionally cytoplasmic expression in adult heart (Dave et al. 2012). However, Sharma et al. detected *ROR1* and *ROR2* mRNA expression in human iPSC-derived cardiomyocytes (Sharma et al. 2017). Although ROR expression is significantly reduced in adult tissues, RORs seem to have central roles e.g. in tissue repair after brain, intestinal and skeletal injuries (Kamizaki et al. 2020).

RORs are implicated in pathogenesis of several cancers, such as leukemias, melanoma and prostate, breast, ovarian and colon cancer (Hojjat-farsangi et al. 2014). ROR1 is being assessed as a target for cancer therapy. Cirmtuzumab, a ROR1-targeting monoclonal antibody, has been developed primarily for the treatment of chronic lymphocytic leukemia and has been tested in phase I trials (Choi et al. 2015). Furthermore ROR2-WNT5A signaling is centrally involved in pathogenesis of bone diseases, such as for osteoporosis (Kamizaki et al. 2020). Finally, given the central role of RORs in skeletal and limb development, mutations in *ROR2* cause recessive Robinow syndrome with abnormal facial features, short limbs, genital and skeletal anomalies (Bokhoven et al. 2000), and Brachydactyly type B with distally shortened fingers and toes (Oldridge et al. 2000).

### 2.3.6 RTKs in cardiac development

Several RTKs are centrally involved in the embryonic development of the heart, as is the case e.g. with the whole ErbB family. Loss of *Erbb2* is lethal at embryonic day E10.5 in mice due to defects in cardiac trabeculation (Chan et al. 2002; Lee et al. 1995). Knockout of *Erbb4* in mice leads to a similar phenotype (Gassmann et al. 1995) and concordantly, a similar phenotype was detected in *Nrg1*-deficient mice which also died at E10.5 (Meyer & Birchmeier 1995). Loss of *Egfr* in mice results

in defects of semilunar (i.e. aortic and pulmonary artery) valve development which causes thickened valves leaflets, aortic stenosis and regurgitation and subsequent cardiac hypertrophy (Chen et al. 2000). *Erbb3*-knockout mice die at E13.5 due to poorly developed cardiac valves and blood regurgitation (Erickson et al. 1997).

Both members of the ROR family are also involved in heart development. *Ror2*-deficient mice die postnatally, are cyanotic and have a ventricular septal defect (VSD) (Takeuchi et al. 2000). Loss of *Ror1* alone in mice produces no heart defects but the mice die after birth due to respiratory problems (Nomi et al. 2001). Loss of both *Ror1* and *Ror2* in mice leads, however, to more severe heart phenotype than *Ror2*-knockout alone, as VSD is accompanied with transposition of the great arteries (Nomi et al. 2001).

Other examples of RTKs involved in the heart development include members of anaplastic lymphoma kinase / leukocyte tyrosine kinase (ALK/LTK), Eph, platelet-derived growth factor receptor (PDGFR), angiopoietin (TIE) and vascular endothelial growth factor receptor (VEGFR) families. Transgenic overexpression of LTK in mice results in cardiac hypertrophy, reduced cardiac output and degeneration of cardiomyocytes (Honda et al. 1999). Loss of *Epha3*, a member of the Eph receptor family, in mice generates hypoplastic valves and atrial septal defect and leads to lethality of approximately 75% of the mice shortly after birth (Stephen et al. 2007). On the other hand, loss of Ephrin-A1, a ligand of EphA3 among other receptors, leads to thickened aortic and mitral valves in mice which are viable but have problems in cardiac function (Frieden et al. 2010). As a sign of necessity of PDGFR-related signaling, *Pdgfb*-knockout mice die perinatally with problems in kidney development, dilated heart and hemorrhages (Levéen et al. 1994). Mice lacking PDGFRβ (Mellgren et al. 2008; Van Den Akker et al. 2008) or PDGFB (Van Den Akker et al. 2008) have been shown to develop thinned, hypoplastic myocardium and VSD. The phenotype in PDGFRβ-deficient mice is accompanied with abnormalities in coronary development (Mellgren et al. 2008). Homozygous deletion of *Tie2* in mice leads to embryonal lethality at E9.5 and serious defects in development, which is likely due to loss of interaction between endocardium and myocardium as TIE2 is expressed in endothelial cells (Dumont et al. 1994). Knockout of *Vegfb*, encoding a ligand for VEGFRs, has been shown to cause reduced heart size in mice and vascular dysfunction after coronary occlusion (Bellomo et al. 2000).

### 2.3.7 RTKs with therapeutic potential in myocardial ischemia-reperfusion

Regulation and therapeutic potential of ErbB family members EGFR, ErbB2 and ErbB4 in the ischemic heart have been widely studied. In ischemic human heart

compared to normoxic control heart, expression of *ERBB2* and *NRG1* have been shown to be decreased at mRNA level, while expression of *EGFR* and ligands *HBEGF* (heparin-binding EGF-like growth factor) and *NRG2* is increased (Munk et al. 2012). In the same study, EGFR phosphorylation was decreased while ErbB2 and ErbB4 phosphorylation were increased by hypoxia in HL-1 cardiomyocytes, and administration of HB-EGF rescued the cells from reduced viability caused by ErbB2 inhibition with trastuzumab in hypoxia (Munk et al. 2012). Moreover, ErbB2 protein expression is reduced in hypoxia-reoxygenation treated neonatal rat cardiomyocytes (Viswanath et al. 2011) and in human hearts with ischemic cardiomyopathy (Gordon et al. 2009). Cardioprotective effects of exogenously administrated NRG-1 in isolated, infarcted rat heart has been demonstrated (Wang et al. 2018). Similar effect of endothelial cell-derived NRG-1 in isolated, infarcted mouse heart has been shown (Hedhli et al. 2011). The mechanism seems to be ErbB4-mediated activation of the RISK pathway and reduction of apoptosis (Wang et al. 2018). Activation of ErbB2 and ErbB4 also confer cardioprotection by activating regeneration and proliferation of cardiomyocytes, as discussed later.

Transactivation of EGFR by various G protein-coupled receptors (GPCR) is central in many cardioprotective approaches. EGFR transactivation is necessary e.g. in ischemic preconditioning and adenosine-mediated cardioprotection as shown in a perfused mouse heart model (Williams-Pritchard et al. 2011), in bradykinin-mediated cardioprotection demonstrated in a perfused rat heart model (Methner et al. 2009), as well as in opioid receptor-mediated cardioprotection in isolated rabbit heart (Förster et al. 2007). EGF administration has been shown to be cardioprotective in ischemia in isolated hearts of both mouse (Lorita et al. 2010) and type 1 diabetic rats (Akhtar et al. 2012). Contradictory to these findings, two-week inhibition of EGFR with a (non-specific) inhibitor AG1478 before ischemia reduced infarct size and apoptosis in rats with type 2 diabetes (Mali et al. 2018). Amphiregulin signaling through EGFR has been shown to contribute to cardiac fibrosis and shRNA-mediated amphiregulin knockdown improved cardiac functionality after infarction in mouse (Liu et al. 2018). Role of EGFR thus seems to be complex in ischemic cardioprotection, while a large proportion of the evidence so far has shown EGFR activity to be beneficial for cardioprotection (Reichelt et al. 2017).

Insulin receptor (INSR) is involved in ischemic cardioprotection, and administration of insulin at reoxygenation has been shown to protect isolated rat cardiomyocytes from apoptosis (Jonassen et al. 2000). Insulin, given at early reperfusion, was also shown to reduce infarction size in isolated rat hearts through Akt-p70s6 kinase dependent signaling (Jonassen et al. 2001). Protein levels of VEGFR1, VEGFR2 and IGF-1R, and their ligands VEGFA and IGF-1, have been shown to be upregulated in porcine heart after ischemia-reperfusion (Infanger et al. 2007). Administration of quinaprilat, an ACE inhibitor, further increased IGF-

1/IGF-1R expression which correlated with reduced laminin, collagen type IV and chondroitin sulfate expression, potentially thus mediating the attenuation of cardiac remodeling (Infanger et al. 2007).

TIE receptors are expressed in the endothelial cells but they affect myocardial cells through paracrine signaling and vascularization. In a rat model of myocardial infarction, TIE2 phosphorylation has been shown to be increased, and protein and mRNA expression of its ligand angiopoietin-1 were decreased while those of angiopoietin-2 were increased (Sandhu et al. 2004). Administration of angiopoietin-1 has been shown to protect cardiac contractility in a mouse infarction model (Lee et al. 2011). As cardiomyocytes themselves do not express TIE receptors, the cardioprotective signaling in cardiomyocytes was shown to be mediated through β1 integrin (Lee et al. 2011).

Yet another RTK involved in ischemic cardioprotection is EphA2 receptor with its (non-exclusive) ligand Ephrin-A1. In a non-reperfused mouse infarction model, infarct size was greater and ischemic cardiomyopathy was more severe four weeks after infarction in EphA2-deficient mice than in wildtype controls (O'Neal et al. 2014). On the other hand, intramyocardial injection of chimeric Ephrin-A1-Fc has been shown to preserve cardiac function and reduce infarct size both in non-reperfused mouse heart model (Dries et al. 2011), as well as in a mouse ischemia-reperfusion injury model (DuSablon et al. 2017).

### 2.3.8 Regenerative potential of myocardium conferred by RTKs

In addition to being activated and possibly cardioprotective by reducing infarct size and cardiomyocyte death in ischemia-reperfusion injury, some RTKs may confer further protection through direct myocardial regeneration. Central examples are ErbB2 and ErbB4. Transient induction of constitutively active ErbB2 has been shown to induce redifferentiation, proliferation and regeneration of cardiomyocytes which reduces infarct size and preserves cardiac function in a mouse model (D'Uva et al. 2015). Similarly, activation of ErbB4 by NRG-1 induces proliferation of mononucleated cardiomyocytes and this leads to reduced infarct size and improved cardiac function in mice (Bersell et al. 2009).

Fibroblast growth factor receptors (FGFR) are another family of RTKs involved in cardiomyocyte proliferation. Administration of FGF-1, a ligand to all FGFRs, together with a p38 inhibitor has been shown to induce cardiomyocyte mitosis, decrease infarct scar and improve cardiac function in a permanent coronary artery ligation model in rats (Engel et al. 2006). In a porcine ischemia-reperfusion model, catheter-based injection of microparticles loaded with either FGF-1 or NRG-1

improved cardiac function and increased myocardial vascularization (Garbayo et al. 2016).

Finally, conditional activation of PDGFRβ in cardiomyocytes has been shown promote heart regeneration and preserve cardiac function after non-reperfused infarction in mice (Yue et al. 2019). Similar regenerative activity was shown also with myocardial adenoviral expression of constitutively active PDGFRβ after infarction (Yue et al. 2019). Altogether, utilization of RTKs to promote cardiac regeneration is promising but RTK-based therapies to regenerate myocardium after ischemia-reperfusion injury have not been assessed in clinical trials for the time being.

