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PPM1K, a novel regulator of metabolism and autophagy in the heart.

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Summary

The general aim of this work was to understand the relationship between mitochondria and autophagy, with a focus on heart biology. Autophagy is of extreme importance for cardiac function. Animal models of impaired autophagy display cardiac phenotypes at basal levels as well as when stressed. In this work we dissected molecularly two different faces of mitochondrial biology related to autophagy: BCAA-catabolism mediated regulation of mTORC1, and the role of the deubiquitinating enzyme USP8, involved in EGFR signaling and mitophagy, in heart mitochondrial function and more generally in heart function. Tissues can adapt to availability of different substrates by activating specific catabolic pathways that in most instances converge on mitochondria. Yet, how fluxes of metabolites through these organelles affect cellular processes is unclear. Here we show that PPM1K, a mitochondrial matrix protein phosphatase that controls the rate limiting step of branched chain amino acid (BCAA) catabolism, modulates mTORC1 activation and autophagy. PPM1K levels directly correlated with increased autophagy and reciprocally, PPM1K was induced upon starvation in vitro and in vivo, including in tissues where BCAA catabolism is considered marginal, like heart. Steady state metabolomics of labeled Leucine metabolites revealed that in the absence of PPM1K, TCA cycle intermediates were as expected decreased, whereas Leucine, its ketoisocaproic ketoacid and surprisingly methionine were increased,

potentially explaining the mTORC1 dependent autophagy inhibition. Our data suggest how mitochondrial BCAA catabolism can be sensed by mTORC1 to modulate autophagy.

Activating mutations in the USP8 gene, coding for ubiquitin-specific protease 8, a deubiquitinase involved in endocytic trafficking and mitophagy, can cause Cushing's syndrome. Usp8 inhibitors are therefore scrutinized to treat Cushing's pituitary adenomas. However, because heart function requires mitophagy, it is unclear if Usp8 inhibitors could be detrimental for the already failing hearts of Cushing's patients. Here we show that acute Usp8 genetic ablation in the mouse heart impairs mitochondrial function and autophagic clearance. Myocardial Usp8 deletion in adult mice resulted in cardiomyopathy associated with the accumulation of damaged and dysfunctional mitochondria. Mechanistically, we found that USP8 interacted with, and stabilized PINK1 that senses dysfunctional mitochondria and activates Parkin dependent mitophagy. Consequently, in cardiomyocytes and cells lacking USP8, PINK1 was not stabilized upon mitochondrial dysfunction, mitophagy was not activated in response to mitochondrial depolarization and chemical mitochondrial uncouplers led to cell death. Our data not only shed light on the mechanisms of mitophagy regulation, but also recommend caution in investigative anti Usp8 therapy for Cushing's syndrome.

INTRODUCTION

AUTOPHAGY

Cellular homeostasis is maintained through intricate mechanisms that control both the quantity and quality of molecules and organelles. Autophagy, a process in which proteins, lipids, and organelles are transported to lysosomes for degradation, is central in accomplishing this objective. Autophagy was originally described as a recycling mechanism to help cells cope with limited nutrient availability, but it is now clear that it is critical in multiple cellular functions, including quality control of proteins/organelles, defense against pathogens, immunity, avoidance of inflammation, metabolism, and the whole cell death and life.

The first observation of autophagy was reported by Thomas P. Ashford and Keith R. Porter, from the Rockefeller Institute. They observed in electron microscopy of rat hepatic parenchymal cells the presence of single-membranous organelles similar but distinct from lysosomes which contained cytoplasmic and organellar components. These organelles were observed to be increased after glucagon perfusion of the liver and were called “microbodies”. One year after, Alex B. Novikoff, at the Ciba Foundation Symposium on Lysosomes in London, described for the first time a mechanism by which cellular organelles and debris were degraded with the involvement of the so called “cytolysosomes”, a mechanism that he called “autolysis”. At the same symposium though, Christian De Duve clarified that the cytolysosomes where lysosomes and named the process “autophagy”. After these first observations and mainly in the 90’s the autophagy field grew up very fast by the independent discoveries of many groups that worked on the

budding yeast. Yoshinori Oshumi, Michael Thumm and Daniel J Klionsky identified and put together most of the autophagy related genes, which had different names before the unified nomenclature, advocated in 2003, of ATG genes (Klionsky *et al.*, 2003; Harnett *et al.*, 2017). The official recognition of autophagy as a crucial pathway for cellular life and death came with the Nobel Prize in Medicine of 2016 to Yoshinori Ohsumi for his seminal discoveries concerning this mechanism.

There are three main types of autophagy: Macroautophagy, microautophagy and chaperone mediated autophagy, based on the mechanism of transport of the material to be degraded to lysosomes (Figure 1). During macroautophagy (which is usually referred to as “autophagy”) double membranous vacuoles named autophagosomes are formed for cargo transport. They are afterwards fused with lysosomes resulting in the degradation of the cargo, which could be cytoplasmic material like proteins and lipids, but also whole organelles such as mitochondria. During microautophagy, portions of the cytoplasm are directly engulfed by invaginations of the lysosomes without a double membrane organelle to mediate the delivery. And finally, during Chaperone Mediated Autophagy (from now on referred as CMA), proteins that harbor a specific sequence (KFERQ-like motif) are specifically targeted to lysosomes through a different mechanism.

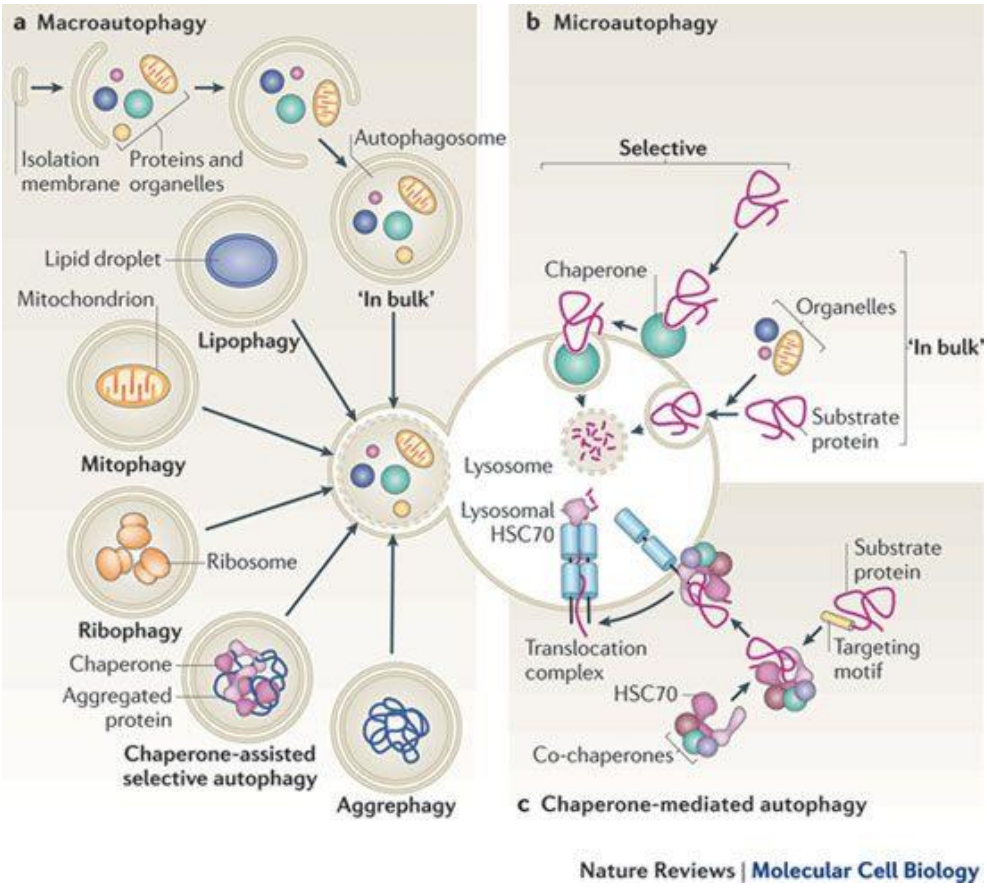

Figure1: Schematic representation of the different types of autophagy. In macroautophagy cytoplasm and/or organelles are engulfed in double membranous vesicles named autophagosomes. Autophagosomes then fuse with lysosomes and result in the degradation of the cargo. During Chaperone Mediated Autophagy, proteins harboring a KFERQ-like motif are directly targeted to LAMP2A lysosomes by the Hsc70 complex. In microautophagy the lysosomal membrane invaginates to engulf portions of the cytoplasm to broken down once entirely enclosed (Figure from Cuervo AM, 2011).

MACROAUTOPHAGY

The process of macroautophagy can be divided in six steps:

1. Induction/Initiation
2. Cargo selection and packaging-Selective autophagy
3. Vesicle nucleation
4. Vesicle expansion/elongation and completion
5. Vesicle targeting, docking and fusion with lysosomes
6. Vesicle breakdown for the recycling of macromolecules

1. Induction/Initiation of autophagy

Initiation of autophagy is triggered mainly by amino acid nutrient sensing (Efeyan, 2015). There are three main complexes that begin the autophagic process: the mTOR complex, the ULK1 complex and the class III Vps34 PI3K complex.

mTOR (mechanistic Target Of Rapamycin), a serine/threonine kinase that inhibits autophagy in normal or nutrient rich conditions, is one of the most important regulatory elements of autophagy (Noda & Ohsumi, 1998). The mTOR protein, together with the regulatory-associated protein of mTOR (Raptor) (Hara *et al.*, 2002; Kim *et al.*, 2002), mammalian lethal with SEC13 protein 8 (MLST8) (Loewith *et al.*, 2002) and the recently identified PRAS40 and DEPTOR is part of the mTOR complex I which is a sensor for amino acids (Oshiro *et al.*, 2007; Peterson *et al.*, 2009). mTOR causes direct and indirect phosphorylation of the Atg13 protein. Phosphorylated Atg13 has a reduced capacity of binding its partners Unc-51 Like Autophagy Activating Kinase 1 (ULK1), resulting in a

decrease in autophagy (Jung *et al.*, 2009). Inhibition of the mTOR activity by nutrient deprivation or the rapamycin leads to partial dephosphorylation of ATG13 and allows the initiation of autophagy. Moreover, mTOR directs a cascade of signaling pathways, as the inhibitory phosphorylation of ULK1 by which it regulates the phosphorylation of many activators of autophagy such as Tap42, Sit4, Ure2 and Gln, which act at the level of transcription and translation of necessary for autophagy proteins (Cherkasova e Hinnebusch, 2003).

Gcn2 kinase is another nutrient sensor for amino acids, that induces autophagy upon depletion of nutrients. Gcn2 works by acting on eIF2a and on the transcriptional regulator of autophagic genes Gen4 (Cherkasova e Hinnebusch, 2003).

ULK1 (UNC-51-like kinase -1, homologue of the yeast Atg1) and ATG13 are the main players of the second complex and give rise to two regulatory feedback loops of autophagy. First, as said above, it acts downstream of the mTOR complex I. However, it also negatively regulates it, through its interaction with and possible phosphorylation of RAPTOR (component of the mTOR complex I), giving rise to one of the regulatory feedback loops of autophagy (Dunlop *et al.*, 2011). Second, ULK1 is phosphorylated and activated b by AMPK, a kinase mostly known for its role in sensing glucose levels and ATP depletion (AMPK kinase is directly activated by a low ATP:ADP ratio) (Mao e Klionsky, 2011). On the other hand, the ULKI complex negatively regulates AMPK activity by phosphorylating some of its subunits (PRKAA1, PRKAB2 and PRKAG1) (Lee *et al.*, 2010).

The third main complex of the initiation of autophagy in mammals is the PI3KC3-C1. It contains Vps34, Beclin-1 (mammalian homolog of yeast Atg6), p150 (mammalian

homolog of Vps15) and Atg14-like protein (Atg14L or Barkor) or ultraviolet irradiation resistance-associated gene (UVRAG). PI3K class III, which is similar to fungi Vps34, leads to the production of PtdIns-3-P, and activates autophagy, whereas the class I enzyme converts the PtdIns-4,5-P_2 to PtdIns-3,4,5-P_3 and activates the mTOR kinase, inhibiting autophagy. The signaling molecules of class I PtdIns3 kinase complex connect tyrosine kinase receptors with the activation of mTOR and autophagy is inhibited as a response to factors like insulin and other growth factors (Yu *et al.*, 2015). PTEN, a 3' PI3K phosphatase is a positive regulator of autophagy (Errafiy *et al.*, 2013). Both mTOR and PDK1 activate the kinase of the ribosomal subunit S6 (p70S6k), which is needed for the maximal autophagic activity. The decreased activity of p70S6k, because of the inhibition of the TOR protein might attenuate excessive autophagy. This process is conserved in the vertebrate phylum, but mammals need the presence of other factors like eIF2a, Ras proteins and G heteromeric proteins to regulate autophagy (Galluzzi *et al.*, 2014). Autophagy is also one of the outcomes upon the activation of other signaling pathways like the tyrosine kinase receptors, Protein Kinase A, casein II kinase, MAP kinases and calcium, with their mechanisms of action still being under investigation.

Signals other than the nutrient sensing, such as hypoxia, ER stress, DNA damage and other forms of stress, can also trigger autophagy (Kroemer *et al.*, 2010). The tumor suppressor protein p53, which upon activation by genotoxic and metabolic stress blocks cell cycle and might lead to apoptosis, is an interesting example of this autophagy activation. In this case, the mTOR activity is inhibited by different mechanisms, like the phosphorylation by AMPK (as said above), the activation of downstream targets of p53 like TSC-2 and PTEN (White, 2016). In addition, products of sestrin1 and sestrin2 (Maiuri

et al., 2009), substrates of p53, are modulators of autophagy. DRAM (damage-regulated autophagy modulator) mediates apoptosis in a p53 dependent manner apart from its role in autophagy. In contrast to autophagy related p53 actions that are dependent on cellular stress, in normal condition this protein inhibits autophagy (Levine e Abrams, 2008). It is also important to mention one more connection between cell cycle and autophagy. This connection is given by the tumor suppressor ARF ($p14^{ARF}$) which interacts with Bcl-X_1 on mitochondria, inhibiting its binding to Beclin1 (Pimkina *et al.*, 2009). Lastly, the inhibitor of cyclin dependent kinase p27 modulates autophagy upon its stabilization in low energy conditions and amino acid depletion.

An interesting inducer of autophagy is ammonia, an intermediate product of glutamine metabolism (Cheong *et al.*, 2012). Cancer cells can increase their survival exploiting this metabolic pathway. The regulation of stresses and autophagy is bidirectional, only to mention one example, the mTOR kinase induces the transcription of HIF1a, inducing this way its activity (Land e Tee, 2007).

2. Cargo selection and packaging- Selective autophagy

Macroautophagy is generally thought as a non-selective process during which a quantity of cytoplasm is isolated and engulfed into the autophagosome. In addition to bulk autophagy, there is also selective autophagy which needs to meet at least three criteria for an efficient process to happen: First, the cargo has to be specifically recognized; second, the cargo has to be effectively tethered to a nascent autophagosome; and third, non-cargo material has to be excluded from the autophagosome. In *S. cerevisiae* and *P. pastoris*, the selective autophagy process is called CVT (cytoplasm to vacuole targeting).

This pathway has not been identified in any other organism and involves the recruitment of Atg11 and Atg19 in yeast, and PpAtg26 and PpAtg28 in fungi (Lynch-Day e Klionsky, 2010). These genes are not otherwise necessary for autophagy but are important for the transport of the cargo to the forming autophagosome. Higher eukaryotic cells lack homologues for these genes. In their case selectivity is achieved by several autophagy receptors and involves mainly the degradation of damaged organelles or protein aggregates. Usually the receptors recognize the cargos by their LC3 interacting regions (LIR motifs) and bind to autophagy modifiers of the LC3/GABARAP family (Schaaf *et al.*, 2016). An autophagy receptor is defined by its ability to bridge cargo and autophagosomal membrane, leading to the engulfment of cargo by the autophagic membrane, so not all proteins interacting with modifiers are receptors.

The most common autophagy targeting signal in mammals is the modification of cargos with Ubiquitin which mobilizes massively also the Ub-proteasome degradatory system. Indeed, most of the autophagy receptors harbor both Ub-binding domains (UBDs) and LIRs (Shaid *et al.*, 2013). To note, that while ubiquitination of receptors is required to induce autophagic clearance, their proteasomal degradation is not.

The best studied and understood mechanism of selective autophagy that involves massive ubiquitination of proteins is mitophagy, the degradation of mitochondria by autophagy. Depolarization of mitochondria causes the accumulation of the kinase PINK1 (PTEN-induced putative kinase protein1) from the intermembrane space of mitochondria to the outer mitochondrial membrane (OMM) (Matsuda *et al.*, 2010). PINK1 subsequently causes the recruitment of the E3 Ub-ligase Parkin on the OMM (Matsuda *et al.*, 2010). The mechanism of Parkin translocation is still unknown but involves also the

phosphorylation of Ubiquitin by PINK1 (Kane *et al.*, 2014). Parkin causes then the ubiquitination of many OMM proteins (Chan *et al.*, 2011), and this Ubiquitination acts as a signal for the autophagy receptor p62/SQSTM1, which is present on the phagophore, the newly synthesized autophagic vesicle (Peng *et al.*, 2017). P62 harbors both the LIR domain, which is necessary for interaction with the autophagic machinery, and a Ub-binding domain. Not always though Ubiquitination of receptors is necessary for the recruitment of the autophagic machinery and degradation. Mitophagy again is the best example, since alternative PARKIN independent mechanisms exist that involve receptors (like NIX and FUNDC1) that lack Ub-binding domains, have only the LIR one, and cause the direct binding of mitochondria to the autophagic machinery (Schweers *et al.*, 2007). Lastly, cardiolipin has been shown to link mitochondria to the phagophore without UB or LIR domains.