### 2.3.9 RTKs implicated in heart failure

A few RTKs have been implicated in heart failure pathogenesis and as potential targets for cardioprotection. ErbB2 and ErbB4 expression are decreased both on mRNA and protein level in rats with pressure-overload hypertrophy, as the disease develops from compensatory hypertrophy to heart failure (Rohrbach et al. 1999). Similarly, cardiac ErbB2 and ErbB4 expression are decreased in patients with heart failure, both on mRNA and protein level (Rohrbach et al. 2005). Additionally decreased ErbB2 phosphorylation has been observed, while NRG-1 protein expression is increased (Rohrbach et al. 2005). Moreover, ventricular assistance device (VAD) treatment, used to unload a failing heart, was shown to increase *ErbB2* and *ErbB4* expression (Rohrbach et al. 2005). Similar effect of VAD on *ErbB2* and *ErbB4* mRNA expression was shown by Uray et al., especially in ischemic cardiomyopathy, though they contradictorily reported increased *ErbB4* expression in failing hearts compared to controls (Uray et al. 2002). As discussed earlier, enhancing ErbB signaling through recombinant NRG-1 has been tested as a heart failure treatment modality in clinical trials with positive results (Lenihan et al. 2016).

VEGFR-mediated signaling governs angiogenesis and through this it affects cardiovascular disease progression (Ky et al. 2011). Soluble VEGFR1 (FLT1) in blood has been shown to be associated with severity of heart failure and short transplant-free and VAD-free survival in the patients with soluble VEGFR1 levels in the highest quartile (Ky et al. 2011). Furthermore, higher plasma levels of VEGF ligand (subtype not specified) have been observed in heart failure patients, together with higher levels of TIE2 and angiopoietin-2 (Chong et al. 2004). Contradictory to this, in mice with transverse aortic constriction (TAC)-induced heart failure, decreased levels of *VEGFB* mRNA after four weeks of TAC have been observed in the heart, while increased levels of *VEGFC* and *VEGFD* mRNA, together with increased level of *VEGFR3* mRNA, were observed after two weeks (Huusko et al. 2012). However, adenoviral delivery of VEGFB preserved heart function, promoted

angiogenesis, and reduced cardiomyocyte apoptosis in the mouse model of TAC-induced heart failure (Huusko et al. 2012). Similarly, adenoviral delivery of VEGFB prevented heart failure in tachypaced dogs through inhibition of apoptosis and oxidative stress in cardiomyocytes (Pepe et al. 2010).

AXL, a member of TAM receptor family, has been shown to be upregulated at protein level in human myocardium with heart failure compared to control hearts, and, concordantly, high soluble AXL levels in serum were observed in heart failure patients (Batlle et al. 2014). Disease progression as estimated by hospitalizations, all-cause mortality, and transplantations correlated with high soluble AXL levels at one-year follow-up (Batlle et al. 2014). However, AXL or any other RTK has not yet progressed to actual clinical use as a biomarker or therapeutic target for heart failure.

### 2.3.10 Cardiotoxicity of RTK inhibitors

Multiple RTKs are involved in cancer pathogenesis and they are targeted with small molecule tyrosine kinase inhibitors (TKI) or antibodies in cancer therapies. Given the central role of RTKs in heart development and diseases as discussed above, it is not surprising that certain RTK-targeting therapies produce cardiotoxicity as adverse drug effects. A hallmark example of this is trastuzumab, an ErbB2-targeting antibody used to treat breast cancer and other cancers with ErbB2 amplification. Trastuzumab produces heart failure, which is at least to some extent reversible (Nemeth et al. 2017; Slamon et al. 2001). Toxicity is especially observed when trastuzumab is combined with anthracyclines (Nemeth et al. 2017). Potential mechanisms include impaired NRG-1/ErbB signaling, increased ROS production and apoptosis (Nemeth et al. 2017). As expectable, pan-ErbB inhibitors which target multiple EGFR family members are potentially cardiotoxic through ErbB2 inhibition, such as has been shown for lapatinib (Jerusalem et al. 2019) and afatinib (Schuler et al. 2012). However, in a review of cardiac safety data from afatinib trials, cardiac failure adverse events were not increased by afatinib as compared to placebo (Ewer et al. 2015).

EGFR inhibition alone has been considered relatively safe from the cardiac point of view (Chen et al. 2008). However, a case-control study revealed increased risk for heart failure in patients treated with cetuximab or panitumumab, both EGFR-targeting antibodies, while there was no increased risk observed with erlotinib and gefitinib, small molecule inhibitors of EGFR (Gronich et al. 2017).

VEGFRs are targeted with many multi-target TKIs. Sunitinib, which targets e.g. PDGFR family receptors in addition to all VEGFRs, has been shown to predispose to hypertension, left ventricular dysfunction and reversible heart failure (Chu et al. 2007). Apoptosis and mitochondrial injury were suggested as possible mechanisms (Chu et al. 2007). Pazopanib is a TKI which inhibits e.g. FGFRs and PDGFRs in

addition to VEGFRs. Pazopanib use has been shown to cause hypertension, conduction problems and ventricular dysfunction (Pinkhas et al. 2017). A meta-analysis of VEGFR TKIs (axitinib, cediranib, pazopanib, ramucirumab, sorafenib, sunitinib, vandetanib) found evidence of increased heart failure risk associated with the use of VEGFR TKIs (Qi et al. 2014).

Preclinical models have been developed to predict cardiotoxicity of TKIs. Cheng et al. demonstrated that a zebrafish model could discriminate a non-cardiotoxic TKI, the EGFR inhibitor gefitinib, from known cardiotoxic TKIs sunitinib and sorafenib (Cheng et al. 2011). The mechanism of sunitinib-induced cardiotoxicity has been shown to be partly mediated by inhibition of AMPK, an intracellular kinase activated by e.g. energy depletion (Kerkelä et al. 2009). Sharma et al. used human iPSC-derived cardiomyocytes to screen cardiotoxicity of TKIs in a high-throughput setting with multiple readouts (Sharma et al. 2017). They identified VEGFR2/PDGFR-inhibiting TKIs as highly cardiotoxic and showed that exogenous insulin or IGF-1 could attenuate the cardiotoxicity (Sharma et al. 2017). Elmadani et al. screened a wide compound family of kinase inhibitors, including several TKIs, on neonatal rat ventricular cardiomyocytes and IGF-1R, PI3Kα and mTOR were identified as central kinases regulating cardiomyocyte viability (Elmadani et al. 2019).

## 2.4 TRIM proteins

### 2.4.1 Protein control by ubiquitination

Ubiquitination is a process where a small modifier protein called ubiquitin is covalently attached to another protein, which is referred to as the substrate. Ubiquitin is a 76-amino acid protein (approximately 8.6 kDa) which is conjugated via its carboxy terminus to a lysine on the substrate through a three-step process (Hatakeyama 2017). First, ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme called E1 by conjugating the C-terminal glycine of ubiquitin to the E1 itself. Subsequently, ubiquitin is transferred to a ubiquitin-conjugating enzyme belonging to the E2 class. Finally, by collaboration of the E2 and an E3 a ubiquitin ligase, ubiquitin is conjugated to a lysine side chain of the substrate or another ubiquitin, already attached to a substrate (Hatakeyama 2017). E3 ligases are divided into three classes: Homologous to E6AP Carboxy Terminus (HECT), U-Box, and RING finger-containing E3s (Hatakeyama 2017; Meroni & Diez-Roux 2005). E3s identify the substrate and the substantially large number of E3s is possibly due to the needed specificity for a large and diverse group of substrates (Meroni & Diez-Roux 2005).

Ubiquitin may be conjugated to the substrate as a single ubiquitin (monoubiquitination, taking place on one or multiple sites per substrate) or as a

polyubiquitin chain. There are several lysines on the ubiquitin through which they can be conjugated to each other forming a polyubiquitin chain. The lysine 48 (K48)-conjugated ubiquitin chains mainly target the substrate protein to proteasomal degradation by the 26S proteasome, a large protein complex which degrades proteins (Hatakeyama 2017). Additionally, proteins with ubiquitin chains conjugated by the other available lysines (K6, K11, K27, K29, K33, K63) or the amino-terminal methionine (M1) are implicated in signal transduction, DNA repair, or membrane trafficking (Hatakeyama 2017; Venuto & Merla 2019). The events of ubiquitination are schematically illustrated in Figure 3.

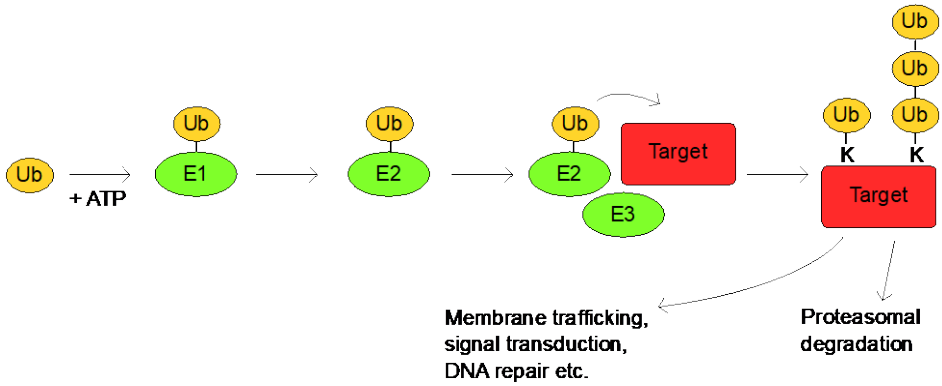


**Figure 3.** Events of ubiquitination of a protein. Ubiquitin is first activated in an ATP-dependent manner by ubiquitin-activating enzyme E1. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme E2, which together with a ubiquitin ligase enzyme, E3, transfers the ubiquitin to the target protein. Mono- and polyubiquitination target proteins to different destinations.

### 2.4.2 TRIM proteins are ubiquitin E3 ligases with various functions

TRIMs are a large family of proteins, and most of them function as E3 ubiquitin ligases. There are currently more than 80 known *TRIM* genes in human (Hatakeyama 2017). The name of the family stems from their characteristic TRIpartite Motif which consists of a RING finger domain, one or two B-box zinc finger domains, and a coiled-coil region (Meroni & Diez-Roux 2005). Another name of the family is RBCC (RING-B-Box-coiled-coil). The RING finger domain is a zinc atom-binding motif which is present in many E3 ubiquitin ligases and it is responsible for binding both RING-containing proteins as well as versatile ubiquitination substrates (Meroni & Diez-Roux 2005). The B-box motif binds also zinc and it may be involved in creating higher order structures of TRIM proteins (Watanabe & Hatakeyama 2017). The Coiled-coil domain is involved in homodimerization of TRIMs (Meroni & Diez-

Roux 2005; Watanabe & Hatakeyama 2017). TRIMs are divided into 11 subclasses according to their C-terminal domains, which are diverse and potentially add to the specificity of interactions between other proteins (Borlepawar et al. 2019).