3. Vesicle nucleation

It is still not clear whether autophagosome biogenesis or cargo recruitment happens first. In principle, it appears that only autophagosome biogenesis is required for bulk autophagy, whereas selective autophagy might require first cargo recruitment that acts as a signal for the formation of isolation membranes. In yeast, where autophagy happens as said above by the CVT pathway, there is a peri-vacuolar, dot-like structure where cargo and ATG proteins colocalize. This structure is called PAS (Pre Autophagosomal Structure) and it is where autophagosome formation happens (Suzuki *et al.*, 2001). Depending on the stimulus of autophagy, this formation can be upstream or downstream of cargo recruitment. Generally, for all organisms, it seems that starvation induced

autophagy where mTOR is inhibited, isolation membranes are generated independently of bulky cargo material, whereas for selective autophagy the cargo material induces membrane biogenesis.

Until recently it was thought that the vesicle is synthesized completely de novo due to the unique protein composition of the autophagosomal membrane that is not encountered in other compartments of the cell. But a compartment of lipid bilayer membranes enriched for PtdIns(3)P (phosphatidylinositol 3-phosphate) was identified as a subdomain of the ER (endoplasmic reticulum) (Axe *et al.*, 2008). This is the site from which phagophores form and is called Omegasome due to its form which is similar to the Greek letter omega (Ω) (Figure 2). This membrane protrusion generates the double membranous autophagic vesicle. Ptdlns(3)P is accumulated on the Omegasome from “cradles” of the DFCP1 binding protein (Axe *et al.*, 2008). The pool of Ptdlns(3)P of the Omegasome, is responsible for priming it with the three complexes already mentioned in the initiation step, and then recruits an effector protein, the WIPI2b (ATG12), which together with the t-SNARE (target membrane—soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor) protein syntaxin 17 and VMP1 acts as a platform for the recruitment of the ATG12-5-16L1 complex (Polson *et al.*, 2010). For this complex to be assembled, ATG12 is conjugated to ATG5 through activation by the E1 enzyme ATG7 and transfer to the E2 like enzyme ATG10 (Otomo *et al.*, 2013). The formed multimeric complex is an E3-ligase and links covalently proteins of the LC3/GABARAP family to the lipid phosphatidylethanolamine (PE). What happens is that ATG4 cleaves the carboxyl-terminus of LC3 resulting in it cytosolic form LC3-I. Upon autophagic activation, ATG7 and ATG3 (E2 like ligase) cause the conjugation of LC3-I to the lipids, resulting in its

membrane bound form, LC3-II. LC3-II can be recycled by cleavage of LC3 from the autophagosome by the ATG4 enzyme (Satoo *et al.*, 2009; Mizushima e Komatsu, 2011).

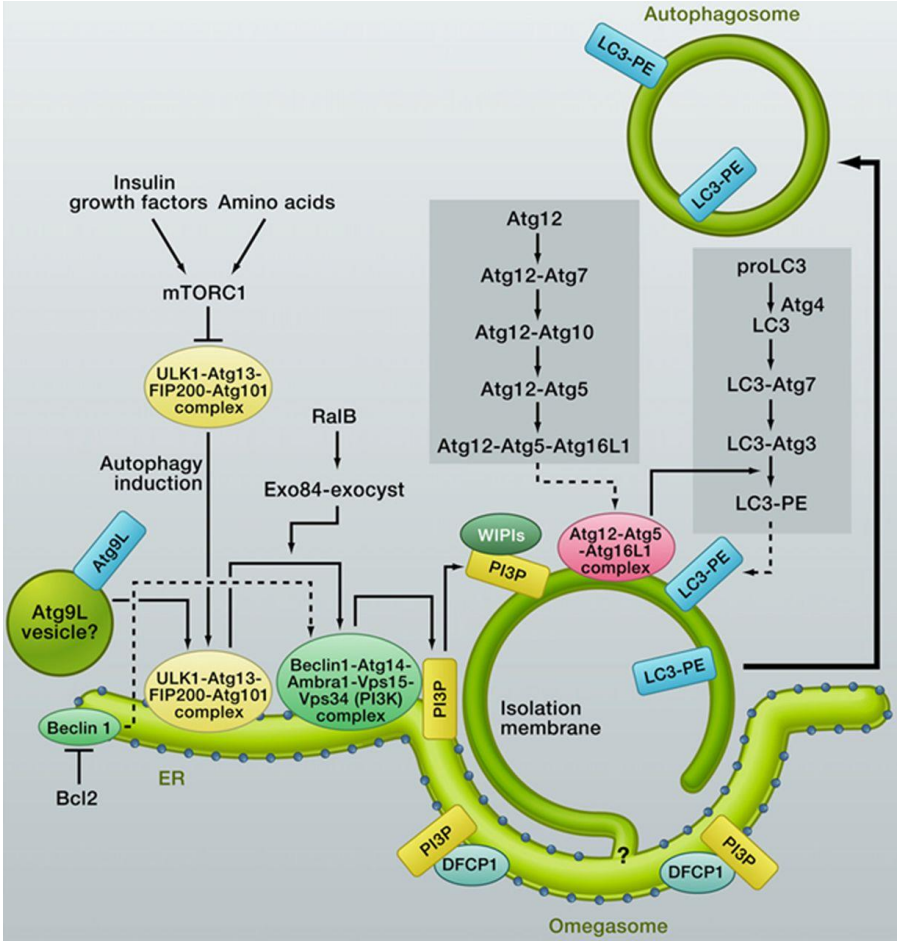

Figure 2: Omegasome/autophagosome formation on the ER requires the recruitment of the ULK complex and the Vps34 complex I. These are required for PI3P generation and WIPI2 and DFCP1 recruitment. An autophagosome is finally generated upon the recruitment of the Atg12–Atg5–Atg16 complex, and LC3–PE (Mizushima e Komatsu, 2011).

4. Vesicle expansion/elongation and completion

After the formation of the Omegasome, the recruitment of the complexes and an initial phagophore formation, the new vesicle needs to expand. The elongation is found to rely

on input from several compartments of the endomembrane system (like Golgi end ER) and mitochondria. The transport seems to happen in form of vesicles, even though the vesicle that have been observed is not clear whether are membrane or cargo providers. However, during starvation induced autophagy (thus not a selective autophagic condition) lipids from mitochondria are transported to autophagosomes, contributing to the elongation of the autophagosome. Also, transport of proteins like COP-1 and COP-2 between the ER and the Golgi through the ERGIC seems to be important in autophagosomal formation (Ge *et al.*, 2014). From the ATG family ATG9 seems to be the only transmembrane protein of the lipid bilayer. Due to the localization of ATG9 in the Golgi, it seems clear the contribution of this compartment to the formation of the vesicle. Importantly, Golgi is the only organelle contributing to autophagosome formation (Yamamoto *et al.*, 2012) in an ATG5/ATG7 independent autophagic pathway. Finally, it seems that also the contribution of the plasma membrane is important for autophagosome formation; first, because it has been shown that ATG16L interact with PM proteins such as clathrin and AP-2 and inhibition of clathrin endocytosis causes a decrease in mature autophagosomes, and second because again ATG9 is present in PM and is also internalized by clathrin mediated endocytosis. ATG16L and ATG9 positive endosomes meet after recycling endosomes and are afterwards added to the phagophore membrane (Ravikumar *et al.*, 2010).

Functional autolysosome formation requires membrane closure. This closure requires membrane scission and the ESCRT components have been suggested as potential regulators of this scission. However, technical difficulties in finding a reliable assay to define the exact components of the machinery have made it difficult to understand the

exact mechanism. Recently, the endosomal sorting complexes required for transport (ESCRT)-III component CHMP2A was identified as a critical regulator of phagophore closure. During autophagy, CHMP2A translocates to the phagophore and regulates the separation of the inner and outer autophagosomal membranes to form double-membrane autophagosomes. Consistently, inhibition of the AAA-ATPase VPS4 activity impairs autophagosome completion. The ESCRT-mediated membrane abscission appears to be a critical step in forming functional autolysosomes (Takahashi *et al.*, 2018).

5. Vesicle targeting, docking and fusion with lysosomes

After enclosure of the cargo to the autophagosome -in case of selective autophagy as said above by receptor proteins- and successful membrane closure, the autophagosome must fuse with late endosomes or lysosomes in order to degrade its cargo. This step is also known as maturation, and the final vesicle formed is called autolysosome. This step depends mostly on lysosomal membrane proteins: the transmembrane lysosome-associated membrane protein 2 LAMP2 and the small GTPase Rab7. Also, it depends on proteins of the SNARE family like syntaxin 17 and VAMP3, the former forming a complex with homo-oligomerized ATG14L, to stabilize VAMP8, an important player in the fusion process (Furuta *et al.*, 2010; Abada *et al.*, 2017). It is interesting to note how ATG proteins that are important in the first stages of autophagosome formation, are also important for the maturation and their fusion with lysosomes. Apparently, some of them are even more important during the fusion, when the inner autophagosomal membrane has to be degraded together with the rest of the cargo. The most recent finding though is BIRC6/BRUCE, as necessary interactors with sintaxin17 for autophagosome-lysosome

fusion (Ikeda, 2018). Other components have been also found to be important for the completion of the fusion step, such as other proteins of the Rab family, the Hippo kinase STK3-4 and the calcium pump SERCA (Mauvezin e Neufeld, 2015; Mcewan *et al.*, 2015; Wilkinson *et al.*, 2015).

6. Vesicle breakdown for the recycling of macromolecules

The degradation of the autolysosomal cargo depends on the acidic pH of the vesicle and is achieved by acidic hydrolases and cathepsins of the lysosomal lumen. The products of degradation, such as amino acids, sugars, fatty acids, and nucleotides, are then transported back to the cytosol by diffusion, or with the aid of specialized transporters such as lysosomal permeases, providing precursors for the synthesis of ATP, proteins, and other essential macromolecules under stress conditions. In turn, some products of autophagic degradation, such as amino acids, exert a negative feedback on autophagy initiation, serving as a homeostatic mechanism required to prevent prolonged or overactivation of autophagy. As mentioned above, the most prevalent form of this negative regulation are free amino acids that directly activate the mTORCI as an autophagic gatekeeper.

MICROAUTOPHAGY

During microautophagy, portions of the cytoplasm are directly engulfed by invaginations of lysosomes, late endosomes or multivesicular bodies without a double-membranous organelle to mediate the delivery. It was first described in yeast, where serves both bulk

cargo degradation and selective autophagy of organelles, like micropexofagy (peroxisomal microautophagy), micromitophagy (mitochondrial microautophagy), microlipophagy and piecemeal microautophagy (for the degradation of potions of the nucleus) (Roberts *et al.*, 2003; Sakai *et al.*, 2006; Uttenweiler *et al.*, 2007; Vevea *et al.*, 2015).

In mammals on the other hand, the essential yeast microautophagy genes have not been found to have conserved functions. The degradative processes similar to the ones of yeast microautophagy occurs in late endosomes (LE) and multivesicular bodies (MVB) and not in lysosomes. This was called eMI (endosomal microautophagy) and it contributes to both bulk autophagy, as well as to selective autophagy, through the recognition of a specific pentapeptide (the KFERQ motif) by HSC70, a mechanism similar to the one of CMA (Chaperone Mediated Autophagy) that will be discussed below (Sahu *et al.*, 2011). Components of the three ESCRT complexes are necessary for this form of autophagy (TSG101 from complex I, CVPS25 from complex II and VPS32 from complex III), whereas VPS4 and Alix are accessory molecules to the machinery. Microautophagy and CMA share many of their substrates to be degraded, such as GAPDH, RNase A and TAU. The KFERQ motif though is not enough for mammalian proteins to be degraded by eMI, whereas it is for CMA. Upon binding the eMI substrate protein through their KFERQ motif, HSC70 targets them to the LE, and more specifically to the phosphatidylserine (PS) on their surface (and not to LAMP2A like in CMA). This causes internalization of the cargo bound to HSC70, and their subsequent degradation, as opposed to CMA, here HSC70 is recycled (Tekirdag e Cuervo, 2018). Not much is known about eMI regulation, but vesicle

formation is the limiting step of the mechanism in both yeast microautophagy and mammalian eMI.

CHAPERONE MEDIATED AUTOPHAGY

Chaperone mediated autophagy is a selective type of autophagy where the cargo proteins need to 1, have a KFERQ-like motif and 2, be amenable for unfolding, meaning that aggregates, oligomers and complexes to be degraded by this mechanism must disassemble completely. The process starts with binding of HSC70 to the consensus peptide; proteins are then targeted to the lysosomal lumen, a process that requires LAMP2A reversible multimerization in the CMA translocation complex and a luminal form of HSC70. LAMP2A and therefore CMA have so far been described only in birds and mammals. However, what defines the CMA capability is the presence of HSC70 and not LAMP2A (Orenstein e Cuervo, 2010)

In the long list of experimentally validated substrates for CMA there are proteins involved in many cellular processes, such as glycolysis, lipogenesis, transcription and immunity. Together with HSC70, co-chaperones like Hop, Hip and BAG-1 contribute to CMA in both the targeting and the unfolding steps. The rate limiting step is given by the levels of LAMP2A on the lysosomal membranes. LAMP2A regulation (multimeric assembly and disassembly) is dependent on GFAP and EF1. The former is regulated by the phosphorylation of Akt1 by the mTORCII, which inhibits the assembly of LAMP2A into the CMA translocation complex. On the contrary, when CMA (and thus the translocation

complex) is needed, the PHLPP1 phosphatase dephosphorylates Akt1. Both the mTORCI and II are present on the lysosomal membranes, but only the latter is able to regulate CMA.

CMA is observed to occur at basal conditions but is increased upon different kinds of stress like nutrient deprivation, oxidative stress, exposure to genotoxic or proteotoxic stressors, hypoxia and lipid overload. It is needed to regulate cellular energetics and proper protein quality control (degradation of misfolded proteins and toxic aggregates).

AUTOPHAGY IN HEALTH AND DISEASES

Autophagy is closely related to both normal physiology as well as disease. When it is impaired, diseases like neurodegeneration, infections, cancer, aging and heart disease can occur. Whole body and tissue specific knockout studies in mice indicate its important role in different contexts.

PHYSIOLOGICAL ROLES

Autophagy is critical in regulating metabolic homeostasis, by the mobilization of intracellular energy resources to meet metabolic demands. First, amino acids from autophagic degradation are used with fatty acids to feed the tricarboxylic acid cycle (TCA)

to sustain ATP production. Indeed, adding TCA substrates like pyruvate is sufficient to reverse the phenotype of autophagy deficient cells (Guo *et al.*, 2016) .

Also, during metabolic stress autophagy can promote survival. Yeast cells and plants lacking ATG genes have reduced tolerance to nitrogen or carbon deprivation; slim molds limited viability and differentiation upon nutrient deprivation; nematodes display decreased survival under the same conditions. Finally, mice knockout for Atg5 or Atg7 die shortly after birth because they cannot cope with the period of physiological neonatal starvation.

Autophagy is also essential for protein quality control. Ubiquitin-positive diffused proteins and aggregates accumulate in ATG5 and ATG7 deleted cells (Riley *et al.*, 2010). Accumulation of diffused proteins precedes that of aggregates in some contexts, suggesting that not only preexisting aggregates might not be properly cleared out, but also that new aggregates form as a result of impaired autophagy, leading to protein damage, misfolding and aggregation.

AUTOPHAGY IN DISEASES

1. Neurodegenerative diseases

Aggregation of misfolded proteins and loss of certain neuronal population is a most prevalent pathological feature of many neurodegenerative diseases. Accumulation of autophagic vacuoles and dysfunctional lysosomes in the brains of patients with

Alzheimer's (Nixon *et al.*, 2005), Parkinson's (Stefanis, 2012) and Huntington's diseases (Shibata *et al.*, 2006) led to the hypothesis that impaired autophagy contributes to the development of these disorders. Indeed, autophagy exerts a key role in degrading aggregate-prone proteins, which have been implicated in the pathogenesis of various neurodegenerative diseases, such as mutant α-synuclein in PD (Stefanis, 2012), mutant huntingtin in HD (Zheng e Diamond, 2012), and mutant TDP-43 in ALS (Wilson *et al.*, 2011). Autophagy modulation can prevent the occurrence and progression of these diseases, and indeed there is a big number of clinical trials to test the use of autophagic modulators to treat them (reviewed recently in (Towers e Thorburn, 2016).

2. Liver disease

The most common genetic cause of human liver disease is the deficiency of a1-antitrypsin and autophagic protein turnover might be important for its pathogenesis. a1-antitrypsin is an aggregate prone protein that normally is degraded by the proteasome. However, its gain of function mutated form "α1-ATZ" is normally degraded by autophagy. When the autophagic clearance is impaired (e.g. by depletion of Atg5), the mutated form aggregates within the hepatocyte ER causing hepatotoxicity (Perlmutter, 2006). Apart from a1-antitrypsin deficiency, dysfunctional hepatic autophagy seems to be associated with the pathogenesis of steatohepatitis, alcoholic hepatitis, viral hepatitis, NAFLD and hepatic fibrosis, hallmark of which is the accumulation of SQSTM1/p62 inclusion bodies (reviewed in (Rautou *et al.*, 2010).