As for other E3s, ubiquitination by TRIMs may, in addition to classical targeting for proteasomal degradation, lead to altered transcriptional regulation, membrane trafficking or subcellular localization of the substrates (Hatakeyama 2017; Meroni & Diez-Roux 2005). Various TRIMs are involved in regulation of the cell cycle, and while some function as oncogenes, others act as tumor suppressors (Venuto & Merla 2019). Furthermore, TRIMs are key factors in autophagy, which is an intracellular lysosome-dependent protein degradation pathway. In autophagy, TRIMs interact with target-recognizing proteins such as p62/SQSTM1 which add substrate specificity to the process (Hatakeyama 2017).

TRIMs are involved in pathogenesis or control of many diseases, such as Opitz syndrome (a congenital state causing midline defects including hypospadia, cleft lip and palate, and laryngotracheal abnormalities) and other developmental disorders, innate immune response to viral infections by for example HIV and influenza A virus, and muscle atrophy (Watanabe & Hatakeyama 2017). Moreover, dysregulation of TRIM expression or chromosomal aberrations which affect *TRIM* genes have been shown to be involved in cancer, e.g. acute promyelocytic leukemia and parathyroid, liver (Watanabe & Hatakeyama 2017), prostate, and breast cancer (Hatakeyama 2017).

The physiology of the heart requires a balance of protein synthesis and degradation. Complex role of ubiquitination in the heart is underscored by the contradictory evidence from animal models where both prevention and exacerbation of hypertrophy and heart failure have been observed as an effect of proteasome inhibitors (Barac et al. 2017). Moreover, the ubiquitin-proteasome system is involved e.g. in regulation of β-adrenergic signaling and pathogenesis of ischemia-reperfusion injury (Barac et al. 2017). Sixty E3 ligases have been identified to have functional roles in the heart, out of which eight are TRIMs (Borlepawar et al. 2019). These TRIMs take part in essential cardiac functions, such as differentiation, apoptosis, hypertrophy, atrophy, and ischemia (Borlepawar et al. 2019). Their roles can be either inhibitive or promoting for pathological cardiac conditions, as has been shown e.g. for TRIM24 and TRIM32. TRIM24 binds and prevents the degradation of dysbindin which is targeted for degradation by TRIM32 (Borlepawar et al. 2017). Dysbindin activity leads to hypertrophy, and TRIM24 and TRIM32 have opposing roles in its regulation (Borlepawar et al. 2017). TRIM32 prevents hypertrophy of cultured cardiomyocytes but on the other hand, overexpression of TRIM32 increases apoptosis of cardiomyocytes (Borlepawar et al. 2017). TRIM8 is another example of a TRIM protein whose function predisposes to hypertrophy, as shown in knockout mice which were protected from hypertrophy after TAC (Chen et al. 2017).

### 2.4.3 The muscle ring finger family members are central TRIMs in the heart

The most studied subgroup of TRIMs in the heart are TRIM54, TRIM55 and TRIM63, the members of the muscle ring finger (MURF) family. MURF family consists of three proteins, MURF1 or TRIM63, MURF2 or TRIM55 and MURF3 or TRIM54. TRIM54 and TRIM63 are specifically expressed in striated muscle while TRIM55 expression has been additionally observed in liver (Centner et al. 2001). TRIM55 is expressed strongly in embryonic mouse heart and its expression begins at E8.5 and peaks at E18.5 (Perera et al. 2011). TRIM55 expression is later reduced in adult myocardium (Perera et al. 2011). Contrary to this, expression of TRIM63 increases after birth and TRIM54 is virtually expressed only after birth (Perera et al. 2011). MURFs have been shown to colocalize with and affect the organization of both M- and Z-bands of the sarcomeres, TRIM63 showing colocalization with both and TRIM55 with M-bands (Centner et al. 2001; Perera et al. 2011).

Mice lacking either *Trim55* or *Trim63* alone do not demonstrate developmental abnormalities while double-knockout (DKO) of the both genes causes massive cardiac hypertrophy and is lethal to majority of the mice pre- or perinatally (Willis et al. 2014). Furthermore, the DKO mice demonstrate mild skeletal muscle hypertrophy (Witt et al. 2008). Although TRIM55 and TRIM63 seem to have redundant functions in embryonic development, it has been shown that knockout of *Trim63* in mice causes exaggerated hypertrophic response to TAC while knockout of *Trim55* does not (Willis et al. 2007). Antihypertrophic activity of TRIM63 has been shown be mediated through inhibition of PKCε translocation to focal adhesions (Arya et al. 2004). Furthermore, TRIM63 overexpression has been detected in multiple situations of skeletal muscle atrophy and its deficiency protects from skeletal denervation atrophy (Bodine et al. 2001). On the other hand, transgenic overexpression of TRIM63 in mice causes thinning of ventricular wall, inhibition of creatine kinase activity and rapid progression of heart failure after TAC (Willis et al. 2009). Knockout of *Trim54* in mice causes no apparent phenotype in normal conditions while increased tendency to cardiac rupture, ventricular dilatation and reduced contractility are observed after myocardial infarction (Fielitz et al. 2007b). DKO of *Trim54* and *Trim63* in mice causes myocardial hypertrophy, decreased ventricular function and skeletal myopathy (Fielitz et al. 2007a). Finally, DKO of *Trim54* and *Trim55* causes aggregate myopathy, impaired cardiac function and disturbed calcium handling in cardiomyocytes (Lodka et al. 2016). Balance of MURF expression in the heart or skeletal muscle is thus crucial for normal muscular function.

### 2.4.4 Ubiquitination targets and interaction partners of MURFs

Direct ubiquitination targets of TRIM55 are poorly characterized while there is more evidence on direct targets of TRIM63. TRIM63 has been shown to ubiquitinate cardiac troponin I (cTnI) (Kedar et al. 2004; Witt et al. 2005) and target it to degradation (Kedar et al. 2004). Witt et al. also showed that both TRIM55 and TRIM63 interacted, in addition to cTnI, with MLC2, myotilin, nebulin, NRAP, T-cap and troponin T, but ubiquitination selectively by TRIM63 was only shown for cTnI and MLC2 (Witt et al. 2005). Moreover, TRIM54 and TRIM63 have been shown to ubiquitinate and mediate degradation of myosin heavy chain β (Fielitz et al. 2007a). The signaling protein phospho-c-Jun is another ubiquitination target of TRIM63 (Li et al. 2011) and signaling of peroxisome proliferator-activated receptor alpha (PPARα) has been shown to be reduced by monoubiquitination by TRIM63 (Rodríguez et al. 2015). Ubiquitin-proteasome-dependent degradation of p62 has been shown to depend on TRIM55, specifically on its MURF2A isoform (Pizon et al. 2013). PPARα and PPARγ are monoubiquitinated by TRIM55 (He et al. 2015).

### 2.4.5 Specific role of TRIM55 in the heart

TRIM55 is involved in sarcomere assembly, it colocalizes with tubulin during myofibrillogenesis, and its functions are partly redundant with TRIM54 (Perera et al. 2011). TRIM55 has been shown to interact with NBR1, LC3 (Pizon et al. 2013), p62 (Lange et al. 2005; Pizon et al. 2013), titin, serum response factor (SRF) (Lange et al. 2005), microtubules and myosin (Pizon et al. 2002). In cardiomyocytes, TRIM55 has been shown to translocate into the nucleus by cellular stress caused by starvation (Pizon et al. 2002) or contractile inactivity, which resulted in concomitant nuclear exclusion of SRF (Lange et al. 2005). Localization of TRIM55 has been shown to be dependent on tyrosine kinase activity of titin, likely controlled by mechanical strain (Lange et al. 2005).

While functions of TRIM55 seem to be largely redundant with other MURFs, there is, however, evidence on its independent significance in cardiac disorders. *Trim55*-knockout mice have more severely impaired cardiac function in diabetic cardiomyopathy, caused by high-fat diet, which is potentially mediated through increased PPARα and PPARγ signaling (He et al. 2015). Moreover, in rats with spontaneously hypertrophic hearts, *Trim55* expression is negatively correlated with left ventricle mass, and in human idiopathic DCM, *TRIM55* expression is reduced (Prestes et al. 2016). Finally, HCM patients with rare, nonsynonymous mutations in *TRIM55* or *TRIM63* are generally younger and their left ventricular wall thickness is greater compared to the patients without these mutations, suggesting pathogenic,

disease-modifying consequences of dysfunctional *TRIM55* or *TRIM63* (Su et al. 2014). However, a more recent larger study did not report as high excess of *TRIM55* or *TRIM63* variants among HCM patients as compared to controls (Walsh et al. 2017).

# 3 Aims

1) To identify RTKs with potential as novel therapeutic targets for myocardial ischemia-reperfusion injury using human gene expression data, a pig model of ischemia-reperfusion, and cardiomyocyte models.

2) To identify novel therapeutic targets for myocardial ischemia-reperfusion injury using drug and shRNA libraries.

3) To identify novel genetic markers predicting risk of heart failure in the FINRISK population and functionally validate candidate variants *in vitro* and *in vivo*.

# 4 Materials and Methods

## 4.1 Cell culture (I-III)

The cell lines used in the study were HL-1 mouse atrial cardiomyocytes (Claycomb et al. 1998) and H9c2 rat cardiomyoblasts (Kimes & Brandt 1976). HL-1 cells were grown in Claycomb medium (Sigma), supplemented with 0.1 mM norepinephrine. H9c2 cells were grown in Dulbecco's Modified Eagle Medium. Both media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine or UltraGlutamine (Lonza) and 50 U/ml of both penicillin and streptomycin (Lonza). Culture dishes for HL-1 cells were coated with a solution of 10 μg/ml fibronectin (R&D Systems) and 0.02% gelatin in distilled water at 37 °C for at least one hour. Trypsin inhibitor was used after trypsin-mediated detachment of HL-1 cells.

### 4.1.1 Hypoxia-reoxygenation treatment (I-II)

Ischemia-reperfusion injury was simulated on cells using hypoxia and reoxygenation. Cells were exposed to hypoxia (1% $O_2$) in a hypoxic workstation ($InVivo_2$, Ruskinn Technology Ltd.) one day after plating for 1 to 48 hours, depeding on experimental setting and readout. For reoxygenation, cells were restored to normal cell incubator (21% $O_2$) for 1 to 24 hours before sample collection or experimental measurements. Samples collected in the end of hypoxic treatment for Western blotting were lysed while the cells still remained in the hypoxic workstation.

### 4.1.2 Transient transfection (III)

HL-1 cells were transiently transfected with expression plasmids (described in 4.2) using Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM (Gibco) medium 24 hours after plating according to manufacturer's instructions.

### 4.1.3 Fluorescence-activated cell sorting (FACS) (III)

Green fluorescent protein (GFP)-positive HL-1 cells transfected with GFP-encoding plasmids were sorted 24 hours after transfection with SH800 cell sorter (Sony) to

enrich transfected cells for downstream experiments. For sorting, cells were diluted in a solution of 2% FBS and 25 mM HEPES buffer in phosphate buffered saline (PBS) and kept at room temperature. Cells were sorted to Claycomb medium supplemented with 20% FBS.