3. Muscle disease

Proper autophagic flux is vital for skeletal muscle function, and metabolism. The importance of autophagy in skeletal muscle health has been proved by the conditional muscle knockout mouse for ATG7, which results in profound muscle atrophy and age-dependent decrease in force (Masiero *et al.*, 2009).

Impaired skeletal muscle autophagy has a key pathogenic role in muscular dystrophies: most of them are characterized by the accumulation of autophagosomes in muscle cells, a condition called autophagic vacuolar myopathy. The first genetic disease caused by a mutation in an autophagy-related gene, EPG5, is the Vici syndrome. Vici syndrome is a recessive inherited multisystem disorder characterized amongst others by myopathic features, including atrophy of type 1 fibers, centrally nucleated fibers and abnormal glycogen accumulation (Cullup *et al.*, 2013).

Danon' s disease, a condition characterized by weakening of cardiac and skeletal muscles is caused by mutations in the LAMP2 gene. In Pompe disease, an inherited disorder caused by the buildup of glycogen, the muscle weakening is caused by mutations of the GAA gene. GAA encodes the acid maltase, is found in lysosomes and normally breaks down glycogen during autophagy (Cullup *et al.*, 2013).

Importantly, proper degradation of the ECM matrix is also important to sustain proper muscle function. Collagen VI deficiency (Bethlem myopathy and Ullrich congenital muscular dystrophy) causes inefficient removal and persistence of altered organelles in myofibers, a condition confirmed also in collagen VI deficient (*Col6a1*$^{-/-}$) mice. Even though the mechanism is not well understood, lack of collagen VI impacts on protein levels of Beclin1 and Bnip3 and the activation of the Akt/mTOR pathway, suggesting that the myopathic phenotype is due to autophagic impairment (Grumati *et al.*, 2010).

4. Cancer

Due to the big overlap between the signaling pathways that regulate autophagy and cancer (like the mTOR/AMPK pathway) there is a strong link between them. It is generally accepted that autophagy is a bona fide tumor suppressor pathway. Monoallelic deletion of BECN1 has been detected in human breast, ovarian, and prostate tumor specimens and the aberrant expression of Beclin 1 in many kinds of tumor tissues correlates with poor prognosis (Qu *et al.*, 2003). Apart from this, many autophagy related genes seem to act as tumor suppressors, including ATG5, ATG4c, UVRAG, Bif-1 and Ambra1. The mechanism is still not well understood, but there are two major hypotheses to explain the tumor protecting role of autophagy: 1. Upon impaired autophagy and due to the overlap with apoptotic pathways, there is impaired apoptosis but stimulation of necrotic cell death, inflammation and general induction of a chronic wound-healing response; 2. Metabolic stress in autophagy-deficient tumor cells can lead to genome damage and tumor progression which could occur through protein, organelle and DNA damage, or insufficient ATP levels for cellular functions required to maintain genome integrity, such as mitosis and DNA replication and repair (Sui *et al.*, 2013).

5. Ageing

Autophagy declines physiologically with age. It has been shown in many different animal models that intermittent fasting promotes longevity because it induces potently autophagy. It is then to be clarified if age related diseases depend on this physiological decline. The reason of the decline is not known, but there is evidence showing that could

be due to change in responses to hormonal regulation of autophagy, to defective autophagosome degradation, or just to cumulative effects of autophagic clearance over prolonged periods of ageing (Rubinsztein *et al.*, 2011). To get the beneficial effects of caloric restriction by fasting, there are alternative approaches that can mimic it by avoiding its adverse effects (e.g. the polyamine spermidine) (Madeo *et al.*, 2018).

6. Infection, immunity and inflammation

Autophagy is important in host defense against intracellular pathogens by the mechanism of xenophagy. Even though there is a plethora of in vitro data, *only* tobacco mosaic virus in plants and HSV-1 and Sindbis virus in mice have been shown to be degraded by xenophagy in vivo (Bauckman *et al.*, 2015).

With respect to pathogen infection and autophagy, also immunity is affected. On innate immunity, for example, *atg5* is required for the delivery of viral nucleic acids from Sendai virus and vesicular stomatitis virus to the TLR7, and type I interferon signaling in plasmacytoid dendritic cells. Whereas, on adaptive immunity, autophagy is involved in the delivery of certain endogenously synthesized microbial antigens (e.g., Epstein Barr viral antigens) to MHC class II antigen-presenting molecules, leading to the activation of $CD4^+$ T lymphocytes (Zhou e Zhang, 2012).

Recently, emerging evidences have indicated that the process of autophagy may play an essential role in acute and chronic inflammatory processes, and thereby potentially impact the outcome of disease progression. The most known case is Crohn's disease, Genome-wide association studies of non-synonymous SNPs have linked ATG16L1 variants with susceptibility to this major type of inflammatory bowel disease that can affect

any part of the digestive tract (Salem *et al.*, 2015). With respect to inflammatory symptoms, autophagy has been found to be involved also to the pathogenesis of pulmonary hypertension, cystic fibrosis, chronic obstructive pulmonary disease and systemic lupus erythematosus.

7. Cardiovascular disease

As it will be discussed further below, autophagy seems to play an important role also in cardiovascular disease. In the myocardium, defects in autophagy cause cardiac dysfunction and heart failure, like in the aforementioned Danon and Pompe diseases. Under stressful conditions, autophagy is rapidly increased as a response and plays a protective role to promote cell survival, like during ischemia and pressure overload.

AUTOPHAGY IN THE CARDIOVASCULAR SYSTEM

1. Genetic models of altered autophagy in the heart

The heart, subjected to continuous mechanical, metabolic and neurohormonal stress, is particularly dependent upon macroautophagy and selective autophagy to maintain its normal function. The importance of autophagy in the heart has been demonstrated from

studies that link the deletion of autophagy-relevant genes in the heart to an accrued propensity of laboratory animals to spontaneously develop cardiodegenerative disorders (Figure 3), as well as in *in vitro* systems, where its role has been dissected in the many different cell types that comprise it.

ATG5 conditional cardiac knockout mice develop cardiac hypertrophy, left ventricular dilatation, contractile dysfunction, and premature death accompanied by disorganized sarcomere structure, mitochondrial misalignment, and aggregation (Taneike *et al.*, 2010). Overexpression of miRNAs that inhibit the transcription of autophagy related genes leads to cardiac hypertrophy and heart failure.

Mice overexpressing the C452F mutation in DNM1L (dynamin 1 like) display mitophagic defects that most probably are the cause of the dilated cardiomyopathy they develop (Ashrafian *et al.*, 2010). Similarly, Tfrc (transferrin receptor) cardiac knockout mice develop cardiomegaly and cardiac dysfunction due to ineffective mitophagy (Xu *et al.*, 2015). Bnip3l knockout mice also exhibit cardiomegaly and contractile dysfunction (Dorn, 2010). Conversely, p53 (Trp53) knockout mice display better cardiac function compared to their wild type counterparts, because cytosolic p53 has been shown to inhibit Parkin mediated mitophagy. Indeed, Parkin deletion in Drosophila leads to cardiac problems, whereas in mice leads to cardiac dysfunction only when combined with the ablation of Mfn2 (Gong *et al.*, 2015). The lack of phenotype in the single knockout mouse for PARK2 is thought to reflect the plethora of alternative mitophagic pathways that compensate when one of them is inhibited.

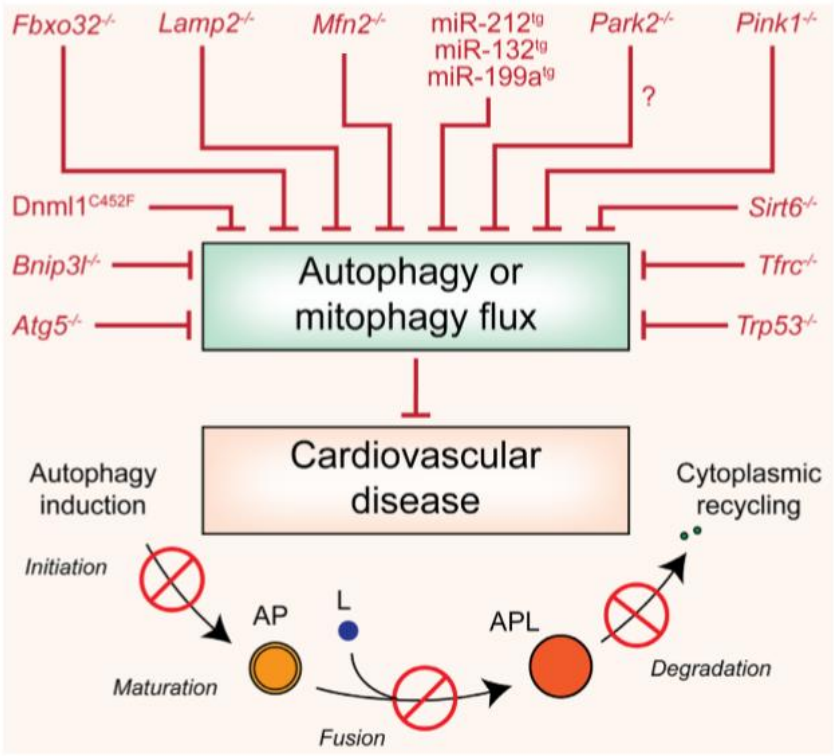

Figure 3: Effects of genetic ablation of genes involved in autophagy and mitophagy on the development of spontaneous cardiovascular disorders. AP, autophagosome; APL, autophagolysosome; Atg5, autophagy related 5; Bnip3l, BCL2 interacting protein 3 like; Dnml1, dynamin 1 like; Fbxo32, F-box protein 32; L, lysosome; Lamp2, lysosomal-associated membrane protein 2; Mfn2, mitofusin 2; Park2, Parkinson disease (autosomal recessive, juvenile) 2, parkin; Pink1, PTEN-induced putative kinase 1; Sirt6, sirtuin 6; Tfrc, transferrin receptor; and Trp53, transformation-related protein 53. Adapted from (Bravo-San Pedro *et al.*, 2017)

2. Autophagy in the different cell types of the cardiovascular system

It is important to take in account the different cell types that comprise the cardiovascular system and dissect their role and their response to autophagic stimuli in health and disease.

Autophagy in the vascular system

There is increasing interest in the role of autophagic flux in maintaining normal vessel wall biology, as well as a growing suspicion that autophagic dysregulation may be a common pathway through which vascular aging and associated pathologies develop.

Vascular autophagy is stimulated by 4HNE lipids that by their autophagic degradation lead to cytoprotection from lipid peroxides, but also from 7-ketocholesterol and ox-LDL that inhibit MTOR in endothelial cells. This seems to be mediated by ER stress response, that activates the MAPK/JNK pathway that results in autophagy.

Macrophages residing in vasculature can store cholesterol esters in lipid droplets. When the lipophagy of these droplets is inhibited, macrophages become foam cells, responsible for atherosclerotic plaque formation and instability. Indeed, Atg5 macrophage knockout mice have more atherosclerotic plaque formation, and additional inhibition of mTOR was beneficial for animal model of atherosclerosis (Liu, K. *et al.*, 2015). Finally, suppression of macrophage- localized autophagy in $Ldlr^{-/-}$ mice increased apoptosis and oxidative stress in plaque macrophages, promoted plaque necrosis, and impaired lesion efferocytosis (Liao *et al.*, 2012).

The beneficial effects of autophagy in vasculature are confirmed also using common supplements like resveratrol, epigallocatehin vitamin C and vitamin D, which attenuate inflammation and cytotoxicity by enhancing autophagy. As said above, autophagy has antiaging properties, which in the vasculature consist in inhibiting large artery stiffening and inhibition of endothelial relaxation. Both phenotypes come from ageing-dependent changes in the redox state of vasculature.

<u>Autophagy in fibroblasts/myofibroblasts</u>

Cardiac fibrosis is a hallmark of multiple cardiovascular diseases. While cardiac fibroblasts are relatively quiescent cells that contribute little to matrix remodeling or wound healing, phenoconverted myofibroblasts persist within the infarcted myocardium. The resulting excessive ECM deposition leads to impairment of the contractility of the myocardium, reduced cardiac function and heart failure.

TGF-β treatment of human atrial fibroblasts both induces autophagy and enhances the fibrogenic response supporting a linkage between the myofibroblast phenotype and autophagy (Ghavami *et al.*, 2015). Inhibition of autophagy represses fibroblast to myofibroblast phenoconversion, suggesting that this could be used to improve cardiac function upon ischemic damage or infarct.

Until now there are not models of specific fibroblast knockout of autophagy related genes, so it has not been possible to dissect the role of autophagy in this specific cell type.

Autophagy in cardiomyocytes

As a post-mitotic cell, cardiomyocytes retain minimal, if any, proliferative capacity in the adult stage. Myocardial growth (hypertrophic remodeling) derives exclusively from cardiomyocyte enlargement, process determined by the balance of protein synthesis and protein degradation. In the setting of pressure stress, protein synthesis predominates, culminating in a hypertrophic phenotype. Imbalances between anabolic and catabolic mechanisms likely are important contributors to cardiovascular pathology, and one of the major processes that regulate them is autophagy. Indeed, morpholino knockdown of essential autophagy genes resulted in increased cell death, reduced survival, and defects

in morphogenesis in zebrafish. Autophagy has been shown to be also a mechanism regulating cardiomyocyte differentiation (Simon, 2012).

3. Cardiac autophagy upon stress

Pressure overload and myocardial infarction

The heart undergoes hypertrophy in response to hemodynamic overload, such as pressure overload (PO), initially with the purpose of reducing wall stress. The heart also initiates various adaptive mechanisms, including autophagy, to cope with energetic stress, increased oxidative stress, and cell death. PO initially activates general autophagy but suppresses it thereafter, when the mitophagic process starts. Genetic inhibition of autophagy (Drp1 and atg5 ablation) to the TAC pressure overload model led to mitochondrial dysfunction and heart failure, whereas restoration of autophagy levels attenuated the progression of heart failure, demonstrating that autophagy protective to the hypertrophic myocardium (Qin *et al.*, 2016; Shirakabe *et al.*, 2016).

Conversely, in mice with haploinsufficiency for a different gene essential for autophagy (Beclin$^{+/-}$), pressure overload triggered increases in autophagic activity were blunted, and pathological remodeling of the left ventricle was moderately (Zhu *et al.*, 2007). Altogether, these findings led us to conclude that load-induced activation of autophagy is maladaptive.

Ischemia reperfusion injury

Autophagy during ischemia, a condition of limited oxygen and nutrient supplies is cardioprotective and is upregulated by the activation of AMPK that on the one hand inhibits the MTORI and on the other hand activates ULK1. If, however, the heart goes under chronic ischemia, the autophagic-lysosomal pathway is inhibited leading to detrimental effects. In reperfusion injury the role of autophagy is still under debate, with groups sustaining its cardioprotective role and others supporting it is maladaptive or even detrimental. For example, it has been shown that 3-methyladenine enhances cell viability upon induction of I/R injury in cultured neonatal cardiomyocytes, but in contrast other studies report that autophagy is needed for the beneficial effects of cardiac preconditioning against I/R injury (revied in (Ma *et al.*, 2015). During reperfusion there is no energy crisis rather ROS production. In this step there is no activation of AMPK, whereas increase in Beclin1 levels and accumulation of autophagosomes. Again, the presence of these autophagosomes is still not known if occurs because of increased autophagic processes or because of impaired autophagic clearance (impaired autophagic flux), as well as the increase of the autophagic vacuoles in tissue samples from patients with ischemic heart disease is not known that means that a possible increase in autophagy is protective or just a symptom of the pathogenesis of the disease.

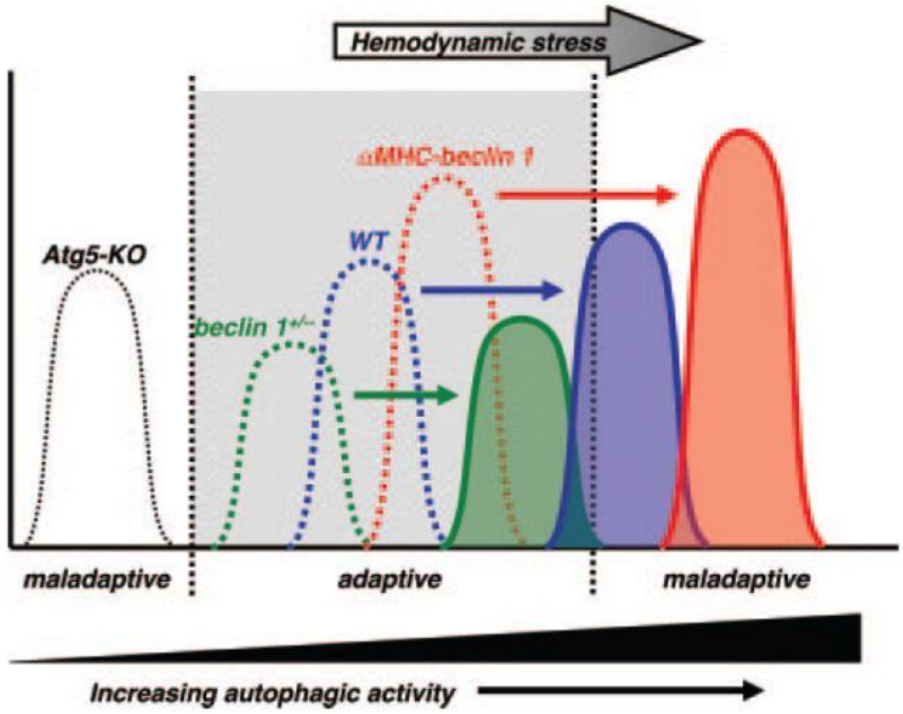

Figure 4: In pressure overload, wild-type mice mount an autophagic response that is sufficiently robust. ATG5-deficient mice cannot increase autophagy and remain in the maladaptive range. In beclin $1^{+/-}$ mice, the increase in autophagic activity is blunted, maintaining activity closer to the adaptive range. In αMHC-beclin 1 transgenic mice, load-induced autophagic activity may shift even farther into excessive autophagy which is maladaptive as well. The physiological impact of autophagy exists as a continuum, where either too much or too little autophagic activity can be detrimental (Rothermel e Hill, 2008)

MITOCHONDRIAL TURNOVER AND MITOPHAGY IN THE CARDIOVASCULAR SYSTEM

Mitochondria are the main providers of energy in the heart but are also responsible for calcium storage and metabolic remodeling, making the strict regulation of their quality essential to proper cardiac function. The mechanisms that regulate mitochondrial health are many and assure a complete mitochondrial turnover on average every 17 days. Among them the most important, interconnected ones are fusion, fission, mitochondrial biogenesis and mitophagy, with the latter having gained a lot of attention in the last years for its possible biomedical impact.

Mitochondrial dynamics

Mitochondria are highly dynamic organelles and their morphology can change from spherical isolated organelles to branched tubular networks, by undergoing fusion and fission, the so-called mitochondrial dynamics. Fusion and fission allow the redistribution of mitochondrial components, which is necessary for a plethora of cellular events. The mechanism of mitochondrial fusion and fission are regulated by large Dynamin-related GTPases. The fusion machinery consists of Mfn1 and Mfn2 for the outer mitochondrial membrane fusion, whereas responsible of the inner mitochondrial membrane fusion and cristae remodeling is OPA1. Drp1 and hFis1 are the ones responsible for mitochondrial fission which occurs upon Drp1 assembly into rings surrounding the outer mitochondrial membrane with the help of Fis1 (Ni *et al.*, 2015). There is an interplay between

mitochondrial dynamics and autophagy, specifically fission; Mitochondrial fission is necessary to produce a daughter unit that will be targeted by the autophagic machinery and “fit” in the autophagic vacuole to be degraded. Upon mitochondrial damage it seems that the dysfunctional parts of the mitochondrion are segregated and then separated from the healthy part by fission to be digested by subsequent mitophagy. Mitochondrial fusion, on the other hand, serves to dilute impaired respiratory components, improve mitochondrial function and thereby prevent removal (Chen e Chan, 2009).

Mitochondrial biogenesis

Mitochondrial biogenesis is the process by which cells increase their mitochondrial mass and DNA. Successful biogenesis relies on a precise cross-talk between mitochondrial and nuclear gene expression. Maintenance of energy metabolism homeostasis is achieved by coordinating the processes of mitochondrial biogenesis and mitophagy processes, which promotes cell survival and stress resistance. Disequilibrium between mitochondrial biogenesis and selective autophagy causes deterioration of cellular function and cell death.

The interplay between all mitochondrial turnover processes is represented in Figure 5.

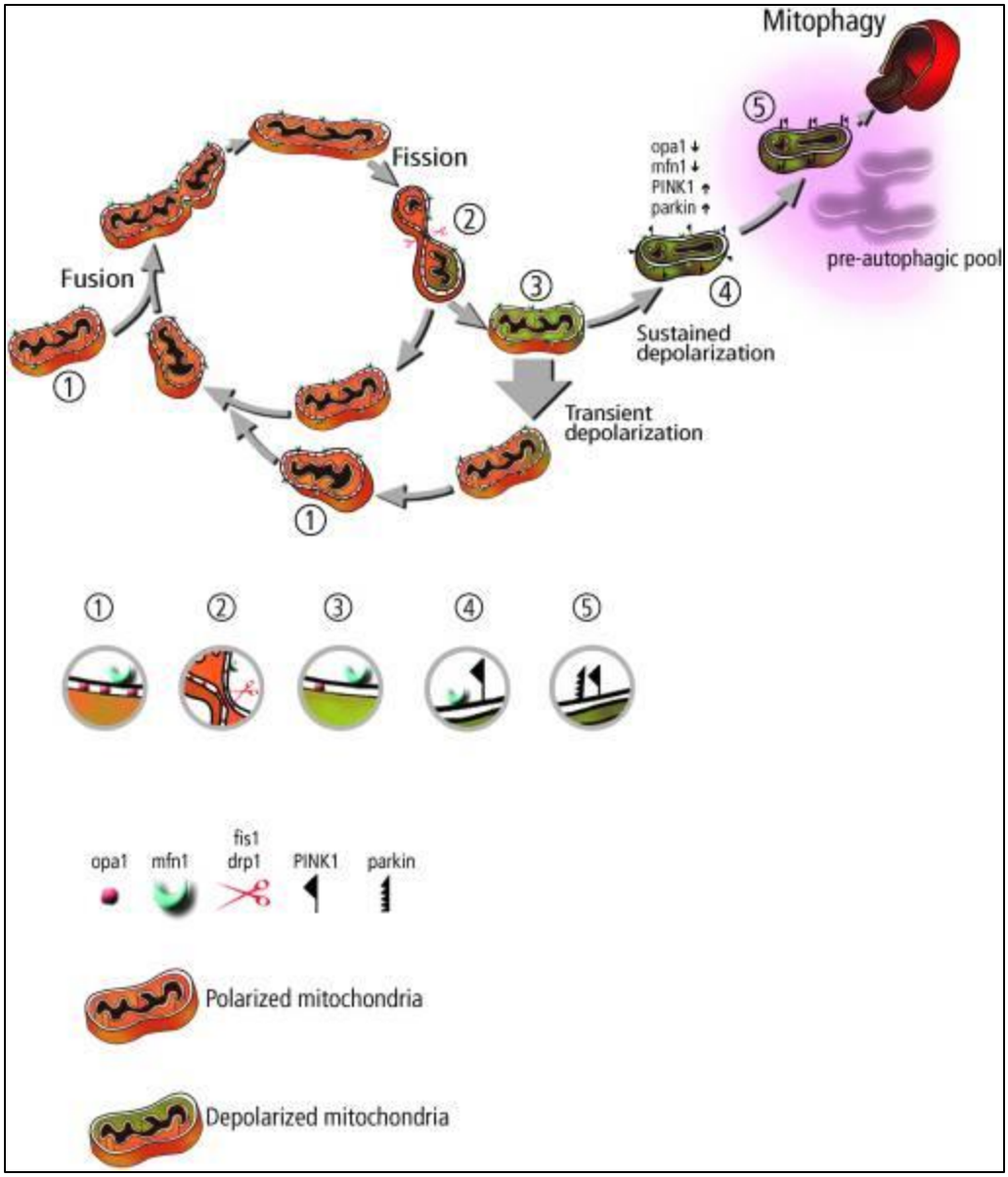

Figure 5: integration of mitochondrial fusion, fission and mitophagy forms a quality maintenance mechanism. Fusion and fission events allow the reorganization and sequestration of damaged mitochondrial components into daughter mitochondria that get degraded by mitophagy (Twig and Shirihai, 2011).

Mitophagy in the heart

Most studies suggest how important general autophagy is for protein quality control in the heart at baseline, but on mitophagy not so much is known. Loss of function studies of PINK1 and Parkin, which are known to be involved in mitophagy, but not general autophagy, give valuable information in understanding the importance of mitophagy in the heart, even though the PINK1-Parkin pathway is not the only one responsible. Parkin KO mice have normal cardiac function, even though mitochondria are morphologically disorganized and some of them dysfunctional. The normal cardiac function is thought to be a consequence of the activation of compensatory mitophagic processes (Song *et al.*, 2015). On the other hand, genetic deletion of PINK1 in mice causes more severe cardiac effects than that of Parkin, such as increased oxidative stress and cardiac hypertrophy since the second month of age (Siddall *et al.*, 2013). Also, since it has not been assessed the specific role of PINK1 in mitophagy in this context, it can be assumed that also its role in phosphorylation of TNF receptor-associated protein 1 (TRAP1) plays a role in cardioprotection. Altogether, the data suggest that mitochondrial clearance by mitophagy or general autophagy is stimulated in the heart during the chronic phase of cardiac remodeling and that they are both protective for the heart.

Ubiquitination and deubiquitination in mitophagy

Ubiquitination was thought at first to be merely targeting proteins for proteasomal degradation. Now it is widely recognized that ubiquitination acts in a number of other cellular processes. Indeed, as said above, Ubiquitination is the most common autophagy

targeting signal in mammals and is really important for the initiation of mitochondrial clearance (Grumati e Dikic, 2018).

Protein ubiquitination is reversible. While the attachment of ubiquitin molecules is catalyzed by the E3 ubiquitin ligases, the hydrolysis of the ubiquitin linkage is conducted by the proteases family of DUBs (DeUBiquitinating enzymes). Deubiquitination can occur in order to supply ubiquitin monomers to the ubiquitin ligases, in order to remove ubiquitin from substrates addressed to the proteasome for degradation (for Ub recycling), or interestingly to alter the fate of a ubiquitinated protein, by stabilizing it or change its conformation.

As already mentioned, upon mitophagy induction, the E3-ubiquitin ligase Parkin translocates from the cytoplasm to the outer mitochondrial membrane, where ubiquitinates many substrates that act as selective autophagy receptors. As expected deubiquitination of these substrates would block the mitophagic process, and until now the DUBs USP30, USP35 and USP15 are known to block mitophagy by counteracting Parkin activity (Cornelissen *et al.*, 2014; Wang *et al.*, 2015). For the three of them, it has been shown that their ablation leads to enhanced mitochondrial degradation whereas their overexpression impairs mitophagy. Interestingly, only one DUB has been found to have a positive role in mitophagy, and this is USP8 (Durcan *et al.*, 2014).

USP8

Usp8 is a deubiquitinating enzyme mostly known for its involvement in endosomal ubiquitin dynamics. By regulating the ubiquitination of members of the ESCRT machinery (Berruti *et al.*, 2010; Crespo-Y√†√±Ez *et al.*, 2018) it regulates membrane deformation and scission events. Also, by it deubiquitinating EGFR it regulates trafficking in the endocytic process (Berlin *et al.*, 2010), making it important to study in the context of autophagy, since endocytic trafficking and autophagy are two interconnected processes. Regarding mitophagy, USP8 has been shown to selectively remove K6-linked ubiquitin chains on Parkin, antagonizing its autoubiquitination and enabling its translocation to mitochondria. Indeed, USP8 knockdown leads to impaired Parkin translocation and delayed mitophagy (Durcan *et al.*, 2014). Given the fact that mitophagy is important for proper cardiac function, and ubiquitination-deubiquitination balance dictates the progress of the mitophagic process, it is expected that impairment of this balance might lead to cardiac dysfunction. Of note, USP8 mutations cause in humans Cushing's disease (Reincke *et al.*, 2015), which is a Pituitary adrenocorticotrophic adenoma that leads to death because of cardiovascular events.

CARDIAC METABOLISM

In addition to efficient protein turnover, crucial for a terminally differentiated tissue like the heart is energy production. The heart is the most metabolically active organ in the body, since it must meet the enormous energy need to continuously contract and relax. To this end, the heart turns over its entire metabolite pool every 10 seconds to produce ATP. Approximately 90% of ATP is consumed for sarcomere contraction and relaxation,

supporting the ATP dependent transport of calcium by the sarcoplasmic reticulum; the remaining pool is required for membrane transport systems. To support this ATP production, the heart utilizes oxygen from arterial blood and requires aerobic metabolism. Not surprisingly, the mitochondrial content in the heart is the highest compared to any other tissue.

The heart utilizes primarily fatty acids (70%) and upon damage carbohydrates-mostly glucose- (30%) as substrates for ATP production. Upon exercise, it utilizes also lactate, whereas it can also utilize amino acids and ketones. Amino acids, like glutamate, glutamine, aspartate, asparagine and the branched chain amino acids (BCAAs), have been shown to be preferentially used as metabolic and anaplerotic substrates in the Krebs cycle during anoxia and ischemia. The capacity of the heart to utilize eventually all kinds of substrates makes it a metabolic omnivore.

Fatty acids that come from the degradation of lipids are catabolized by beta-oxidation giving rise to Acetyl-CoA (Acetyl-Coenzyme A), that feeds the tricarboxylic acid (TCA) cycle for energy production. Similarly, carbohydrates and mainly glucose are converted to glucose-6-phosphate that enters glycolysis, and the resulting pyruvic acid again gets converted in Acetyl-CoA. Lastly, amino acids from degraded proteins (that in other tissues enter the urea cycle) are converted in substrates to feed the TCA cycle as well.

We will now briefly review the basic pathways of energy conversion in the heart.

The tricarboxylic acid (TCA) cycle

Also known as Krebs cycle, the TCA cycle leads to the complete oxidation of nutrient derived carbons to CO_2 together with production of three Nicotinamide adenine dinucleotide (NADH), one Flavin adenine dinucleotide ($FADH_2$) and one Adenosine tri-phosphate (ATP) molecule per cycle. Acetyl-CoA is the starting molecule of the TCA cycle and it could originate from glycolysis-derived pyruvate or from β-oxidation of fatty acids. TCA cycle is linked to amino acids biosynthesis via its intermediate metabolites. Also, it provides the reducing agent NADH that is used in other biochemical reactions, one of the most important being the oxidative phosphorylation.

Oxidative phosphorylation-OXPHOS

Electron transport chain (ETC) and Oxidative phosphorylation (OXPHOS) is the process whereby the electrons stripped from the reducing equivalents extracted from the catabolic intermediates by the TCA are transferred to molecular oxygen and the resulting energy is used to drive ATP biosynthesis. The electron transport chain (ETC), composed by multiprotein complexes localized within the IMM, is the enzymatic machinery behind OXPHOS.

Complex I, the NDH-CoQ oxidoreductase or NADH dehydrogenase, is the main entry point of electrons into the ETC. It oxidizes NADH, derived from TCA cycle, to NAD^+ and transfers electrons to coenzyme Q (CoQ), inducing the pumping of 4 protons across the IMM. Complex II, the succinate dehydrogenase complex, oxidizes carbons from succinate and transfers electrons through $FADH_2$, also derived from TCA cycle, to CoQ. Complex II does not pump protons into the IMS. Complex III, the cytochrome bc1 complex, is collecting electrons from $CoQH_2$ and transfers them to cytochrome c while pumping 4

protons across the IMM. Complex IV or the cytochrome c oxidase, transfers electrons from cytochrome c to the final electron acceptor O_2. This reaction is accompanied by the pumping of 2 protons. At the end of the ETC, ATP synthase generates ATP. The protons pumped into the IMS backflow through the ATP synthase into the matrix generating a rotation of the ATP synthase that initially produces mechanical force converted into chemical energy and allowing the coupling of adenosine di-phosphate (ADP) with inorganic Phosphor into adenosine tri-phosphate (ATP).

For a long time, respiratory chain complexes were considered as individual complexes distributed randomly throughout the IMM – this was referred to as the fluid model. However, using Blue Native PAGE (BNGE) experiments in yeast, it has been demonstrated that respiratory complexes are organized into supercomplexes (SCs) (plasticity model). After construction of each single complex, respiratory chain complexes assemble into dynamic functional structures depending on the energetic needs of the cell.

Fatty acid oxidation (FAO)

Fatty acid oxidation (FAO) is the catabolic process by which fatty acid molecules are broken down to generate Acetyl-CoA, which enters the TCA cycle, and NADH and $FADH_2$, used in the ETC (Bartlett e Eaton, 2004). FAO is a key metabolic pathway for energy homoeostasis in organs such as the liver, heart and skeletal muscle. During fasting, when glucose supply becomes limited, FAO is essential. Mitochondria, as well as peroxisomes, harbor all enzymes necessary for FAO. Importance of FAO is supported by the fact that almost each enzyme of the pathway is related to a pathological condition (Rinaldo *et al.*, 2002).

CARDIAC METABOLISM AND AUTOPHAGY

Autophagy impacts on cardiac disease. However, metabolism itself can impact on autophagy, suggesting that metabolism can regulate cardiac function in an autophagy-dependent manner. Fatty acids and carbohydrates, the main fuels of the heart are likely to participate in this potential regulatory network. We will now review the existing evidence supporting such a crosstalk between metabolism and autophagy in the heart.

Fatty acids and autophagy

Fatty acids are aliphatic carboxylic acids consisting of a hydrocarbon chain and a terminal carboxyl group. Based on their structure and biological functions they are classified in various groups. Saturated fatty acids (the ones without a double bond between the carbon atoms of the hydrocarbon chain) are divided in short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA), and very long-chain fatty acids (VLCFA). The Monounsaturated fatty acids (MUFA) have only one carbon–carbon double bond, whereas the Polyunsaturated fatty acids (PUFA) contain two or more double bonds along their carbon backbones. PUFAs are then subdivided in ω-3 and ω-6 according to the position of the first double bond starting from methyl end.