### 4.1.4 Generation of HL-1 cell lines carrying *Trim55* variants with CRISPR-Cas9 (III)

HL-1 cells carrying mutations in *Trim55* gene were generated with clustered regularly-interspaced-short palindromic repeats (CRISPR)-Cas9 using homology-directed repair (HDR) method. Single guide RNA (sgRNA) (sequence: 5'-GGCGCCAAAAACCTTGCACA-3') targeting *Trim55* was cloned in to the plasmid encoding Cas9 enzyme together with GFP (4.2) using oligo inserts (5'-CACCGGGCGCCAAAAACCTTGCACA-3' (top) and 5'-AAACTGTGCAAGGTTTTTGGCGCCC-3' (bottom)) according to the protocol by Ran et al. (Ran et al. 2013). The plasmid was transfected to HL-1 cells together with the HDR template containing the specific coding mutation (TRIM55 E140K) to be introduced to *Trim55* (5'- TTATCTCCTCCCAGGCCAGAAAAAAAATTGGAC CAGCCCATGTGTGAAGAGCATGAAGAGGAACGCATCAACATCTATTGT CTGAACTGTAAAGTGCCCACATGCTCTTTGTGCAAGGTTTTTGGCGCCC ATAAGGACTGCCAGGTGGCTCCCCTGACTCATGTGTTCCAGAGGCAGA AGG-3'). Two days after transfection, 16,000 GFP-positive cells were sorted to a 24-well plate well using FACS and seven days later, cells were seeded as single cells to 384-well plate wells. Clones were expanded and genotyped with sequencing around the mutation site (single alleles of the selected clones cloned into plasmids for sequencing each allele separately). One clone with the desired TRIM E140K variant, in combination with a deletion allele (E140K/–), and additionally, clones with homozygous (–/–) and heterozygous (WT/–) deletions of *Trim55* were used in the validations.

### 4.1.5 Ligands, chemical compounds and inhibitors (I-III)

Ligands, chemical compounds and inhibitors used in the study are listed in Table 3. Concentrations and timing of the treatments are described for each experiment in the later sections.

**Table 3.** Ligands, chemical compounds and inhibitors used in the study

| REAGENT | APPLICATION | VENDOR | USED IN |
|---|---|---|---|
| Wnt-5a | ROR1 stimulation | R&D Systems | I |
| Gefitinib | EGFR inhibition | Cayman Chemicals | II |
| Afatinib | EGFR-ErbB2-ErbB4 inhibition | SCBT | II |
| Isoproterenol | Hypertrophic stimulus | Sigma-Aldrich | III |
| MG132 | Proteasome inhibition | Sigma-Aldrich | III |

## 4.2 Expression plasmids (III)

Expression plasmids described in Table 4 were generated for this study using general molecular cloning techniques. Site-directed mutagenesis was used to generate the mutated TRIM55 E140K plasmids. Generated constructs were confirmed with sequencing. Mammalian expression plasmids were used to express the proteins in mammalian cells. Zebrafish expression plasmids were used for expression of the proteins in zebrafish embryos (see 4.16). References of the background plasmids used for cloning these constructs are described in III.

**Table 4.** Expression plasmids generated for this study

| INSERT | BACKBONE | PURPOSE |
|---|---|---|
| hSpCas9, GFP, sgRNA | pSpCas9(BB)-Cas9-2A-GFP | Mammalian expression |
| GFP-TRIM55 WT | pEZYegfp | Mammalian expression |
| GFP-TRIM55 E140K | pEZYegfp | Mammalian expression |
| GFP-Luciferase | pEZYegfp | Mammalian expression |
| TRIM55 WT, GFP | pDESTTol2pA2 | Zebrafish expression |
| TRIM55 E140K, GFP | pDESTTol2pA2 | Zebrafish expression |
| GFP | pDESTTol2pA2 | Zebrafish expression |

## 4.3 Primary antibodies

**Table 5.** Primary antibodies used for Western blotting (WB), immunoprecipitation (IP) or immunofluorescence (IF) experiments. Abbreviations: CST, Cell Signaling Technology; DSHB, Developmental Studies Hybridoma Bank; SBCT, Santa Cruz Biotechnology; Sigma, Sigma-Aldrich;Thermo, Thermo Fisher Scientific; Ubiquitin, poly- and monobiquitinated proteins.

| ANTIGEN | CATALOG ID OR CLONE | VENDOR | HOST SPECIES AND TYPE | APPLI-CATION | USED IN |
|---|---|---|---|---|---|
| actin | sc-1616 | SCBT | goat polyclonal | WB | I |
| Akt | 9272 | CST | rabbit polyclonal | WB | I |
| α-actinin | 710947 | Thermo | rabbit polyclonal | IF | III |
| α-tubulin | sc-5546 | SCBT | rabbit polyclonal | WB | I |
| cTnI | ab209809 | Abcam | rabbit monoclonal | WB | III |
| GAPDH | G8795 | Sigma | mouse monoclonal | WB | I, III |
| GFP | ab290 | Abcam | rabbit polyclonal | IP | III |
| myosin | MF 20 | DSHB | mouse monoclonal | IF | III |
| p21 | sc-6246 | SCBT | mouse monoclonal | WB | III |
| p38 | 9212 | CST | rabbit polyclonal | WB | I |
| p62 | 23214 | CST | rabbit monoclonal | WB, IP | III |
| phospho-tyrosine | 4g10 | produced in house | mouse monoclonal | WB | I |
| phospho-Akt (S473) | 4060 | CST | rabbit monoclonal | WB | I |
| phospho-p38 | 9211 | CST | rabbit polyclonal | WB | III |
| ROR1 | sc-83033 | SCBT | goat polyclonal | WB | I |
| ROR1 | sc-130386 | SCBT | goat monoclonal | WB, IP | I |
| SRF | 5147 | CST | rabbit monoclonal | WB, IP | III |
| TRIM55 | PA5-62826 | Thermo | rabbit polyclonal | WB | III |
| TRIM55 | ab4387 | Abcam | goat polyclonal | WB | III |
| Ubiquitin | FK2 | Sigma | mouse monoclonal | WB | III |

## 4.4 Small interfering RNAs (I, III)

Small interfering RNAs (siRNA) were used in knockdown of the expression of mouse *Ror1*, *Ror2* (I) or *Trim55* (III) in HL-1 cells (Table 6). Lipofectamine 2000 (Thermo Fisher Scientific) was used for siRNA transfections 24 hours after plating. The final concentration of siRNAs on the cells was 100 nM. Knockdown efficacy was confirmed with Western blotting. All siRNAs were acquired from Qiagen.

**Table 6.** SiRNAs used in the study

| TARGET | CATALOG ID | USED IN |
|---|---|---|
| *Ror1* | SI01404655 | I |
| *Ror1* | SI01404662 | I |
| *Ror2* | SI01404683 | I |
| *Ror2* | SI01404690 | I |
| *Trim55* | SI04422859 | III |
| *Trim55* | SI00972853 | III |
| *Trim55* | SI00972860 | III |
| NEGATIVE CONTROL | SI03650318 | I, III |

## 4.5 Cell lysis, immunoprecipitation and Western blotting

Cells were lysed for immunoprecipitation and Western blotting, after washing with PBS, with lysis buffer containing 1% Triton X-100, 10 mM Tris-Cl pH 7.4, 150 mM NaCl, and 1 mM EDTA. Lysis buffer was supplemented with either separate protease and phophatase inhibitors (5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na3VO4, 2 mM PMSF, and 10 mM Na4P2O7) or with Pierce Protease Inhibitor Mini Tablets (Thermo Fisher Scientific). Lysates were centrifuged for 15 min at 16,000 x g and protein concentration of the recovered supernatants was measured with Bradford assay (Bio-Rad).

Immunoprecipitation was performed using 1–2 mg of total protein by incubating the lysates overnight at +4 °C with target specific antibodies. Subsequently, the lysates were incubated with Protein G PLUS-Agarose beads (sc-2002, Santa Cruz Biotechnology) for 60-90 minutes and the beads were washed three times with lysis buffer. Elution and denaturation of the precipitated proteins was carried out by heating the beads in Laemmli buffer at 95 °C for five minutes.

For Western analyses, lysates were heated at 95 °C for five minutes before loading to 8-15% polyacrylamide gels. Proteins were separated on the gels with electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were blocked with a solution of 5% milk or bovine serum albumin (BSA) in TBS-T solution (50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% Tween-20) and incubated overnight with the primary antibodies. After incubation of the membranes with horseradish peroxidase (HRP)-conjugated (Santa Cruz Biotechnology) or IRDye secondary antibodies (LI-COR)), signals were detected either with WesternBright ECL (enhanced chemiluminescence) HRP substrate reagent (Advansta) and imaging with ImageQuant LAS 4000 (GE Healthcare Life Sciences) for HRP-conjugated secondary antibodies, or imaging with Odyssey imaging system (LI-COR) for IRDye antibodies. Densitometric quantitation of the signals was performed with Fiji/NIH ImageJ v. 2.0.0.

Total protein levels of ROR1 and ROR2 were studied in HL-1 cells and H9c2 cells after hypoxia-reoxygenation treatments (I). Cell lysates were collected after hypoxic treatment for one, three or 24 hours, and additionally after subsequent reoxygenation for three or 24 hours and were analyzed by Western blotting. ROR1 phosphorylation in HL-1 cells after one-hour hypoxia, and additionally after 24-hour reoxygenation, was studied with immunoprecipitation of ROR1 and subsequent Western blot analysis of tyrosine phosphorylation (I). Moreover, total protein levels and phosphorylation levels of signaling proteins Akt and p38 were studied in HL-1 cells after siRNA-mediated knockdown of ROR1 (cell lysates collected 48 hours after transfection), and after Wnt-5a treatment at 400 ng/ml for 30 or 60 minutes (I). Lysates were analyzed with Western blotting. Finally, ROR1 total protein levels in pig myocardium samples (both normoxic and ischemia-reperfusion-injured) were studied with direct Western blotting.

Total protein levels of TRIM55, p21, p62, SRF, cTnI and ubiquitinated proteins were studied in CRISPR-edited HL-1 cell lines carrying *Trim55* variants (III). Lysates were collected three days after plating and analyzed with Western blotting. Additionally, ubiquitination of SRF and p62 was studied in the edited cell lines with immunoprecipitation of the target proteins, followed by Western analysis of poly- and monoubiquitinated proteins (III). MG132 was used on wild-type HL-1 cells at 50 or 100 μM for 3 or 5 hours before cell lysis to block proteasomal degradation of ubiquitinated proteins to demonstrate specifity of the anti-ubiquitin antibody (III).