Fatty acids are broken down by the digestive tract into free fatty acids and monoglycerides and are absorbed by the small intestine. They are re-esterified in triglycerides and transported by the lymphatic system to circulation as chylomicrons, and finally accumulate in many cell types including muscle fibers, where they are stored until used

for energy supply, membrane structural lipids, signal transduction pathways, eicosanoids metabolism, and gene expression.

Palmitic acid is the most abundant free saturated fatty acid in bloodstream. Palmitic-acid-mediated autophagy induction seems to be protective free fatty acids-induced β-cell dysfunction, apoptotic cell death, and insulin resistance by different publications (Martino *et al.*, 2012; Liu, J. *et al.*, 2015). However, until now the specific impact of this mechanism in cardiomyocytes and heart has not been studied.

Myristic acid role in autophagy has been well studied in isolated mouse cardiomyocytes. Upon treatment cardiomyocytes increase their LC3-II levels as well as expression of beclin1. Mechanistically, myristate increases C14 ceramide and ceramide synthase 5, required in sphingolipid-induced autophagy in cardiomyocytes. Indeed, siRNA for endogenous CerS5 abrogates the autophagic effects of myristic acid. Myristate treatment increases the size of isolated cardiomyocytes, suggesting that myristate is responsible for lipid overload-caused cardiac hypertrophy. Interestingly, inhibition of autophagy by 3MA or LC3 knockdown prevents the myocyte hypertrophy, showing that autophagy is necessary for myristate role in hypertrophy (Russo *et al.*, 2012).

Omega-3 polyunsaturated fatty acids are known to reduce the incidence and progress of many diseases. Docosahexaenoic acid and eicosapentaenoic acid have been reported to induce autophagy in different cell types including myocardioblasts (Hsu *et al.*, 2014). The molecular mechanism underlying DHA-triggered autophagy is related to the p53/AMPK/ mTOR signal pathway.

Trans fatty acids (TFA) like vaccenic acid and elaidic acid are known to be deleterious for human health due to the decrease of LDL/HDL levels upon their consumption, causing

among others coronary heart disease. TFAs led to increased autophagy and autophagy-mediated apoptotic death of primary myofibroblasts, death that was significantly reduced in ATG5 and ATG3 knockout MEFs. Again, in this case autophagy seems not to be a cytoprotective mechanism rather than a cause of cell death (Ghavami *et al.*, 2012).

Carbohydrates and autophagy

As said above, the main carbohydrate utilized in the heart is glucose. Generally, low blood glucose elicits a systemic response due to the production of glucagon by pancreatic islets alpha cells and high blood glucose to the production of insulin by pancreatic islets beta cells. These two hormones modulate autophagy in manners that have been well studied, but environmental glucose itself can modulate autophagy because of its role in homeostasis regulation (Moruno *et al.*, 2012). It is also well known that, for example in liver, glucagon activates autophagy while insulin inhibits it.

The role of insulin in the initiation of autophagy is well known and is tightly connected to the one of amino acids. Insulin is not sufficient for mTOR activation in absence of amino acids, meaning a synergic effect is necessary. Its contribution to the amino acid mediated activation of mTOR is tissue dependent; hepatic tissue needs less insulin than skeletal muscle for autophagy induction. What insulin does is to activate phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt). Akt then phosphorylates and inhibits tuberous sclerosis complex (TSC2) and relieves inhibition on Rheb (Ras homologue enriched in brain), allowing the activation of mTOR and the subsequent inhibition of autophagy.

It is difficult to reach a consensus on the effects of environmental glucose to autophagy. On one hand glucose promotes the assembly of V-ATPase, enhancing the activity of the lysosomal hydrolases important for lysosomal degradation of autophagic cargo (Mcguire *et al.*, 2016). Glucose also inhibits mTORCI activity (Miniaci *et al.*, 2015). Conversely, extracellular glucose is generally considered to inhibit autophagy, since glucose deprivation, which causes an increase in AMP/ATP ratio, leads to AMPK activated autophagy. In vivo data interestingly shows trehalose to activate autophagy and reverse the adverse structural remodeling of the heart following myocardial infarction, markedly attenuating cardiac dysfunction. These effects are not observed in Beclin$^{+/-}$ mice, meaning that the trehalose effect is indeed autophagy mediated. Importantly, the effect was due to mitophagy activation and improved mitochondria quality control (Sciarretta *et al.*, 2018).

Amino acids and autophagy

Amino acids (leucine in particular) are known inducers of autophagy, converging at the level of mTOR activation (reviewed in (Meijer *et al.*, 2015). The amino acid activation of mTOR is independent of Akt. There are two parallel pathways that activate mTOR dependent on amino acids, the Rag GTPase-Ragulator and the Vps34-phospholipase D1 (PLD1) pathways.

Other than mediating mTOR dependent inhibition of autophagy, there are other ways amino acids could lead to autophagic modulation. One of them includes the amino acid dependent inhibition of JNK1, which leads to the formation of a stable complex between Beclin1 and Bcl-2 which sequesters Beclin1 and results in inhibition of autophagy (Hsu *et al.*, 2018).

Finally, amino acid catabolism may actually result in increased cytosolic Acetyl-CoA. The increase of cytosolic Acetyl-CoA has been shown to inhibit autophagy by stimulating mTORC1 signaling. More specifically the acetyltransferase EP300, responsible for the inhibitory acetylation of several ATG proteins, because of its low affinity (high *K*m) for Acetyl-CoA, acts as a sensor of cytosolic Acetyl-CoA which translates increases in cytosolic Acetyl-CoA into inhibition of autophagy. Thus, mitochondrial amino acid catabolism would control the inhibitory acetylation of ATG proteins and regulate the autophagic flux.

Amino acid metabolism and cardiac diseases

The contribution of amino acid metabolism to cardiac diseases has been understudied. Most studies concerning amino acids and cardiac function show how their supplementation in ischemic conditions might impact on cardiac physiology. However, not much is known about endogenous amino acid metabolism and a possible cardioprotective effect.

In the last years the role of a specific subset of amino acids, the Branched Chain Amino Acids (BCAAs) to the development of heart failure has been studied. Branched chain amino acids are leucine, isoleucine and valine, and they are catabolized in a complicated two step pathway.

BRANCHED CHAIN AMINO ACIDS

Branched chain amino acids (BCAAs) are essential amino acids that are not synthesized in our bodies but must be acquired by diet. L-type amino acid transporters and

bidirectional transporter for l-glutamine and l-leucine/EAA appear to play a major role in BCAA entry and activation of downstream signaling (Yanagida *et al*., 2001, Bodoy *et al*., 2005, Verrey *et al*., 2004). BCAA are catabolized in a strictly regulated, two-step process, and their catabolic products are Acetyl-CoA and Succinyl-CoA, making them important to fuel the TCA cycle. Apart from being building blocks and energy sources, BCAA regulate the release of hormones (for example, leptin, GLP-1 and ghrelin) that can potentially affect food intake and glycaemia levels and are known activators of the mTOR complex I and glucose signaling (Zhang *et al*., 2017). The tight regulation of BCAA levels is important to maintain cellular homeostasis and health. Indeed, impaired BCAA metabolism causes genetic disorders.

In the beginning it was controversial whether BCAA rich diets where beneficial in regard to lean body mass in obesity and metabolic disorders where good or bad for metabolic disease. In clinical studies increased levels of BCAA are positively correlated with insulin resistance and metabolic diseases, and the association was even more clear after unbiased proteomic approaches in a big cohort of patients and healthy individuals. Even though it is not yet proven completely, evidence supports that the correlation is causative. BCAA infusion in humans worsens insulin sensitivity and when combined with high fat diet also in rodent models. Also, importantly, a study on people that at the time were completely healthy, showed that elevated BCAA levels was the most strongly associated signature of the individuals that after 10 years developed diabetes. Finally, pharmaceutical induction of BCAA catabolism and reversal of elevated levels of BCAA in a rat model of diabetes improved insulin resistance (Lynch e Adams, 2014; Bloomgarden,

2018). In any case the mechanism or mechanisms that promote insulin resistance upon BCAA increase is not fully understood, but there are many valid hypotheses.

BCAA activate the mTOR kinase and downstream targets that might induce the insulin resistance (Yoon, 2016). Indeed, the effect was partially reversed by the use of sirolimus, a specific inhibitor of mTOR. The second main hypothesis is BCAA dysmetabolism, thus the elevations in BCAA are not per se what result in insulin resistance, but the accumulation of their catabolic intermediates, which on one hand could be toxic if excessive, or could mean enhancement of BCAA catabolism and improper beta-oxidation, accumulation of fatty acid intermediates and subsequent mitochondrial dysfunction that results in stress and insulin resistance. It is however true that the increase of BCAA catabolism theory is opposite to the fact that that insulin resistant models have a decrease in the transcript and protein levels of genes involved in BCAA catabolism in liver, skeletal muscle and adipose tissue.

Mutations in BCKDHA, BCKDHB and DBT genes, all involved in the catabolism of the BCAA, have been found to cause an autosomal recessive disease called Maple Syrup Urine Disease (MSUD), name given by the characteristic smell of patients' urine (Stojiljkovic *et al*., 2016). Patients with this disorder have increased levels of BCAAs in the plasma and increased a-ketoacids in the urine and can be easily treated by simple BCAA dietary restriction. There are different clinical variants of the disease. The most frequent is also the most severe, and includes metabolic acidosis, encephalopathy, feeding difficulties, liver failure, and early death. Besides the biochemical hallmarks of MSUD, patients have increased levels of lactate, alanine, and α-ketoglutarate, which are related to mitochondrial dysfunction. The neurological features of the disease are mostly

due to the importance of BCAA metabolism in glutamate homeostasis, important for brain development and cognitive functions. Also, excessive leucine affects water homeostasis within the subcortical grey matter resulting in brain swelling and oxidative stress, whereas, a-ketoisocaproic acid, intermediate in leucine metabolism, acts as a neurotoxin in the encephalopathic syndrome (Sgaravatti *et al, 2003*). Intermediates of BCAA catabolism are also found accumulated in the methylmalonic and propionic organic acidurias (Manoli e Venditti, 2016). Interestingly, apart from the major clinical features that are similar to the ones of MSUD patients suffer also of dilated and hypertrophic cardiomyopathies (Bhan e Brody, 2001; Prada *et al.*, 2011). The mechanism is uncertain but may result from energy deprivation or toxic accumulation.

Catabolism of Branched Chain Amino Acids

Unlike many of the other amino acid metabolic/catabolic activities that take place in liver, the first step of BCAA catabolism occurs in the cytosol of brain, muscle, and many non-hepatic cells where BCAA are converted into branched-chain α-keto-acids (BCKA) by branched-chain amino-transferase (BCAT). BCKA are then transported to the mitochondrial matrix and are oxidized by the branched-chain α-keto acid dehydrogenase (BCKD) complexes and eventually degraded into acetyl-CoA or succinyl-CoA to feed the TCA cycle. The BCKD-mediated reaction is the rate-limiting step in the BCAA catabolic pathway and its activity determines the overall level of BCAA/BCKA. Therefore, BCKD expression and activity is subjected to tight regulation to maintain BCAA homeostasis in response to external nutrient and growth signals. BCKD holoenzyme activity is

determined by the phosphorylation status of its regulatory subunit E1α. When BCAA levels are low, E1α is phosphorylated by a specific BCKD kinase, leading to inhibition of BCKD activity and preservation of free BCAA. When BCAA levels are high, E1α is dephosphorylated by a BCKD phosphatase, leading to BCKD activation and to the reduction in total BCAA. A mitochondrial-targeted 2C-type ser/thr protein phosphatase named PP2C in mitochondria (PP2Cm or also PPM1K) was discovered based on genome scanning (Lu *et al.*, 2009).

PPM1K is a serine/threonine protein phosphatase that resides in the inner mitochondrial compartment, the mitochondrial matrix. Proteomic and biochemical analyses established that PPM1K is the endogenous BCKD phosphatase responsible for BCAA-induced dephosphorylation and activation of BCKD. Apart from the BCKD E1 subunit there is no other known substrate for PPM1K, thus up to date its only role regards the BCAA catabolism. Its orthologue in yeast is Aup1p (autophagy-related protein phosphatase), which was identified in a screening for protein phosphatases that functionally interact with Atg1p, an autophagy related protein kinase in S. cerevisiae. Indeed, Aup1p is localized in mitochondria and is required for survival in stationery phase cells by being necessary for proper mitochondrial clearance though autophagy (Tal *et al.*, 2007). In mammals it is not yet known a similar role for PPM1K.As expected, PPM1K -deficient mice have impaired regulation of BCKD activity, leading to significantly higher plasma levels of BCAA and BCKA either at basal or following ingestion of a high dose of BCAA.

In zebrafish embryos and adult mice, PPM1K is highly expressed in cardiac muscle cells as well as in the central nervous system, a pattern which correlates well with BCKD activities observed in these tissues. Interestingly, in the mouse failing hearts BCAA catabolism scores among the most significantly altered metabolic changes (Sun *et al.,* 2006). BCAA catabolic gene expression diminishes and intramyocardial BCKAs accumulate. The expression of PPM1K in heart is indeed dynamically regulated by stress, as measured at both mRNA and protein levels, with significantly reduced expression in hypertrophic and failing hearts (Lu *et al,*. 2007). However, turning-off BCAA catabolic flux can be a compensatory step to preserve free amino acids to provide building blocks for protein synthesis required for cellular growth during cardiac hypertrophy.

Interestingly, impairment of BCAA catabolism by genetic ablation of PPM1K gene per se impairs cardiac function in mice and promotes pressure overload–induced heart failure, associated with elevated superoxide production, oxidative injury, and profound metabolic changes in the heart. Supplementation with BCAAs after pressure overload protects cardiac function in PPM1K deficient mice. In zebrafish, PPM1K genetic inactivation leads to a dose-dependent loss of cardiac contractility and premature death (Sun *et al.*, 2016). These data suggest the importance of BCAA catabolism to the metabolic reprogramming of the stressed heart. However, the exact mechanisms by which PPM1K and BCAA catabolism controls heart function is unknown.

Potential mechanisms of PPM1K-dependent cardiac regulation

The central role of PPM1K in BCAA metabolism posits that the cardiac changes observed in PPM1K deficient mice might be due to an alteration in BCAA catabolism. However,

several possible mechanisms exist to explain the connection between PPM1K and heart function:

1. Increase of BCAA levels due to their impaired catabolism might lead to chronic induction of mTOR activity. In the heart, mTOR activity is directly implicated in cardiac hypertrophy. In addition, BCAA-induced mTOR activation suppresses autophagy, that as discussed above is crucial for cardiac physiology.
2. Altered BCAA/BCKA levels can inhibit pyruvate and fatty acid transport and utilization, sensitizing the heart to ischemia reperfusion insult. Because cardiac tissue has a constant high demand for pyruvate and fatty acid as its main fuel source, chronically elevated BCAA/BCKA can potentially impair the bioenergetic homeostasis of the heart, leading to contractile dysfunction and accelerated heart failure under increased afterload or during aging.
3. Defects in the mitochondrial respiratory chain due to impaired flux of BCAA intermediates.

In addition, because PPM1K-deficient mitochondria are more susceptible to calcium-induced permeability transition pore opening in the absence of external BCKA/BCAA challenge and generate elevated levels of reactive oxygen species (ROS), PPM1K defects in cardiac function observed in these knockout mice might not be dependent on PPM1K role in BCAA catabolism regulation (Lu *et al.*, 2009).

Aim of the thesis

The general aim of this thesis is to understand the relationship between mitochondria and autophagy, with a focus on heart biology. In this work we dissect molecularly two different facets of mitochondrial biology related to autophagy: BCAA-catabolism and its role in autophagy, and the role of the deubiquitinating enzyme USP8, involved in EGFR signaling and mitophagy, in heart mitochondrial function and more generally in heart function.

The mitochondrial matrix phosphatase PPM1K controls autophagy by regulating branched chain amino acid catabolism.

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Summary

Tissues can adapt to availability of different substrates by activating specific catabolic pathways that in most instances converge on mitochondria. Yet, how fluxes of metabolites through these organelles affect cellular processes is unclear. Here we show that PPM1K, a mitochondrial matrix protein phosphatase that controls the rate limiting step of branched chain amino acid (BCAA) catabolism, modulates mTORC1 activation and autophagy. PPM1K levels directly correlated with increased autophagy and reciprocally, PPM1K was induced upon starvation *in vitro* and *in vivo*, including in tissues where BCAA catabolism is considered marginal, like heart. Steady state metabolomics of labeled Leucine metabolites revealed that in the absence of PPM1K, TCA cycle intermediates were as expected decreased, whereas Leucine, its ketoisocaproic ketoacid and surprisingly methionine were increased, potentially explaining the mTORC1 dependent autophagy inhibition. Our data suggest how mitochondrial BCAA catabolism can be sensed by mTORC1 to modulate autophagy.

Introduction

Autophagy, a process in which proteins, lipids and whole organelles are transported to lysosomes for degradation, is central in quantity and quality control of molecules and organelles. Beyond quality control, autophagy is required for numerous functions, from defense against pathogens, to innate immunity, inflammation and metabolism. Because of these housekeeping functions, autophagy is not surprisingly regulated at transcriptional, posttranscriptional, and posttransductional level and key autophagy regulators respond to changes in metabolite and energy levels, to fulfill the archetypal and ancestral function of autophagy of responding to nutrient availability changes.

Initiation of autophagy mainly depends on two kinases: AMP activated kinase (AMPK), whose activation triggers it (Mao e Klionsky, 2011), and the mammalian target of rapamycin (mTOR) complex I (mTORC1) which exerts the opposite function (Noda e Ohsumi, 1998; Russell *et al.*, 2014). Both act upstream of the major complexes responsible for autophagosome formation: AMPK works as an energy sensor, monitoring the AMP/ADP ratio; mTORC1 as a nutrient and growth factors sensor. mTORC1 is a multiprotein complex formed by mTOR, by the regulatory associated protein of mTOR (Raptor), by the mammalian lethal with SEC13 protein 8 (mLST8), by the 40-kDa proline-rich Akt substrate (PRAS40), and by the DEP domain-containing mTOR-interacting protein (DEPTOR) (Laplante e Sabatini, 2012). mTORC1 can localize in different cellular compartments to respond to different stimuli (Zhou *et al.*, 2015). For example, amino acid (AA) sensing by mTORC1 occurs on the surface of the lysosome, where AAs activate the Rag GTPase complex to recruit mTORC1 in a Ragulator dependent manner (Sancak *et al.*, 2008; Sancak *et al.*, 2010; Bar-Peled *et al.*, 2012; Kim e Kim, 2016); when AAs are

absent, the Rag GAP complex (GATOR1) inactivates the Rag GTPases, leading to mTORC1 dissociation from the lysosomes (Bar-Peled *et al.*, 2013). Early studies indicate that mTORC1 is stimulated mainly by branched chain amino acids (Leucine, isoleucine and valine, BCAA) and particularly by Leucine (Lynch, 2001; Jewell *et al.*, 2015). Of note, while BCAA can be for sure generated by lysosomal hydrolases during autophagy, their catabolism (that controls their availability for mTORC1 activation) occurs in the mitochondrial matrix, raising the question of whether also mTORC1, like AMPK, can be controlled by mitochondrial inputs.

BCAA are central in systemic metabolism: their plasma levels regulate body weight, glycemia and satiety (Lynch e Adams, 2014) and their level are strictly regulated, because their deficiency leads to developmental impairment and their accumulation because of defective catabolism to metabolic diseases like Maple Syrup Urine Disease (MSUD) (Burrage *et al.*, 2014). Dietary BCAA are catabolized in a two-step process. In skeletal muscle they are converted to their corresponding branched chain α-Keto acids by the branched chain aminotransferase (BCAT). Branched chain α-Keto acids are then transported mainly to the liver, where they enter the mitochondrial matrix to be converted to acetyl- and succinyl-CoA that eventually feed the TCA cycle. This step is catalyzed by the branched chain amino acid dehydrogenase (BCKD), which is activated by its dephosphorylation by PPM1K (Lu *et al.*, 2009).

PPM1K is a serine threonine phosphatase of the PP2C family localized in the mitochondrial matrix, where it dephosphorylates the E1 subunit of the BCKD complex. Additionally, PPM1K can perhaps dephosphorylate, en route to mitochondria, the cytoplasmic enzyme ATP-citrate lyase (ACL) (White *et al.*, 2018). Models of PPM1K

depletion or downregulation have been used to study the systemic effects of impaired catabolism of BCAAs. Unexpectedly, these animal models develop cardiovascular disease apart from the metabolic one, but the molecular mechanism is yet unknown, albeit activation of the permeability transition pore (PTP), an unselective inner mitochondrial membrane large conductance channel (Bernardi, 1999), has been implied (Lu *et al.*, 2007). Interestingly, Aup1p, the yeast orthologue of PPM1K, regulates mitophagy (Tal *et al.*, 2007), suggesting a role for this phosphatase in autophagy. Yet, whether and how the key regulator of BCAA catabolism controls autophagy in the heart and in other tissues has not been investigated. We therefore set out to test if PPM1K participates in autophagy. We show that PPM1K stimulates autophagic flux in all the systems tested, including adult cardiomyocytes, by modulating BCAA accumulation and mTORC1 activation. Our data suggest a role for impaired autophagy in the cardiac defects associated to PPM1K deletion and imply mitochondrial BCAA catabolism in mTORC1 dependent autophagy.

Results

PPM1K levels control autophagy

Because Aup1p, the yeast orthologue of PPM1K, affects mitophagy, a form of selective autophagy, we wished to investigate if any relationship between PPM1K and autophagy was retained also in higher eukaryotes. To this end, we transiently overexpressed increasing amounts of V5-tagged PPM1K in different cell types (HeLa, HepG2, MEFs) and observed by immunoblotting a dose dependent increase in the levels of the autophagic marker LC3II (Fig. 1A and 1B). 3D volume rendering of stacks of confocal images of mitochondrially targeted dsRED (mitoRFP) and YFP-LC3 confirmed the accumulation of LC3 positive puncta, a bona fide marker of autophagosomes, that partially colocalized with mitochondria in cells overexpressing PPM1K (Fig. 1C). Because LC3-II accumulation can result from impairment of autophagic flux, or from stimulation of autophagy, we blocked autophagic flux by treating cells with the lysosomal inhibitor chloroquine. While as expected chloroquine resulted in the accumulation of LC3II in control transfected cells, it did not further increase the levels of LC3II in PPM1K overexpressing cells, suggesting that PPM1K overexpression inhibits autophagic flux (Fig. 1D). To further confirm that PPM1K levels positively correlate with autophagic flux, we turned to a silencing approach. Efficient shRNA mediated PPM1K downregulation (Suppl. Fig. 1A) inhibited the physiological increase of LC3-II normally observed upon starvation-induced autophagy (Fig. 1E) and led to accumulation of the autophagic cargo P62 (Suppl. Fig. 1D). LC3-II levels remained lower also when PPM1K-downregulated and starved cells were treated with the lysosomal acidification inhibitor bafilomycin (Fig. 1F), further supporting that PPM1K downregulation impairs autophagosome formation. Similar

results were obtained by imaging of a mCherry-GFP-LC3 sensor that detects autophagic flux based on the different stabilities of mCherry and GFP in the acidic lysoautophagosome environment. Indeed, upon autophagosome fusion with lysosomes, the GFP moiety attached to LC3 is readily degraded, leading to the "switch" of the biosensor from the green to the red channel. Conversely, when lysosomal acidification is inhibited (e.g., by chloroquine or bafilomycin), GFP is not degraded and yellow lysoautophagosomes are retrieved. In PPM1K silenced cells, the number of yellow LC3 puncta (representing autophagosomes) was not significantly altered in basal conditions, whereas they greatly decreased upon starvation-induced autophagy. In starved cells, PPM1K silencing led to the almost complete disappearance of the red LC3 puncta, corresponding to the lysoautophagosomes. Blockage of autophagic flux with chloroquine did not restore autophagosome number in PPM1K silenced cells to the wild type one, confirming that early steps of autophagy signaling and autophagosome formation are inhibited by PPM1K downregulation (Fig. 1G). We recapitulated the effects of PPM1K overexpression and downregulation on autophagy also in the human cell line HepG2, indicating that the effect is not specific for mouse (Suppl. Fig. 1B and 1C).

Because PPM1K deletion in vivo affects heart development and function (Lu *et al.*, 2007), we wished to verify if PPM1K modulated autophagy also in isolated adult cardiomyocytes. To this end, we developed tools to overexpress and downregulate PPM1K by adenoviral transduction and we devised a protocol to gently isolate adult mouse cardiomyocytes so that they could survive in culture for 5 days (Suppl. Fig. 1E). When we infected them with the adenoviruses coding for PPM1K shRNA as well as for Flag-tagged PPM1K, leading to the expected changes in PPM1K levels (Suppl. Fig. 1F), we confirmed by quantification

of LC3-positive puncta measured by confocal microscopy in cardiomyocytes starved for 2 h that PPM1K levels positively correlate with autophagosome number (Suppl. Fig. 1G, 1H). These data indicate a linear relationship between PPM1K levels and the stimulation of autophagy in all the systems tested.

PPM1K regulates autophagy via mTORC1

We next wished to address how PPM1K depletion could inhibit autophagy. Given its known role as a modulator of PTP, deletion of PPM1K might lead to mitochondrial dysfunction and ATP depletion, impinging on the master autophagy regulator AMPK. We therefore first investigated whether upon PPM1K ablation cellular ATP levels were perturbed. However, PPM1K silencing did not alter ATP levels in cells cultured in glucose or galactose containing media (Fig. 2A). Accordingly, the effect of PPM1K on mitochondrial morphology in nutrient rich media, a sensitive proxy of mitochondrial dysfunction, were marginal and PPM1K knockdown mitochondria could still efficiently elongate upon starvation (Suppl. Fig. 2A), indicating that PPM1K was dispensable for autophagy induced mitochondrial elongation. Finally, unlike mitochondrial fusion deficient cells that die during starvation (Gomes *et al.*, 2011), PPM1K deficient cells survived to limited nutrient availability (Suppl. Fig. 2B). Indeed, even during starvation, levels of phosphorylated AMPK, reflective of AMPK activation, were not affected by PPM1K knock-down (Fig. 2B). These results indicate that PPM1K downregulation does not impinge on AMPK activity.

We then measured mTORC1 activation by inspecting the dephosphorylation of phosphorylated S6 (p-S6) ribosomal protein and we noticed that levels of p-S6 were

increased upon PPM1K silencing, even upon starvation when they are known to decrease because of mTORC1 inhibition (Fig. 2C). To verify if the observed reduction in LC3-II and the increase in p-S6 caused by PPM1K depended on mTOR activity, we treated cells with the mTORC1 inhibitor rapamycin. Rapamycin fully inhibited S6 phosphorylation and normalized LC3-II levels in PPM1K silenced cells, suggesting that PPM1K deletion inhibits autophagy by causing mTORC1 hyperactivation (Fig. 2D).

To understand if the mTORC1 hyperactivation recorded upon PPM1K silencing was a consequence of decreased BCAA catabolism, we resorted to a metabolomics-based analysis of Leucine metabolites in cells where PPM1K was genetically silenced/overexpressed. For this purpose, we measured by gas-chromatography mass spectrometry (GC-MS) steady state levels of 33 different metabolites (Table 1) upon feeding ^{13}C-Leucine to control and PPM1K silenced cells undergoing 2 hours of starvation While we could not measure any significant change in the metabolic profile of PPM1K overexpressing cells (Fig. 3B), probably because the transient overexpression strategy yielded a mixed population of transfected and untransfected cells that confounded the measurements, the amount of several metabolites was different in cells lacking PPM1K (Fig. 3A). Levels of the TCA cycle intermediates succinate, oxoglutarate and malate were significantly reduced in starved PPM1K silenced cells, compared to control starved MEFs. This is not surprising, given that cells are starved of glucose to drive TCA anaplerosis via pyruvate carboxylation and that Leucine catabolism, blocked by PPM1K silencing, represents a major source of acetyl-CoA (Fig. 3C). However, the most surprising finding, in addition to the non-significant, yet evident accumulation of Leucine and of its ketoacid a-ketoisocaproic (aKIC) acid, was the retrieval of accumulated Methionine. While it is

unknown that Leucine can be converted by mammalian tissues into the essential aminoacid Met, the latter has a role in the regulation of mTORC1 activity via its recently identified modulator SAMTOR that senses S-adenylmethionine (SAM) levels (Gu *et al.,* 2017). Altogether, these data indicate that PPM1K silencing inhibits starvation induced autophagy by causing accumulation of Leucine, and surprisingly of Met, that can both lead to mTORC1 persistent activation.

PPM1K expression is induced in response to fasting.

Our data suggest that PPM1K deletion might impair heart function by impinging on autophagy. However, whether and to which extent PPM1K is expressed in tissues other than liver and how starvation impacts on PPM1K levels is poorly characterized. We therefore decided to analyze PPM1K expression levels in different mouse organs and observed that PPM1K is differentially, yet ubiquitously expressed in all tissues checked (Fig. 4A), and particularly in the heart (Fig 4A), as previously reported (Zhou et al., 2012). However, the original report that levels of PPM1K were known to decline during starvation, when mTORC1 is inhibited and autophagy are activated is in contrast with our finding that PPM1K reduction inhibits autophagy. We therefore starved adult mice and found that contrary to what expected, at 24, but especially 48 h PPM1K protein levels increased in all tissues examined: skeletal muscle, liver and heart (Fig. 4B), where the increase happened in cardiomyocytes, as indicated by immunoblotting for PPM1K of heart muscle cells purified from fed and fasted animals (Fig. 4C). We confirmed that this increase was functional, by measuring phosphorylated BCKD that decreased concomitant to PPM1K increase upon starvation (Fig. 5A). Mechanistically, the

accumulation was due to an increase in PPM1K mRNA levels in heart (Fig. 5B) and liver (data not shown) during the first 24 hours of fasting. Because PPM1K protein is stable for more than 12 hours, as determined by a classic cycloheximide protein stability assay (Suppl. Fig 3A), PPM1K accumulates not only at early timepoints of fasting, but even at 48 h, when PPM1K transcript levels returned to baseline. We further investigated how PPM1K levels can be physiologically induced. *In vivo* rapamycin administration did not increase PPM1K levels (Fig. 5C), ruling out a role for mTOR activity in PPM1K levels regulation. On the other hand, PPM1K protein levels were higher in fed mice drinking water supplemented with BCAAs (Fig. 5D), suggesting that levels of dietary BCAAs can modulate their own catabolism.

Discussion

Metabolic reprogramming is well recognized as an integral part of cardiac pathological remodeling, but limited knowledge is available on the role of amino acids catabolism in the failing heart (Huang *et al.*, 2011; Drake *et al.*, 2012). The catabolism of branched chain amino acids is essential for on cardiac health. In humans, mutations in genes involved in BCAA catabolism cause different genetic disorders (Chuang *et al.*, 2006; Manoli e Venditti, 2016). Even though their clinical manifestations are mainly neurological, propionic and methylmalonic acidemia are associated with dilated and hypertrophic cardiomyopathies (Prada *et al.*, 2011; Riemersma *et al.*, 2017). In addition to that, animal models with impaired BCAA catabolism develop cardiac diseases. Specifically, loss of PPM1K, the key phosphatase of the rate limiting step of BCAA catabolism, apart from causing the expected metabolic syndrome, leads to progressive heart failure in mice and zebrafish (Lu *et al.*, 2007; Sun *et al.*, 2011)

We provide evidence that PPM1K modulates autophagy by impinging on mTORC1.

Deletion of PPM1K can trigger PTP opening and mitochondrial dysfunction (Lu *et al.*, 2007); however, in our experimental setting mitochondria lacking PPM1K can still elongate upon starvation and the mitochondrial dysfunction sensor AMPK is not activated, ruling out that the observed change in autophagy is a consequence of mitochondrial dysfunction. We therefore set out to investigate if autophagy was inhibited because mTORC1 was activated in cell lacking PPM1K. Importantly, pharmacological mTOR

inhibition could normalize autophagic flux even in the absence of PPM1K, substantiating a role for this key energy sensor in PPM1K mediated autophagy.

How deletion of a mitochondrial BCAA catabolism gene impacts on mTORC1 regulation is still unclear. Our targeted metabolomics data point to a reduction in the TCA cycle intermediates succinate, oxoglutarate and malate, and to an accumulation of Met (together with Leucine and its α-ketoacid α KIC) in cells lacking PPM1K. Met and its downstream metabolite SAM have been recently identified to activate mTORC1 via the SAMTOR. Further experiments are required to understand how Met could accumulate in cells lacking PPM1K and whether the observed effects on autophagy are caused by Met and/or SAM accumulation via SAMTOR (Gu *et al.*, 2017). Irrespectively, our data place mitochondrial BCAA catabolism in the context of autophagy regulation and indicate that inhibition of this catabolic pathway might lead to increased autophagy, calling for an analysis of BCAA metabolism in conditions where autophagy is augmented.

While the common notion is that BCAA catabolism is restricted in the liver, our data indicate that on the other hand PPM1K is expressed in multiple tissues, including the heart, and that it is upregulated upon fasting and by increased BCAA dietary content, even in the fed animal. Because increased PPM1K curtails autophagy, it is tempting to speculate that BCAA-low diets might exert their reported beneficial effects on weight and metabolic parameters by activating autophagy in vivo (Cummings *et al.*, 2018).

Our data suggest that the heart pathology caused by PPM1K deletion might be the consequence of impaired autophagic flux. A wealth of data is accumulating on the role of defective autophagy in heart disease In the myocardium, defects in autophagy cause

cardiac dysfunction and heart failure, like in Danon and Pompe diseases (Fayssoil, 2008; Rowland *et al.*, 2016). Even though autophagy is naturally stimulated upon stress (Kroemer *et al.*, 2010), accumulation of autophagosomes is observed in coronary artery disease, hypertension, aortic valvular disease and congestive heart failure in both humans and animal models, suggesting that impaired autophagic flux correlates well with cardiovascular disease (Tanaka *et al.*, 2000; Miyata *et al.*, 2006; Cebollero *et al.*, 2012). It has been also demonstrated a cardioprotective role of autophagy induction (Sciarretta *et al.*, 2014; Zhang *et al.*, 2017).

It would be interesting then to understand if rapamycin treatment, that fully corrects autophagy in vitro in cells lacking PPM1K, can correct the heart defects caused by targeted PPM1K inhibition in vivo.