## 4.6 Cellular viability assays (I-III)

CellTiter-Glo (CTG) Luminescent Cell Viability Assay (Promega), measuring ATP content of the cells, and CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT), measuring metabolic activity of the cells by assessing reduction activity,

were used to quantitate viability and proliferation of HL-1 cells. Assays were performed on 96-well or 384-well plates according to manufacturer's instructions. Wells containing only growth medium were used for background subtraction.

In the experiments using siRNA-mediated knockdown of ROR1 or ROR2 (I), HL-1 cells were exposed to hypoxia for 24 hours one day after transfection, followed by 24-hour reoxygenation, or grown in normoxia as controls before viability measurements. To study the effect of Wnt-5a on the viability of HL-1 cells (I), Wnt-5a was administered to the cells at 200 or 400 ng/ml at the time of plating for 72 hours. The cells were grown in normoxia as controls or exposed to hypoxia one day after plating for 48 hours, followed by 24-hour reoxygenation before viability measurements.

Gefitinib, used in chemical validation experiments (II), was administered to HL-1 cells at the time of plating at concentrations ranging from 1.6 nM to 5 μM. The following day, the cells were exposed to 24-hour hypoxia treatment, followed by 24-hour reoxygenation, or were grown in normoxia as controls, before viability measurement.

Viability of CRISPR-edited HL-1 cells (III) was assessed three days after plating. TRIM55-transfected cells were let to grow for two days after sorting before viability measurements.

## 4.7 Pig model of myocardial ischemia-reperfusion injury (I)

Pig experiments were approved by the Laboratory Animal Care and Use Committee of the State Provincial Office of Southern Finland (license number: ESAVI/1167/04.10.03/2011). Drs. Christopher Stark and Timo Savunen performed the pig experiments and kindly provided the myocardium samples. Global myocardial ischemia-reperfusion injury was produced by exposing anesthetized pigs (n = 4) to cardiopulmonary bypass with aortic cross-clamping and cardioplegia for one hour, followed by reperfusion for 29–31 hours in anesthesia, before sacrificing the pigs. Control pigs (n = 3) underwent similar anesthetic procedures. For anesthesia, infusion of propofol, midazolam and phentanyl was used and pigs were ventilated with a respirator. Additionally, pigs were administered cefuroxime as antibiotic prophylaxis, heparin, enoxaparin, lidocaine, amiodarone, bupivacaine, and noradrenaline. In the end of the procedures, myocardial samples were collected from left ventricles and snap-frozen. Hematoxylin-eosin staining was used for formalin-fixed, paraffin-embedded tissue samples to study the histological changes of the myocardium. Ischemic injury was confirmed with troponin T measurement (Elecsys Troponin T high sensitive, Roche).

## 4.8 Phospho-RTK array analysis of pig myocardium samples (I)

Pig myocardium samples were analyzed with a phospho-RTK array kit (Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems)) targeting 49 human RTKs. Samples were homogenized and lysed with a lysis buffer provided with the kit, and the analysis was performed according to manufacturer's instructions. Phosphorylation signals were quantitated with ImageJ software. Phosphorylation status of individual RTKs was compared between normoxic controls and ischemia-reperfusion injury samples.

## 4.9 RNA extraction, RNA-sequencing and real-time RT-PCR (I, III)

Total RNA from pig myocardium samples (I), CRISPR-edited HL-1 cell lines (III), or zebrafish embryos (III) was extracted with either TRIsure reagent (Bioline), RNeasy Mini Kit (Qiagen), or with NucleoSpin TriPrep kit (Macherey-Nagel). Samples were treated with DNase I to reduce genomic DNA carryover.

Genome-wide, strand-specific RNA-sequencing (RNA-seq) of CRISPR-edited HL-1 cell lines was performed at FIMM sequencing unit using HiSeq 2500 System (Illumina) with target coverage of 6 million reads per library (III). Quality control of the results was performed with Bluebee platform (Lexogen Gmbh). For differential expression (DE) analysis, raw read counts (excluding genes without more than one count per million per each replicate in at least one cell line) were analyzed with DESeq2 package for R. Absence of batch effects between biological replicates was confirmed with principal components analysis (PCA). Significantly enriched pathways among the differentially-expressed genes were analyzed with gene set enrichment analysis (GSEA) using fgsea package for R.

For generation of cDNA libraries, 200-1000 ng of total RNA was included in reverse transcription reaction, using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) or SensiFAST cDNA Synthesis Kit (Bioline). In real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of pig myocardium samples, Universal Probe Library (UPL) probes (Roche) were used in the reactions with TaqMan Universal Master Mix II (Thermo Fisher Scientific). A QuantStudio 12 K Flex Real-Time PCR System thermal cycler (Thermo Fisher Scientific) was used to carry out the reactions. For HL-1 cell line and zebrafish embryos samples, reactions were performed with a CFX384 Touch Real-Time PCR Detection System (Bio-Rad), using iQ SYBR Green Supermix (Bio-Rad) which requires no probes as accumulation of PCR products is detected with SYBR Green I dye. All reactions were performed with technical triplicates per each biological sample. Used primers and probes are listed in Table 7.

**Table 7.** Primer and probes used in RT-PCR reactions. Probe numbers refer to UPL catalog numbers by Roche.

| TARGET | SPECIES | LEFT PRIMER | RIGHT PRIMER | PROBE | USED IN |
|---|---|---|---|---|---|
| *Ror1* | pig | GCGGCTCGCAATATTCTC | GAAAGCCCAAGGTCTGAAATC | #108 | I |
| *Gapdh* | pig | ACAGACAGCCGTGTGTTCC | ACCTTCACCATCGTGTCTCA | #28 | I |
| *Adra2b* | mouse | CAACGAGCTGCTGGGCTATT | TGTCCAGACTGATGGCACACA | | III |
| *B2m* | mouse | ATTCACCCCCACTGAGACTG | TGCTATTTCTTTCTGCGTGC | | III |
| *Myl7* | mouse | TGACCCAGGCAGACAAGTTC | CGTGGGTGATGATGTAGCAG | | III |
| *Nppa* | mouse | TTCCTCGTCTTGGCCTTTTG | CCTCATCTTCTACCGGCATC | | III |
| *Trim55* | mouse | GAAGAGCATGAAGAGGAACG | CTATCGTTGCTTCCCACAAG | | III |
| *trim55a* | zebrafish | AGCTCATTTGCCCCATTTGCCTTGAG | CGCAGTAGATATTAATCCTCTCGTCCTGATGCTCT | | III |
| *rpl13a* | zebrafish | GGCGGACCGATTCAATAAGGTTCTGATCATTG | CCAGAGATGTTGATACCCTCACACCTCAC | | III |
| *TRIM55* | human | ATCTACAAGCAGGAGTCCACCAGGCCAG | TGAGCTCAGACTTCTGTCTCTGGAACACA | | III |
| *TRIM55* | human | AGGAGTCCACCAGGCCAGAAAAGAAATC | TGGAACACATGAGTGAGGGGAGCCA | | III |

## 4.10 ShRNA library screening on HL-1 cells (II)

A lentiviral shRNA library, DECIPHER Mouse Module 1 shRNA library (Cellecta) which targets 4,625 genes with 27,500 barcoded small hairpin RNAs (shRNA), was transduced to HL-1 cells according to manufacturer's instructions. Transductions were performed in triplicates. Half of the transduced populations were exposed to 96-hour hypoxia, which produced visible cellular death, followed by reoxygenation

for three hours. Half of the cell populations were used as controls grown in normoxia. DNA was extracted from the populations, and replicates within treatments were pooled due to low DNA yield. Barcoded shRNAs were quantitated from the DNA with next generation sequencing (NGS), performed by Cellecta. Data analysis is described in 4.18.

## 4.11 Drug library screening on HL-1 cells (II)

Drug library screening was purchased from Misvik Biology Inc. (Turku, Finland) as a service. In the screening, 689 FDA-approved drugs were plated at concentrations of 40, 200, 1000 or 5000 nM in 384-well plate wells at a density of 1,000 HL-1 cells / well. The following day, the plates were moved to hypoxic chamber for 48 hours, followed by reoxygenation for 24 hours. Control plates were kept in normal cell incubator for the same time. Cellular viability was measured with the CTG assay. Data analysis is described in 4.18.

## 4.12 Cell cycle analysis (III)

Cell cycle progression of CRISPR-edited HL-1 cell lines or cells transfected with TRIM55 plasmids was assessed with propidium iodide (PI) staining and flow cytometry. CRISPR-edited cells were analyzed two days after plating. GFP-positive transfected cells were sorted with FACS two days after transfection for analysis. Cells were trypsinized, pelleted, washed with PBS and rediluted in a solution of 0.1% Triton X-100 and 3.8 mM sodium citrate. Cells were treated with RNase A for 20 minutes and PI was added subsequently at the final concentration of 50 µg/ml. Sample analysis was performed with an Accuri C6 flow cytometer (BD Biosciences) at the HiLife Flow Cytometry Unit, University of Helsinki.

## 4.13 Immunofluorescence microscopy and analysis of hypertrophy (III)

Sarcomere structures of CRISPR-edited HL-1 cells were analyzed with immufluorescence microscopy of α-actinin filaments. Cells were plated on coverslips and were grown for three days before analysis as described below.

CRISPR-edited HL-1 cells or cells transfected with TRIM55 plasmids were stimulated with isoproterenol at the concentration of 100 µM for 48 hours to test their tendency for hypertrophy. Isoproterenol was administered to CRISPR-edited cells one day after plating. GFP-positive transfected cells were sorted one day after transfection and isoproterenol was administered after sorting.

For immunofluorescence analysis, cells were washed, fixed with 3% paraformaldehyde, permeabilized with 0.4% Triton X-100 and blocked with 2% BSA. Incubation with primary antibodies (710947 or MF 20) was carried overnight at +4 °C. Incubation with the secondary antibodies (Thermo Fisher Scientific) and simultaneous counterstaining of the nuclei with Hoechst dye, was performed for 45 minutes. Coverslips were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). α-actinin filaments from confocal microscopy images of 135-615 cells / cell line from two independent experiments were automatically detected and quantitated using Harmony 4.9 software (PerkinElmer) at the High Content Imaging and Analysis (HCA) unit at FIMM.

Hypertrophic response was assessed by measuring cell surface area using immunofluorescence microscopy after staining with anti-myosin antibody. Cell surface areas of 282–484 randomly selected cells per treatment from two independent experiments were manually traced and quantitated with FIJI/ImageJ.

## 4.14 Calcium transient measurements (III)

CRISPR-edited HL-1 cells plated on coverslips were stained two days after plating with calcium indicator dye fluo-4 AM (Invitrogen). Cells were kept in Tyrode's solution with 1.8 mM $CaCl_2$ while imaging spontaneous and paced calcium currents with a fluorescence microscope. Pacing was performed with electrodes generating bipolar pulses at frequencies of 0.5 and 2 Hz. Calcium transients were quantitated relative to the background fluorescence using HCImage (Hamamatsu) and Clampfit (Axon Instruments) softwares.