While we did not investigate the role of PPM1K in the CNS, where most of the manifestations of MSUD occur, our data raise the interesting possibility that defects in BCAA catabolism might be associated to autophagy inhibition. If this was proven true also in the CNS, autophagy reactivation might prove beneficial against the neurological complications of the non-classical MSUD, where residual enzyme activity varies from 2% to 30%. Non-classical MSUD patients are usually diagnosed at infancy or childhood because of feeding problems, poor growth, developmental delays and behavioral problems. Interestingly, the disease might progress to encephalopathy upon prolonged fasting. While this could be the consequence of massive BCAA release during fasted states, it could also suggest a link between MSUD and autophagy that remains to be explored.

Materials and methods

Cell culture and transfection

MEFs, human embryonic kidney 293 cells (HEK293) and HeLa cells were were grown in DMEM supplemented with 10% FBS (Gomes *et al.*, 2011). Unless otherwise stated, glucose was substitued with 0.9 mg/ml galactose to force ATP production by the respiratory chain (Acín-Pérez *et al.*, 2004).

For transient overexpression of PPM1K, cDNA for PPM1K was amplified by PCR and cloned in pCDNA3.1 and pAd-Track vectors. The primers used for PCR amplification were PPM1K FW: 5'ATGTCAACAGCTGCC3' and PPM1K REV: 5'TCAGGCCCATCGTCCACTGGAGG3' (not including the carboxyterminal insertions of FLAG and V5 tags).

For transient downregulation of PPM1K, silencing PPM1K oligonucleotide was cloned in pLKO.1 vector or pRNAT-H1.1/Adeno (Genscript) vector. shScrambled was used as control. Silencing PPM1K oligonucleotide sequence: ATGAAGCTGACCACTGACCAT; scramble sequence: AGCAATATTACGTGTGGACGC.

Transfections of shRNA vectors and pCDNA3.1 vectors were performed using polyethilenimmine (PEI) in a 3:1 PEI:DNA ratio.

For adult murine cardiomyocyte isolation, mice were injected with 20 units of heparin for 30 minutes and then sacrificed. Heart was then isolated and aorta was ligated in order to perfuse it with perfusion and digestion buffers in a constant flow perfusion system with the use of a Stereoscope (Motic SMZ-171). The hearts were then minced and mechanically dissociated. Calcium was then reintroduced and cells were plated in 20µg/lt

laminin (Sigma-Aldrich) coated dishes. After one hour, plating medium was removed and cells were grown in M199 supplemented wit 0,2% BSA, ITS, blebbistatin and antibiotics. When indicated, starvation was achieved by culturing cells in HBSS media (GIBCO) for the indicated time points.

To induce autophagy cells were treated with 200 nM rapamycin while the block of the autophagic flux was achieved supplementing the culture media with 25mM chloroquine or 200nM Bafilomycin A1 as indicated. The measuarement of the ATP levels was performed using ATPlite Luminescence Assay System (Perkin Elmer).

Adenoviral production

Adenoviruses were produced according to manufacturer indications with the protocol of AdEasy Adenoviral Vector system (Agilent).

Real time quantitative PCR

RNA from organs and cells was isolated by TRIZOL-chloroform extraction. RT-PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, 4368814). qRT-PCR analyses were carried out on retrotranscribed cDNAs with QuantStudio5 (Applied Biosystems). Expression levels are given relative to Gapdh. Primers used were: Ppm1kFW:5'TCCTCGGGAGAAAGACTTGGA3 and Ppm1kRV: 5'GAGGTCAGGAGGCTTGCATCT3',

Gapdh FW: 5'TGCACCACCAACTGCTTAGC3' and Gapdh RV: 5'GGCATGGACTGTGGTCATGAG3'.

Immunoblotting

For SDS-PAGE experiments, proteins were separated on Any-KD (BioRad) polyacrilamide precasted gels, transferred onto PVDF membranes (BioRad) and probed using the indicated antibodies and isotype matched HRP-conjugated secondary antibodies. The following primary antibodies were employed at 1:1000 dilution: rabbit anti-PPM1K (Abcam); mouse anti-actin (EMD Millipore); rabbit anti-GRP75 (Santa Cruz Biotechnology); mouse anti-V5 (Invitrogen); mouse anti-Tubulin (Santa Cruz Biotechnology); rabbit anti-LC3/MAP1LC3A (Novus Biologicals); rabbit anti-BCKD E1 total (Abcam)); rabbit anti-BCKD E1-Phospho S293 (Abcam); mouse anti-Phospho-S6 Ribosomal Protein S240/244 (Cell Signaling Technology); rabbit anti-p62/SQSTM1 (MBL International Corporation); rabbit anti- Phospho-AMPKα Thr172 (Cell Signaling Technology). The bands were visualized by enhanced chemiluminescence ECL kit (Pierce). Densitometry was performed using ImageJ gel measure tool.

Immunofluorescence

Cells were fixed in 4% PFA for 20 min at room temperature. After washing in PBS, cells were permeabilized 5 minutes at RT with PBS 1% Triton X-100 and processed for immunofluorescence using the following conditions: blocking in 3% BSA in PBS 0.1% Triton X-100 for 1 hour at RT following by incubation with the primary antibody rabbit anti LC3 (Novus Biologicals) 1:200 dilution overnight at 4°C. The next day cells were washed with PBS 0.1% Triton X-100 and incubated with secondary antibody anti rabbit Alexa-Fluor 568 for 1 hour. Samples were then washed and counterstained with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen) to label cell nuclei. Images were acquired

with Leica TCS SP5 confocal microscope with a CCD camera and analyzed using Image J.

In vivo procedures

All mice procedures were performed according to protocols approved by the local Ethic committees. Mice were sacrificed by cervical dislocation and different tissues were extracted. For the in vivo treatments: 20g/L of BCAAs were added in the water for 48 hours, rapamycin was administered at 1 mg/Kg i.p.. During starvation experiments mice had full access to water.

Metabolomic analysis.

Wild-type and PPM1K knocked-down fibroblasts were seeded and starved culturing them in HBSS. After 4 hours of starvation, heavy (13C) or light (12C) Leucine were added to the culture medium and cells were harvested after 2 hours. Sample were dried and snap frozen at -80°C. Dried samples were then resuspended in methanol, transferred into glass tubes and analyzed by gas-chromatography mass spectrometry (GC-MS) (Enot *et al.*, 2015).

The final data tables are made with the characteristics (peak area, height, m/z, retention time) of the potentially isotopically precursors (labelled as M0) and those of their potential isotopomers (labelled as M1/M2/M3 etc depending on the metabolite). All statistical analyses and data representations were performed on pre-processed log2-transformed peak area. For differential analysis, statistics on the log2(M1+M2+M3 ecc/M0) data

matrices when comparing PPM1K silenced cells to the wild-type ones. Log2 (Fold change) and -log10(p-value) were used to depict the volcano plots on Figure 3A and 3B.

Statistical analysis

Experiments were displayed graphically (bar or line charts) as mean and SEM (Standard Error of the Mean) or S.D. Values were tested for significance by the one-sample T-test or one-way ANOVA with a Tukey's posttest. Results were considered significant when *p< 0.05.

Acknowledgements

This work was supported by Fondation Leducq TNE016004 and by FIRB Automed to LS

Figure legends

Figure 1. PPM1K is required for macroautophagy

(A) Western blot analysis of LC3 levels upon transient overexpression of PPM1K from total mouse fibroblast lysates.

(B) Bar graph represents the densitometric analysis of LC3II levels normalized to tubulin upon PPM1K overexpression.

(C) Representative confocal images of live mouse fibroblasts overexpressing PPM1K or empty vector following transfection of mito-RFP and LC3-GFP.

(D) Mouse fibroblasts were transfected with PPM1K encoding vector or empty vector and treated with chloroquine or vehicle as control. Shown is western blot analysis of LC3 levels from total cell lysates.

(E) Western blot analysis of LC3 levels upon transient downregulation of PPM1K with shPPM1K from total mouse fibroblast lysates. Cells were starved for the indicated time periods. Scrambled was used as control.

(F) Western blot analysis of LC3 levels upon transient downregulation of PPM1K with shPPM1K or scrambled plasmid from total mouse fibroblast lysates. Cells were starved for two hours and treated with bafilomycin.

(G) Representative confocal images of live mouse fibroblasts transfected with ShPPM1K and mCherry-EGFP-LC3. Cells were cultured in full medium or starved and treated with chloroquine as indicated. shScrambled was used as control.

Figure 2. PPM1K regulates autophagy via –mTORC1

(A) Normalized levels of ATP in cells, cultured in glucose and galactose containing media, upon transient downregulation of PPM1K with shPPM1K. Data represent mean ± SEM of 3 independent experiments. shScrambled was used as control.

(B) Western blot analysis of p-AMPK levels in total lysates from mouse fibroblasts transfected with shPPM1K or control shScrambled and starved for the indicated time.

(C) MEFs were transfected with the indicated shRNA and then starved for the indicated time, lysed and proteins were separated by SDS-PAGE and immunoblotted using the indicated antibodies. The same membrane of Fig.1C was used to immunoblot p-S6.

(D) MEFs were transfected with the indicated shRNA and then starved for the indicated time, lysed and proteins were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Where indicated, cells were treated with Rapamycin for 2 hrs.

Figure 3: PPM1K silencing reduces levels of TCA cycle intermediates and increases Methionine levels.

(A) Volcano plots relative to metabolites detected in PPM1K wild-type and knocked down cells after 13C-leucine supplementation in HBSS media. The indicated metabolites were the ones combining a p-value <0.05 and a fold change bigger than 1.2. Quadruplicates per condition were used. Statistical analysis was performed by t-test.

(B) Volcano plots relative to metabolites detected in PPM1K wild-type and PPM1K overexpressing cells after 13C-leucine supplementation in HBSS media. The indicated

metabolites were the ones combining a p-value <0.05 and a fold change bigger than 1.2. Quadruplicates per condition were used. Statistical analysis was performed by t-test.

(C) Schematic representation of Leucine catabolic pathway including the main findings of our metabolomic approach. Arrows indicate which metabolites are decreased and which increased upon PPM1K silencing. The accumulation of a-KIC and Met indicate a possible mechanism for mTOR activation in this condition.

Figure 4. PPM1K is induced in multiple tissues upon fasting.

(A) Western blot analysis for PPM1K levels in total lysates from different mouse tissues as indicated. Bar plots represent the densitometric analysis of the blot bands. N=3.

(B) Western blot analysis for PPM1K levels in total lysates from different tissues of mice starved for the indicated time points. Bar plots represent the densitometric analysis of blot bands. Data represent mean ± SEM of n ≤4 mice for each group. One way ANOVA was performed. P-value: *<0.05, **<0.01.

(C) Evaluation of PPM1K expression in whole cardiomyocyte lysates upon isolation from fed and starved mice. Shown are western blots for the indicated proteins.

(D) Evaluation of the levels of total BCKD and p-BCKD in heart and liver lysates after starvation of mice for the indicated time periods. Shown are western blots for BCKD total and p-BCKD. Bar plots represent the normalized p-BCKD/total BCKD levels after the densitometric analysis of blot bands. Data represent mean ± SEM of n ≤3 mice for each group. One way ANOVA was performed. P-value: *<0.05, **<0.01.

(E) Bar graphs represent the normalized mRNA levels of PPM1K in the heart upon starvation of adult mice for the indicated time periods. One way ANOVA was performed. P-value: *<0.05, **<0.01.

(F) Western blot analysis for PPM1K, total S6 and p-S6 in total lysates from heart and liver after treatment of mice with rapamycin for the indicated time periods. N=2 per condition.

(G) Western blot analysis for PPM1K levels in heart, liver and skeletal muscle total lysates. Where indicated mice where supplemented with BCAAs in their drinking water. N=2 per condition.

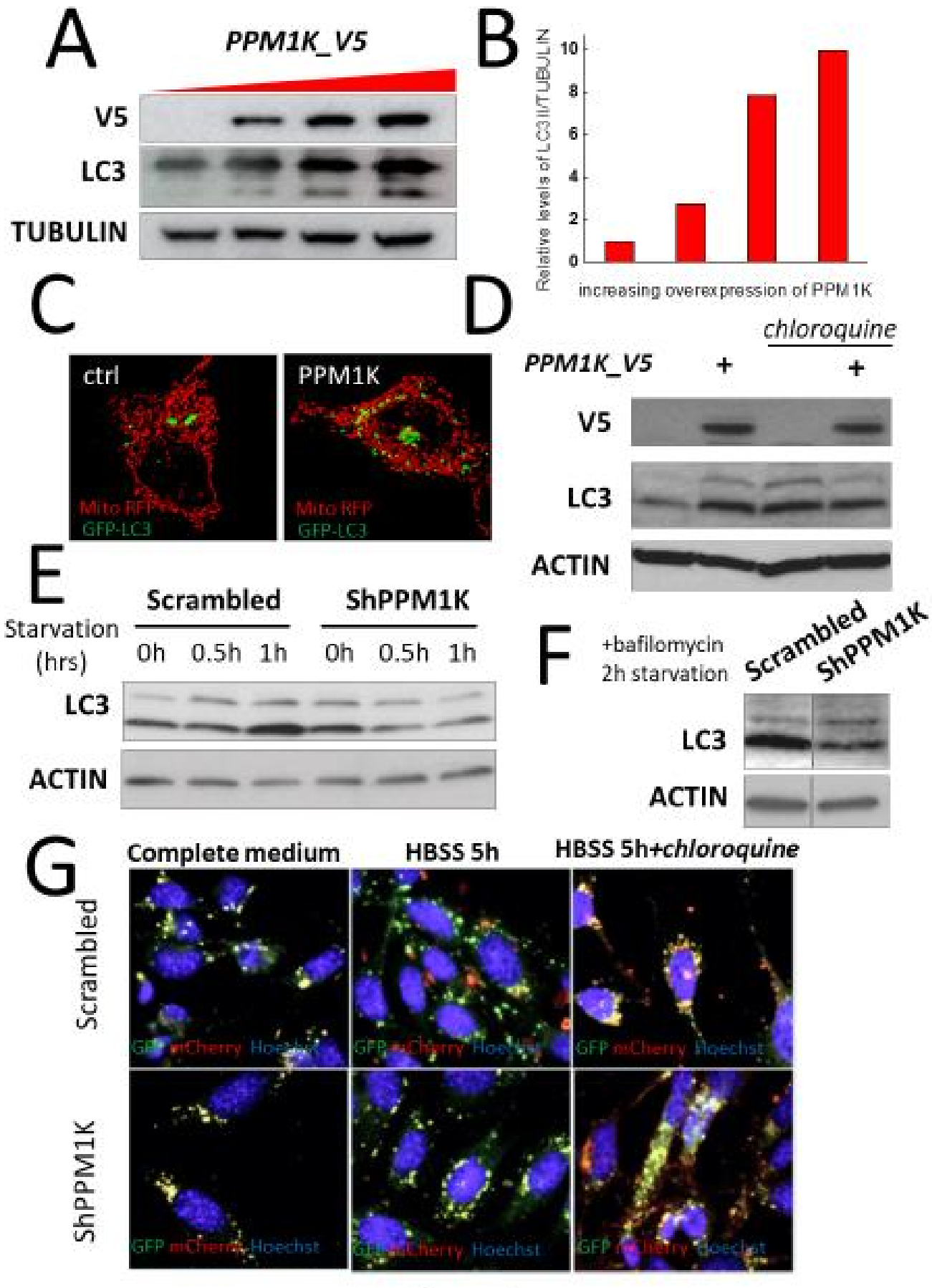
A
PPM1K_V5
V5
LC3
TUBULIN
B
Relative levels of LC3II/TUBULIN
0
2
4
6
8
10
increasing overexpression of PPM1K
C
ctrl
PPM1K
Mito RFP
GFP-LC3
Mito RFP
GFP-LC3
D
chloroquine
PPM1K_V5
+
+
V5
LC3
ACTIN
E
Starvation (hrs)
Scrambled
ShPPM1K
0h
0.5h
1h
0h
0.5h
1h
LC3
ACTIN
F
+bafilomycin
2h starvation
Scrambled
ShPPM1K
LC3
ACTIN
G
Complete medium
HBSS 5h
HBSS 5h+chloroquine
Scrambled
ShPPM1K
GFP mCherry Hoechst

Figure 1.