## 4.15 Zebrafish embryo experiments (II, III)

Pigment-deficient zebrafish (casper strain) were housed and maintained according to normal procedures under license MMM/465/712-93 (issued by the Finnish Ministry of Forestry and Agriculture) at the Zebrafish Core (Turku Bioscience Centre). Zebrafish embryos were cultured in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28.5 °C.

In the ischemia-reperfusion model (II), embryos were placed to deoxygenized water three days after fertilization for 15 or 30 minutes and returned to normal growth conditions for 24 hours for reoxygenation before readout measurements. Embryos were treated with gefitinib (500 or 5000 nM) or afatinib (5, 50, 500 or 5000 nM) administered to the water one hour before hypoxic exposure.

To study the effect of *trim55a* knockout in zebrafish embryos (III), three *trim55a*-targeting sgRNAs (Table 8) were injected either separately or pooled together into 1–4-cell stage embryos together with recombinant Cas9. *Slc45a2*-

targeting sgRNAs were used as negative controls. SgRNAs were synthesized with SgRNA synthesis kit (New England Biolabs) using the oligonucleotides depicted in Table 8. *Trim55a* knockout efficacy was confirmed with sequencing of the edited locus, RT-PCR and Western blotting. To address the effect of human TRIM55 WT or TRIM55 E140K overexpression in the embryos (III), expression plasmids (4.2) were injected together with *in vitro*-transcribed Tol2 mRNA into 1–4-cell stage embryos. Overexpression in the heart was confirmed with fluorescence microscopy for the GFP control plasmid. Additionally, presence of *TRIM55* transgenes in the genomic DNA was confirmed by PCR and their mRNA expression with RT-PCR.

Fractional shortening or ejection fraction of the heart and heart rate were measured with videomicroscopy at the end of 24-hour reoxygenation for ischemia-reperfusion experiments. Measurements were performed four days after fertilization for embryos with *trim55a* knockout or TRIM55 overexpression. Quantifications were performed with FIJI/ImageJ.

**Table 8.** Oligonucleotides used to synthesize the sgRNAs in the zebrafish embryo knockout model (III)

| TARGET | SEQUENCE |
|---|---|
| *trim55a* | TTCTAATACGACTCACTATAGCTCCCCGTGTGGGTAGGTAGGTTTTAGAGCTAGA |
| *trim55a* | TTCTAATACGACTCACTATAGGGCTCTTCACTGGGCTCCGGGTTTTAGAGCTAGA |
| *trim55a* | TTCTAATACGACTCACTATAGCATGTCTGCAGGAGGGACAGGTTTTAGAGCTAGA |
| *slc45a2* | TTCTAATACGACTCACTATAGGTTTGGGAACCGGTCTGATGTTTTAGAGCTAGA |
| *slc45a2* | TTCTAATACGACTCACTATAGTTCTCATCGGGATAAAGAGGTTTTAGAGCTAGA |

## 4.16 Database analyses (I, III)

A few publicly available databases were used in this study. Messenger RNA expression levels of 52 RTKs were studied in the Affymetrix expression data from samples representing healthy heart (n = 62), ischemic cardiomyopathy (n = 63) or acute myocardial infarction (n = 12), available from IST Online database (Medisapiens Ltd., ist.medisapiens.com) (I). Expression of the RTKs between different disease states were compared with Student's t-test, followed by *P*-value

correction with false discovery rate (FDR) method (Benjamini & Hochberg 1995). RTK protein sequence conservation between human and pig was addressed by aligning the RTK amino acid sequences available at UniProt database with EMBOSS Needle and EMBOSS Water tools by the European Bioinformatics Institute (I) (Rice et al. 2000).

Genotype and health status data from the FINRISK study cohort were used for GWAS of heart failure-associated genetic variants (III). FINRISK is a nationwide health survey study initiated in 1972 by the Finnish Institute for Health and Welfare, with new cohorts added every five years (Borodulin et al. 2015). Data from cohorts of the years 1992, 1997, 2002 and 2007 were used in this study. After genotype imputation and removal of data not passing the quality thresholds, 20,118 controls and 994 heart failure patients were included in the analysis. Association of genetic variants with heart failure was tested with logistic regression. Tests were corrected for age, gender, smoking status, hypertension treatment, lipid lowering medications, diabetes, and 10 first principal components. Coding, functional variants in high linkage disequilibrium with the lead variants from GWAS were searched for using PLINK software. Functionality of coding variants was estimated with Variant Effect Predictor software.

## 4.17 3D modeling of TRIM55 (III)

3D models of TRIM55 were generated at the Structural Bioinformatics Laboratory, Åbo Akademi University. After sequence comparisons between TRIM55 and entries in the Protein Data Bank, X-ray structures of B-box 2 domain of TRIM63 were used as templates for modeling the TRIM55 B-box 2 (residues 119-163). Two dimeric models of TRIM55 B-box 2 were generated with the MODELLER software. QMEAN software was used to assess the quality of the models and structural comparisons were performed with VERTAA software. Structural model images were prepared with PyMOL.

## 4.18 Statistical analyses

Experimental data in the original publications are presented as barplots (mean as the bar height and standard error of the mean or standard deviation as error bars); as boxplots (median, red or black horizontal line; first and third quartile, box; range of the data, whiskers; and additionally individual values as overlaid dots for some analyses); as volcano plots; or as dotplots (with median indicated with horizontal line). Normality of the data was assessed with Shapiro-Wilk test and equality of variances with Bartlett's test. Multiple comparisons were performed with one-way analysis of variance (ANOVA) (for normally distributed data) or with Kruskall-

Wallis test (for non-normally distributed data). Two-group comparisons after multiple comparison tests were performed with two-tailed Student's t-test (for normally distributed data) or with Mann-Whitney U-test or Dunn's post-hoc test (for non-normally distributed data). Multiple testing corrections were performed with false discovery rate (FDR) method. Heatmaps and clustering analyses were generated with heatmap.2 function or pheatmap package for R. Statistical analyses were performed with RStudio software. RTK expression analyses from IST Online database (I), DE and GSEA analyses for RNA-seq results (III), and logistic regression analyses (III) are desribed above.

For quantitation of enriched and depleted shRNAs by hypoxia-reoxygenation in the shRNA library screening (II), separate shRNAs targeting the same gene were used as replicates in the analyses. Differences in abundances of shRNAs were assessed with three statistical methods (nonparametric quantile regression (Koenker et al. 2019), DESeq2 (Love et al. 2014) and NBPSeq (Di et al. 2014)). Mean rank of a gene calculated from ranks from all three methods was used as a measure of significance for differential expression. Mean rank of 500 was used as a cutoff.

For the analysis of the drug library screening (II), the highest relative luminescence value of CTG measurements in cells treated with different concentrations of each drug in hypoxia-reoxygenation was used as a representative value for each drug. Drugs inducing representative values of one standard deviation higher than the value for DMSO control-treated cells in the hypoxia-reoxygenation were considered to significantly increase cellular viability.

Mutual interactions of significant hits from drug and shRNA library screens were analyzed with protein-protein interaction analysis (II). Protein targets of the drugs were acquired from the DrugBank database (Wishart et al. 2006). Drugs were categorized as agonists or antagonists according to information in the Drug Gene Interaction Database (Cotto et al. 2018). Experimentally validated protein-protein interactions were acquired using the PSICQUIC package (Shannon 2019). Network graphs were generated by applying the protein-protein interactions to the lists of significantly depleted or enriched shRNAs in hypoxia-reoxygenation, or targets of antagonist or agonist drugs which significantly improved cell survival in hypoxia-reoxygenation.

For pathway analysis of significant drug and shRNA targets, all pathway annotations available at the Molecular Signature Database (Liberzon et al. 2011) were used. All significant hits from the drug library screening, as described above, were included. For the shRNA library screening, median normalized counts of shRNAs targeting the same gene were calculated. Genes with at least 30% enrichment of the shRNAs (according to median count values in the barcode sequencing data) in hypoxia-reoxygenation, compared to normoxic controls, were included. Maximally collapsing metric learning (MCML) (Globerson & Roweis

2005; van der Maaten 2009) method was used to calculate the dimensionality-reduced value for a pathway in control and hypoxia-reoxygenation samples from median-normalized shRNA counts in the barcode sequencing data or representative relative luminescence values. Significance score of a pathway was calculated from the difference between dimensionality-reduced pathway values of control and hypoxia-reoxygenation samples. Statistical significances of the pathway significance scores and of the fitness of each pathway to the data were calculated by randomly choosing protein sets with similar length and fitting a probability distribution function with an Epanechnikov kernel function to the scores of the randomized protein sets. *P*-values were inferred from the cumulative distribution function of the scores.

# 5 Results

## 5.1 Several RTKs are active and regulated in hypoxic heart (I)

RTKs are a potential group of druggable targets for myocardial ischemia-reperfusion injury, as they are involved in signaling processes governing cellular survival and proliferation. Knowledge on RTK activation and regulation in hypoxic or ischemic heart has been scattered and limited to a relatively small subset of the family members. Mainly ErbB2 and ErbB4 have been shown to be robustly involved in ischemic cardioprotection and cardiac regeneration (Bersell et al. 2009; D'Uva et al. 2015; Wang et al. 2018). We studied the mRNA expression of RTKs in ischemic human heart in a family-wide screen using the data available from IST Online database. In acute myocardial infarction, expression of four RTK genes, namely *EGFR*, *ERBB2*, *ERBB3* and *EPHA2* were found to be downregulated compared to healthy heart (I, Fig. 1C). As expected, ErbB receptors were among the RTKs implicated in ischemic injury of the heart.

In our analysis of RTK mRNA expression in hearts with ischemic cardiomyopathy, several RTKs including EPH receptor genes and *ERBB3* were downregulated, while *ROR1*, *KIT* and *TIE1* were upregulated (I, Fig. 1A) when compared to healthy heart. Noteworthy, *EPHA2* was again downregulated as in acute ischemia. Interestingly, while ROR1 was the most significantly upregulated gene, *ROR2* expression was downregulated (I, Fig. 1A). Although ROR receptors are involved in cardiac development, no role for them has been demonstrated in myocardial ischemia before.

Phosphorylation of RTKs is a major event in their activation but RTK phosphorylation in ischemic heart has not been studied systematically at the level of the whole RTK family. To address this, pig myocardial samples with ischemia-reperfusion injury were studied with a phospho-RTK array which covers 49 RTKs. Among the most highly phosphorylated RTKs, EGFR, INSR and several EPH receptors were included, and additionally e.g. ErbB2 phosphorylation was detected (I, Fig. 2). Two receptors demonstrated significant differences between controls and ischemia-reperfusion, as phosphorylation of VEGFR2 was increased and that of ROR1 was decreased in ischemia-reperfusion (I, Fig. 2A). Our result for VEGFR2

are in line with the observation that VEGFR2 is necessary for VEGF-mediated cardioprotection (Messadi et al. 2014). Interestingly, ROR1 phosphorylation was downregulated (I, Fig. 2A and 2B), while its mRNA expression was increased in ischemic cardiomyopathy (I, Fig. 1A). However, in the direct Western blot analysis of ROR1 protein expression and RT-PCR analysis of *ROR1* mRNA expression in pig myocardium, no significant differences between controls and ischemia-reperfusion-injured samples were observed (I, Fig. 3).

## 5.2 ROR1 is downregulated in acutely hypoxic heart and its knockdown improves cardiomyocyte viability in hypoxia-reoxygenation (I)

Further characterization of the role of ROR1 in myocardial ischemia was performed in HL-1 and H9c2 cell lines. A short, one-hour exposure to hypoxia decreased ROR1 total protein levels in both cell lines (I, Fig. 4). In the analysis of ROR1 phosphorylation with immunoprecipitation and subsequent Western blotting of phosphotyrosines in HL-1 cells, decreased phosphorylation of ROR1 was detected, both after hypoxia for one hour, as well as after one hour hypoxia followed by reoxygenation for 24 hours (I, Additional File 6). This observation was concordant with the decreased ROR1 phosphorylation in pigs after one-hour ischemia and approximately 30-hour reperfusion period (I, Fig. 2).

To test whether the observed downregulation of ROR1 had functional significance, viability of HL-1 cells was assessed, both in normoxia and in hypoxia-reoxygenation setting, after siRNA-mediated knockdown of ROR1. ROR1 knockdown improved the viability of the cells (I, Fig. 5A), suggesting that the ROR1 downregulation has protective potential. Concordantly, administration of Wnt-5a, a ligand to ROR1, decreased the viability of the cells (I, Fig. 5C). Phosphorylation of Akt and p38 was decreased by ROR1 knockdown and increased by Wnt-5a treatment, suggesting that these intracellular signaling proteins are involved in mediating the effects of ROR1 signaling (I, Fig. 5B and 5D). The activity of p38 has been shown to suppress cardiomyocyte proliferation (Engel et al. 2005). Here, it was not assessed whether the difference in cellular viability was due to differences in proliferation or cell death. Taken together, however, these findings indicate that ROR1 signaling suppresses cardiomyocyte viability.

## 5.3 Combined shRNA and drug screening reveals novel potential targets for ischemia-reperfusion injury (II)

To search for novel druggable targets for ischemia-reperfusion injury in a high-throughput setting, genetic screening with an shRNA library, targeting 4,625 genes, and drug screening with a drug library, consisting of 689 drugs, were performed on HL-1 cells exposed to hypoxia and reoxygenation. Results were analyzed separately for both screens using a protein-protein interaction (PPI) analysis among the significant hits and a pathway analysis to reveal centrally involved signaling pathways. Both screening methods identified EGFR as the most interconnected node in the PPI analyses, and in both screenings, EGFR inhibition was found to be protective for cardiomyocytes (II, Fig. 2A and 2C). *EGFR*-targeting shRNAs were enriched by hypoxia-reoxygenation (II, Fig. S1) and EGFR inhibitors gefitinib and erlotinib enhanced cellular survival in hypoxia-reoxygenation (II, Tables S1 and S2). Noteworthy, an ACE inhibitor (ramipril) and an ARB (azilsartan) were also detected as compounds supporting cardiomyocyte survival (II, Tables S1 and S2).

Concordant hits from the two screens were identified as follows: If shRNAs targeting a gene were significantly enriched in hypoxia-reoxygenation, and if an antagonist drug targeting the product of the same gene significantly improved cellular viability in hypoxia-reoxygenation, the target was regarded as a concordant hit. Similarly, if shRNAs targeting a gene were significantly depleted in hypoxia-reoxygenation and a significant improvement in viability with an agonist drug targeting a product of the same gene was observed, the gene was also regarded as a concordant finding. Inhibition of the following targets was found out to concordantly improve cardiomyocyte survival in hypoxia reoxygenation: *EGFR*, *PRKACA* (catalytic subunit α of protein kinase A) and *CA2* (carbonic anhydrase 2). Agonism of the following targets was found out to concordantly improve cardiomyocyte survival in hypoxia reoxygenation: *AR* (androgen receptor) and *VDR* (vitamin D receptor).

Furthermore, EGFR-related pathways were significantly enriched in the pathway analyses of both screens (II, Tables 1 and 2). Altogether, both screening methods suggested EGFR inhibition as cardioprotective in hypoxia-reoxygenation.

## 5.4 EGFR inhibitor gefitinib preserves cardiac function in ischemia-reperfusion injury (II)

To validate EGFR as a therapeutic target for ischemia-reperfusion injury, gefitinib was chosen as the candidate drug. Gefitinib increased the cellular viability of HL-1 cells in hypoxia-reoxygenation setting (II, Fig. 3A). To test the activity of gefitinib *in vivo*, a zebrafish embryo model of ischemia-reperfusion was used. After 15

minutes of ischemic exposure followed by reoxygenation for one day, a significant decrease in cardiac contractility was observed in control embryos (II, Fig. 3C). Administration of gefitinib in the water at concentrations of 0.5 and 5 μM before the ischemic exposure protected the hearts from the decrease in contractility (II, Fig. 3C). No differences in heart rate were observed. A longer, 30-minute ischemic exposure was lethal to a significant fraction of the embryos, and reduction in contraction was significantly prevented by gefitinib administered at 5 μM (II, Fig. S2A). The specificity of the effect of gefitinib and validity of the model was controlled by using afatinib, an inhibitor of EGFR, ErbB2, and ErbB4, which is potentially cardiotoxic through ErbB2 inhibition (Nemeth et al. 2017; Schuler et al. 2012). Afatinib administration decreased the cardiac contractility and heart rate in ischemia-reperfusion at concentrations ranging from 50 to 5000 nM (II, Fig. S2C–D). EGFR inhibition with gefitinib was thus validated as a potential mechanism to prevent ischemia-reperfusion injury.

## 5.5 E140K variant of TRIM55 is linked to risk of heart failure (III)

As heritability of heart failure risk is evident (Lee et al. 2006; Lindgren et al. 2018) and as the number of identified causative genetic variants is limited (Shah et al. 2020) we aimed to search for novel genetic variants associated with heart failure in the FINRISK study population. Logistic regression analysis detected a few variants with significant association to heart failure. To identify potential causative variants which may affect the heart muscle itself, i.e. coding, damaging mutations in genes highly expressed in cardiomyocytes, variants in high linkage disequilibrium (LD) with the variants most significantly associated with heart failure were assessed. This analysis revealed a variant in the *TRIM55* gene, rs138811034 in total LD with an intronic variant in chromosome 8 (rs117667921), highly associated with heart failure ($P = 2.45 \times 10^{-7}$) (III, Fig. 1A). Rs138811034 encodes an exonic variant, causing an amino acid change E140K (glutamate 140 to lysine) in the protein (III, Fig. 1B). This was predicted to be a likely damaging variant with the Variant Effect predictor software. Additionally, *in silico* 3D models indicated that the variant potentially affected the surface charge and dimerization of the TRIM55 protein (III, Fig. 1B–C). Although the E140K variant was not independently associated with heart failure in the cohort, its linkage to the lead variant rs117667921 and its predicted damaging role warranted further functional validation.

## 5.6 TRIM55 E140K affects cardiomyocyte-specific functions (III)

To functionally validate the role of TRIM55 E140K in cardiomyocytes, HL-1 cells carrying the *Trim55* variant in the genome were generated using the CRISPR/Cas9 technique. The cell lines used for experimentation included a cell line with a knock-in of the E140K allele together with a deletion allele (E140K/–), and a homozygous (–/–) and heterozygous (WT/–) *Trim55* knockout cell lines (III, Fig. 2). In whole transcriptome-RNA-seq analysis, E140K/– cell line clustered together with the –/– cell line when analyzed according to the most highly differentially expressed genes (III, Fig. 3A). Significantly affected pathways in E140K/– cells included pathways related to muscle contraction and sarcomere structures (III, Fig. 3B). Expression of *Nppa* encoding ANP, a typical heart failure and stress marker, was higher in E140K/– cells compared to WT/WT cells (III, Fig. 3D). E140K/– cells also demonstrated reduced viability and impaired cell cycle progression when compared to wildtype cells (III, Fig. 4A and 4C), and a significant hypertrophic response to isoproterenol (III, Fig. 4D and 4E). Similarly, the –/– and WT/– cells had a significant hypertrophic response to isoproterenol, while WT/WT cells did not (III, Fig. 4D and 4E).

The effect of the variants on ubiquitination of known TRIM55 target proteins was assessed with immunoprecipitation and Western blotting. While reduced protein levels of SRF and p62 were observed in E140K/– cells (III, Fig. S6A and S6B), their ubiquitination could not be detected (III, Fig. S6D and S6E). No differences in total ubiquitination level of the cells were detected, either (III, Fig. S6F). Moreover, E140K/– cells demonstrated increased expression of cardiac α-actinin (III, Fig. S7A) and increased calcium transient amplitudes when paced with 0.5 Hz frequency (III, Fig. S7F).

As *Trim55* E140K/– cells seemed to have a different phenotype from a total knockout of *Trim55*, suggesting a dominant negative effect, activities of the variant were tested with plasmid overexpression of human TRIM55 in HL-1 cells. TRIM55 E140K overexpression reduced the cellular viability, compared to WT overexpression (III, Fig. S8B). Cells overexpressing TRIM55 WT did not have a significant response to hypertrophic stimulus, while TRIM55 E140K-transfected and control cells did (III, Fig. S8D and S8E). Thus, while expressed together with endogenous wild-type TRIM55, TRIM55 E140K produced a different phenotype as compared to overexpression TRIM55 WT, supporting the concept of a dominant negative effect.

To test the effect of the variants *in vivo*, a zebrafish embryo model was used. First, knockout of endogenous *trim55a* gene with CRISPR was shown to reduce cardiac contractility in embryos four days after fertilization (III, Fig. 5B). Heart-specific plasmid overexpression was used to specifically test the effect of the E140K

variant. Both overexpression of E140K variant alone as well as in combination with TRIM55 WT, mimicking a heterozygous state, reduced cardiac contractility (III, Fig. 5D). Additionally, increase in heart rate was observed in E140K-transfected embryos (III, Fig. 5D).

# 6 Discussion

## 6.1 Novel therapeutic targets for ischemia-reperfusion are needed

Although many therapeutic approaches to prevent myocardial ischemia-reperfusion injury have been assessed, none of them have this far been successfully translated into clinical practice. One potential reason for the failure of translation is that cardiomyocyte death can be triggered via multiple, redundant mechanisms which could be acted upon synergistically with multitarget approaches (Davidson et al. 2019; Hausenloy et al. 2017b). In addition to combining the existing cardioprotective modalities, novel therapeutic targets are needed, as they could provide an additional mechanism to prevent cellular death (Hausenloy et al. 2017b).

## 6.2 Novel therapeutic targets for ischemia-reperfusion can be identified with preclinical screening approaches

Chemical and genetic screens on cell models are widely used e.g. in cancer research to identify novel drugs or druggable targets but they have been rarely utilized in the context of heart diseases. High-throughput drug screening methods on cardiomyocytes have been used earlier on H9c2 cells to search for cardioprotective agents against ischemia-reperfusion injury (Guo et al. 2012) and for compounds protecting from cardiotoxicity of doxorubicin (Gergely et al. 2015). Human iPSC-derived cardiomyocytes have been used to identify cardiotoxicity of TKIs (Sharma et al. 2017). Screening with gene silencing, e.g. with RNA interference or CRISPR techniques, is another high-throughput screening method, extensively used e.g. in cancer research (Diehl et al. 2014; Fredebohm et al. 2013). There are no prior publications where such methods have been used in heart diseases such as ischemia-reperfusion injury.

Large-scale preclinical screening methods are an efficient way of identifying potential molecules taking part in disease pathogenesis. Here, we applied multiple different screening methods to search for therapeutic targets of myocardial ischemia-reperfusion. First, the family of RTKs was systematically studied with mRNA

expression data and with a phosphorylation array. Secondly, the drug and shRNA library screenings were used together to cover thousands of targets. These analyses indicated that inhibition of either ROR1 or EGFR could represent novel approaches to prevent ischemia-reperfusion injury.

## 6.3 RTK family members are differentially regulated by ischemia

Regulation of RTK expression at mRNA or protein level in ischemic heart is poorly characterized, although certain RTKs have been studied in more detail, such as ErbBs (Bersell et al. 2009; D'Uva et al. 2015; Munk et al. 2012; Wang et al. 2018) and EPHA2 (Dries et al. 2011; O'Neal et al. 2014). Our mRNA expression analysis of RTKs in ischemic heart indicated downregulation of *EGFR*, *ERBB2*, *ERBB3* and *EPHA2* mRNA expression in acute myocardial infarction. For *EGFR*, this is contradictory to the finding by Munk et al. who reported upregulation of *EGFR* expression in hypoxic hearts of patients undergoing coronary artery bypass grafting (Munk et al. 2012). This difference could be due to the fact that the samples analyzed by Munk et al. were from hearts with chronic ischemia, as compared to acutely ischemic samples in our analyses. On the other hand, both the results by Munk et al. and our analyses demonstrated *ERBB2* downregulation in ischemic heart, in accordance with the observed decrease in ErbB2 protein expression in human ischemic cardiomyopathy (Gordon et al. 2009). Moreover, *EPHA2* expression, along with *EPHA1*, *EPHA3* and *EPHA7*, has been shown to be increased after ischemia-reperfusion injury in mice (Dries et al. 2011), as opposed to our findings. Altogether, these discrepancies point to the importance of sampling time after ischemia-reperfusion injury. The same receptors might undergo different adaptive regulatory events at different timepoints. Differences between species are also an important factor to consider.

In the RTK mRNA expression analysis in ischemic cardiomyopathy, both up- and downregulation of RTK expression was observed. Interestingly, expression was regulated in opposite directions for *ROR1* and *ROR2*. Expression of ROR1 was upregulated while expression of ROR2 was downregulated. RTK gene expression can thus be either upregulated or downregulated in ischemic heart, and it could reflect either protective responses or pathological adaptations.

Our family-wide screening of RTK phosphorylation in ischemic heart is a systematic approach that has not been used this context before. Certain aspects of the screening method should, however, be considered. As the array was designed to detect human receptors, its functionality on pig samples is debatable. To address this, we analyzed the similarity of RTK protein sequences between human and pig and the overall similarity was observed to be high (93.1%). However, direct inferences

from the observed phosphorylation level or its absence cannot be made with certainty while the comparisons of levels between control and ischemia-reperfusion samples are more reliable. On the other hand, the samples were collected approximately 30 hours afer reperfusion, a timepoint at which the changes in phosphorylation might already be over. Significantly decreased phosphorylation of ROR1 in ischemia-reperfusion samples was an intriguing finding as ROR1 expression was the most significantly upregulated RTK in ischemic cardiomyopathy. These differences could be due to different disease state (acute vs. chronic ischemia) and the fact that mRNA expression and phosphorylation are differentially regulated.

## 6.4 ROR1 inhibition is potentially cardioprotective in ischemia-reperfusion injury

The functional role of ROR1 was studied in HL-1 cells. Its knockdown increased the viability of the cells both in normoxia and hypoxia. Consistently, opposite effects were observed upon treatment with Wnt-5a, an activating ligand of ROR1. Wnt-5a is, however, not an exclusive ligand for ROR1 as it can also activate e.g. ROR2, RYK and Frizzled receptors (Oishi et al. 2003; Park et al. 2015; Yoshikawa et al. 2003). In accordance with our findings, inhibitors of Wnt-5 and Wnt-3 have been shown to reduce infarct size (Laeremans et al. 2011; Yang et al. 2019) and promote cardiomyocyte proliferation in mice (Yang et al. 2019). These effects are potentially partly mediated by ROR1. ROR1 inhibition with the targeting antibody cirmtuzumab, originally developed for the treatment of chronic lymphocytic leukemia (Choi et al. 2015), is a therapeutic approach worth assessing in the setting of myocardial ischemia-reperfusion.

Taken together, ROR1 is a key example of an RTK which can exert harmful effects on ischemic heart. This is an unexpected finding as RTKs are often implicated in cellular survival and their activity in ischemic heart has previously been reported to be mainly beneficial. These findings emphasize the multifaceted signaling networks RTKs are involved in and that the net effect of a single receptor's activation can be either desirable or detrimental for the outcome of the disease. Interestingly, we observed similarly beneficial effects of inhibiting EGFR activity in cardiomyocytes, as discussed below.

## 6.5 Role of EGFR differs from that of ErbB2 and ErbB4 in the pathophysiology of ischemia-reperfusion injury

While ErbB2 and ErbB4 are cardioprotective in ischemia-reperfusion injury as discussed above, the role of EGFR is more complex. Although a lot of evidence

indicates that EGFR transactivation by GPCRs is a necessary event e.g. in ischemic preconditioning and cardioprotection by multiple compounds (Förster et al. 2007; Methner et al. 2009; Williams-Pritchard et al. 2011) and that EGF administration protects ischemic heart (Akhtar et al. 2012; Lorita et al. 2010), contradictory evidence have shown the benefit of EGFR inhibition in myocardial ischemia (Liu et al. 2018; Mali et al. 2018). In our combined screening with an shRNA and a drug library, EGFR inhibition was one of the concordant hits from both screenings. Although ErbB receptors share a number of signaling pathways, the net effect of EGFR activity can be different from the effect of ErbB2 or ErbB4 activity. This is also seen in cardiac development, where ErbB2 and ErB4 both affect the development of cardiac trabeculation (Gassmann et al. 1995; Lee et al. 1995) while EGFR is involved in the development of cardiac valves (Chen et al. 2000). Speculatively, as EGFR-EGF and ErbB4-NRG-1 signaling axes could use same coreceptors and signaling cascades, inhibition of EGFR-EGF axis could free the common downstream mediators to be more available for ErbB4-NRG-1 signaling. Additionally, EGFR inhibition could potentially cause a reciprocal upregulation in expression or activity levels of other ErbB family members which could act in a cardioprotective manner. As gefitinib is a clinically used cancer drug, it is a suitable candidate for drug repurposing studies. To this end, further preclinical validations in a mammalian model are necessary. As gefitinib was administered here prior to ischemia, it should be assessed whether the cardioprotective activity also exists if gefitinib was administered during ischemia or reperfusion. If cardioprotection occurs only when administered before an ischemic event, it reduces the usability of gefitinib as an on-demand treatment for acute ischemia-reperfusion injury. In this case, however, prophylactic use as secondary prevention could be considered.

## 6.6 Functional characterization of the damaging TRIM55 E140K variant gives insight into its pathological role in heart failure

Cellular functions of TRIM55 have been characterized relatively extensively (Centner et al. 2001; Lange et al. 2005; Perera et al. 2011; Witt et al. 2005). Pathological role of genetic variants of *TRIM55* in heart failure has also been adressed earlier (Su et al. 2014) but their mechanistic characterization has been lacking. We analyzed the exonic coding variant of *TRIM55*, encoding E140K variant in the protein, and detected that it could modify cardiomyocyte-specific functions, cellular viability and cell cycle progression *in vitro*, as well as cardiac contractility *in vivo*. Interestingly, the variant was able to exert its effects even in the presence of a wild-type allele.

The fact that the variant demonstrated negative effects when expressed together with the wild-type allele may make the observation more clinically relevant, as most of the carriers of the variant are heterozygotes. Even though a wildtype allele is present, E140K variant in TRIM55 could supposedly interfere with sarcomeric assembly during heart development or with regulation of SRF-mediated transcription by TRIM55. The identified non-synonymous variants by Su et al. were regarded to more likely modify the penetrance and disesase severity of HCM, instead of being independent causal mutations (Su et al. 2014; Walsh et al. 2017). Similarly, the TRIM55 E140K variant might not be causative on its own but its presence together with e.g. a sarcomeric mutation could lead to increased susceptibility for heart failure. Genetic diagnostic methods of heart failure are mostly limited to cases of suspected or known familial cardiomyopathies when the prevalence of detectable mutations is high enough (Ponikowski et al. 2016). Increasing knowledge of genetic variants and cellular functions leading to heart failure is expected to lead to improvement of treatment modalities. Additionally, preventive measures could be taken on patients known to carry pathogenic mutations (Czepluch et al. 2018). Significance of TRIM55 E140K in heart failure pathogenesis should be confirmed in larger clinical studies. If it is confirmed to be significant in the clinical setting, it could be used in genetic screening of cardiomyopathies or heart failure, at least as a variant modifying the disease susceptibility or severity. Carriers of the variant could be monitored and treated more rigorously. As one of the findings of the effects of E140K variant in cardiomyocytes was increased hypertrophic tendency, the workload of the heart should presumably be kept as low as possible in the carriers of the variant. This could be done by highly intensive blood pressure lowering treatment and effective use of ACE inhibitors or ARBs.

# 7 Conclusions

Ischemia-reperfusion injury, occurring during myocardial infarction and its treatment, is an undertreated condition. There are opportunities to enhance outcomes of myocardial infarction by preventing ischemia-reperfusion injury. New approaches to prevent and treat the injury are needed.

Heart failure is another major factor contributing to heavy burden of heart diseases. Heritability of heart failure is currently not well known and also presents a ground for discoveries for better treatment and outcome.

This thesis work aimed to find novel therapeutic targets for ischemic heart disease and genetic markers for heart failure using large-scale screening methods to fill gaps in the knowledge of these fields.

The following conclusions can be made based on the results of the study:

1) ROR1 is a potential druggable target for the treatment of myocardial ischemia-reperfusion injury.

2) EGFR is a potential druggable target for ischemia-reperfusion injury. The clinically used EGFR inhibitor, gefitinib, should be assessed further as a candidate for drug repurposing.

3) Exonic E140K variant of *TRIM55* is a potential genetic modifier of heart failure risk.

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