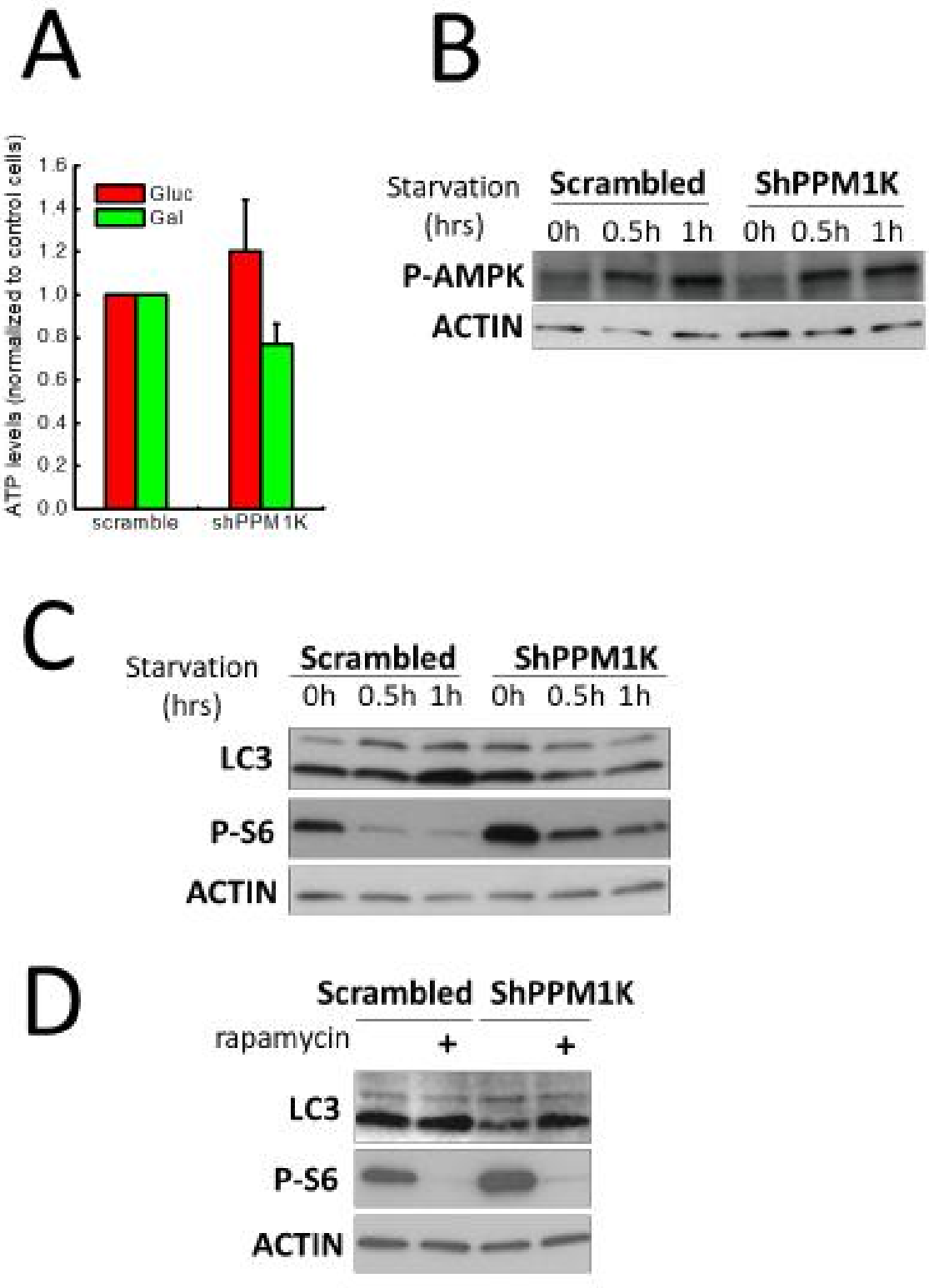
A
B
ATP levels (normalized to control cells)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Gluc
Gal
scramble
shPPM1K
Starvation (hrs)
Scrambled
ShPPM1K
0h 0.5h 1h 0h 0.5h 1h
P-AMPK
ACTIN
C
Starvation (hrs)
Scrambled
ShPPM1K
0h 0.5h 1h 0h 0.5h 1h
LC3
P-S6
ACTIN
D
Scrambled ShPPM1K
rapamycin + +
LC3
P-S6
ACTIN

Figure 2.

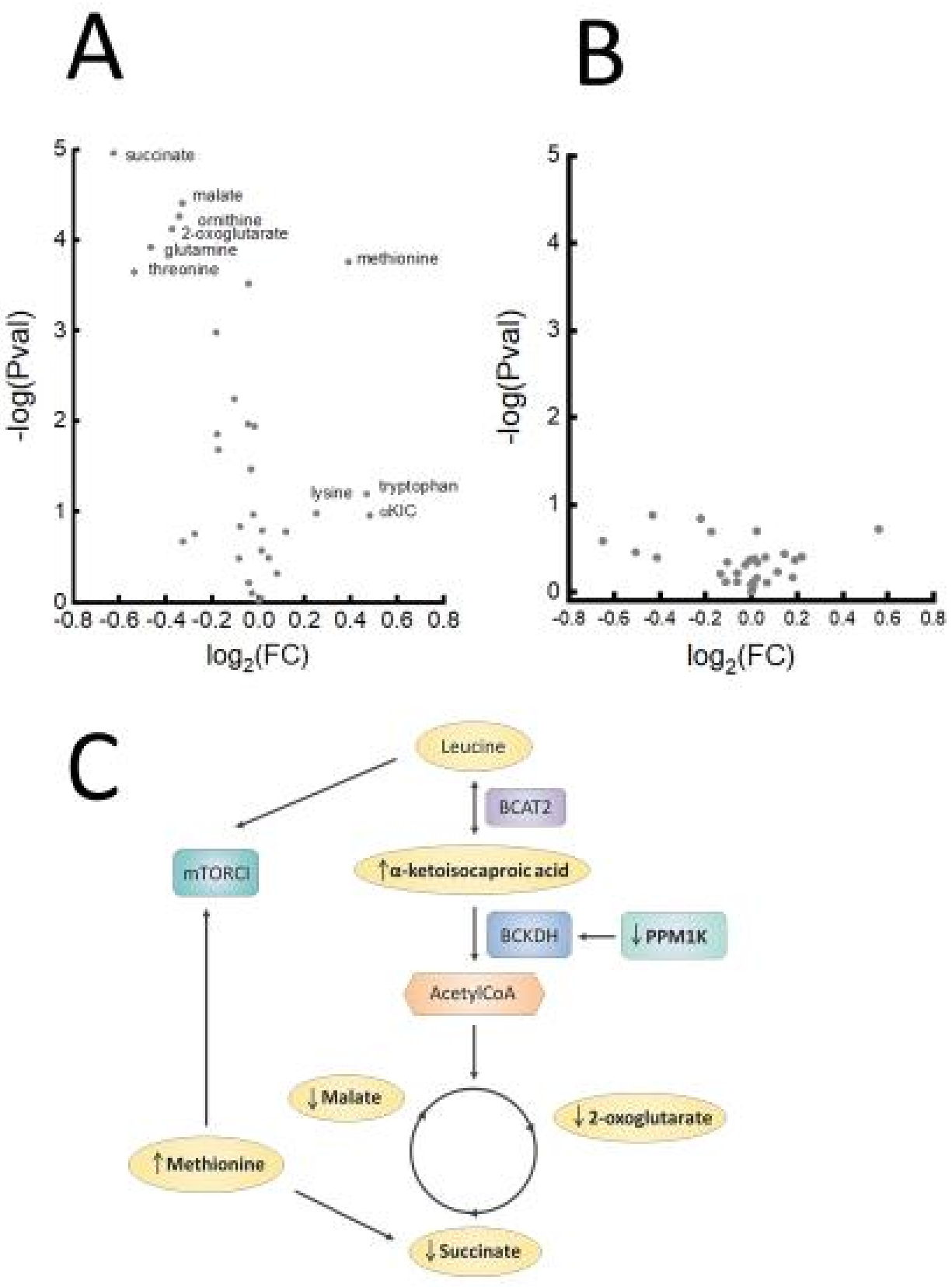
A
B
C
succinate
malate
ornithine
2-oxoglutarate
glutamine
threonine
methionine
lysine
tryptophan
αKIC
-log(Pval)
log2(FC)
Leucine
BCAT2
↑α-ketoisocaproic acid
mTORC1
BCKDH
↓PPM1K
AcetylCoA
↓Malate
↓2-oxoglutarate
↑Methionine
↓Succinate

Figure 3.

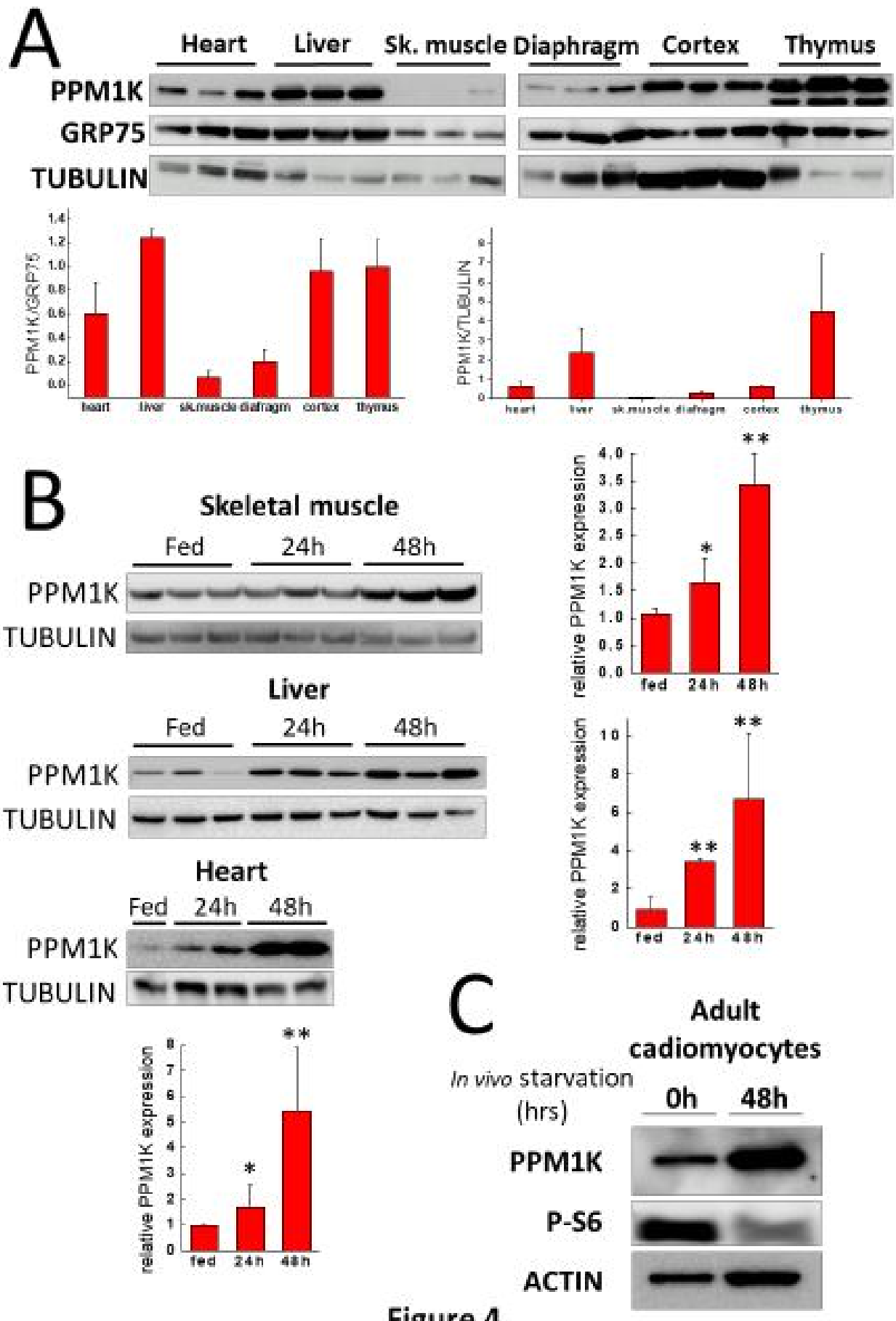
A
Heart
Liver
Sk. muscle
Diaphragm
Cortex
Thymus
PPM1K
GRP75
TUBULIN
PPM1K/GRP75
heart
liver
sk.muscle
diafragm
cortex
thymus
PPM1K/TUBULIN
B
Skeletal muscle
Fed
24h
48h
PPM1K
TUBULIN
relative PPM1K expression
fed
Liver
Heart
C
Adult
cadiomyocytes
In vivo starvation
(hrs)
0h
48h
PPM1K
P-S6
ACTIN

Figure 4.

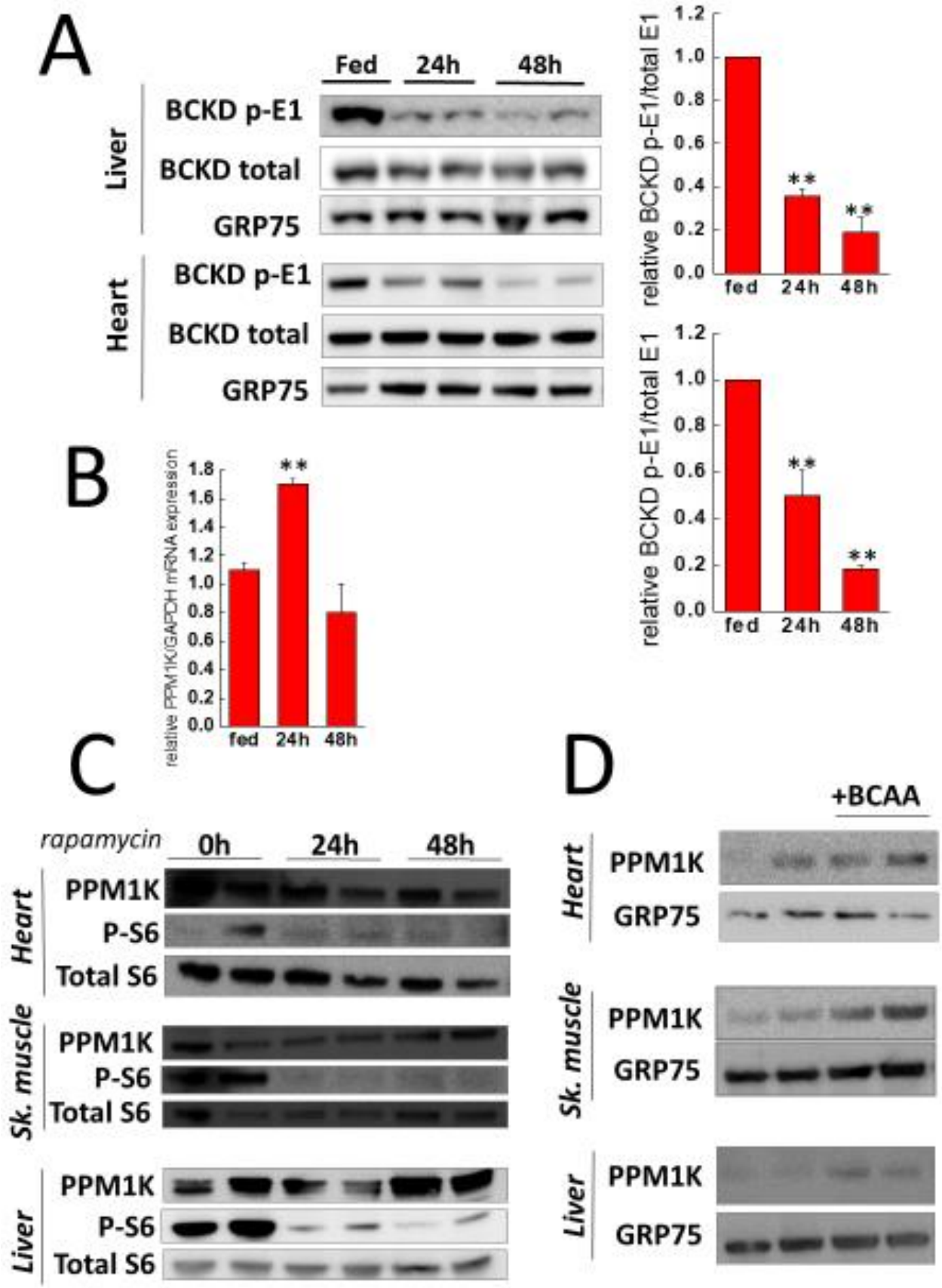
A
Fed
24h
48h
Liver
BCKD p-E1
BCKD total
GRP75
Heart
BCKD p-E1
BCKD total
GRP75
relative BCKD p-E1/total E1
fed
24h
48h
B
relative PPM1K/GAPDH mRNA expression
C
rapamycin
0h
24h
48h
Heart
PPM1K
P-S6
Total S6
Sk. muscle
PPM1K
P-S6
Total S6
Liver
PPM1K
P-S6
Total S6
D
+BCAA
Heart
PPM1K
GRP75
Sk. muscle
PPM1K
GRP75
Liver
PPM1K
GRP75

Figure 5.

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The mitochondrial matrix phosphatase PPM1K controls autophagy by regulating branched chain aminoacid catabolism.

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Supplementary online material

Supplementary Figure legends

Supplementary figure 1. Silencing of endogenous PPM1K by transfection and adenoviral transduction. Confirmation of PPM1K being necessary for starvation induced autophagy.

(A) Western blot analysis of PPM1K levels in mouse fibroblasts transfected with shPPM1K or ShScrambled as control.

(B) Evaluation of LC3 levels upon transient overexpression (left panel) and silencing (right panel) of PPM1K in HepG2 cells. HBSS was used to starve cells where indicated. Shown are western blot for the indicated proteins.

(C) Western blot analysis of PPM1K, P62 and LC3 levels in fibroblasts transfected with shPPM1K. HBSS was used to starve cells.

(D) Representative images of adult cardiomyocytes maintained in culture for up to 5 days. Pictures were acquired with optic microscope.

(E) Adult cardiomyocytes were transduced for 48 hrs with adenoviral vectors to induce PPM1K downregulation (lane1) or overexpression (lane3). Shown is Western blot analysis of PPM1K levels. shScramled was used as control.

(F) Bar graphs represent the quantification of LC3 positive puncta per cell in adult cardiomyocytes starved for 2 hours and stained for LC3. Data represent mean ± SEM of 2 independent experiments (n=30 cells per condition).

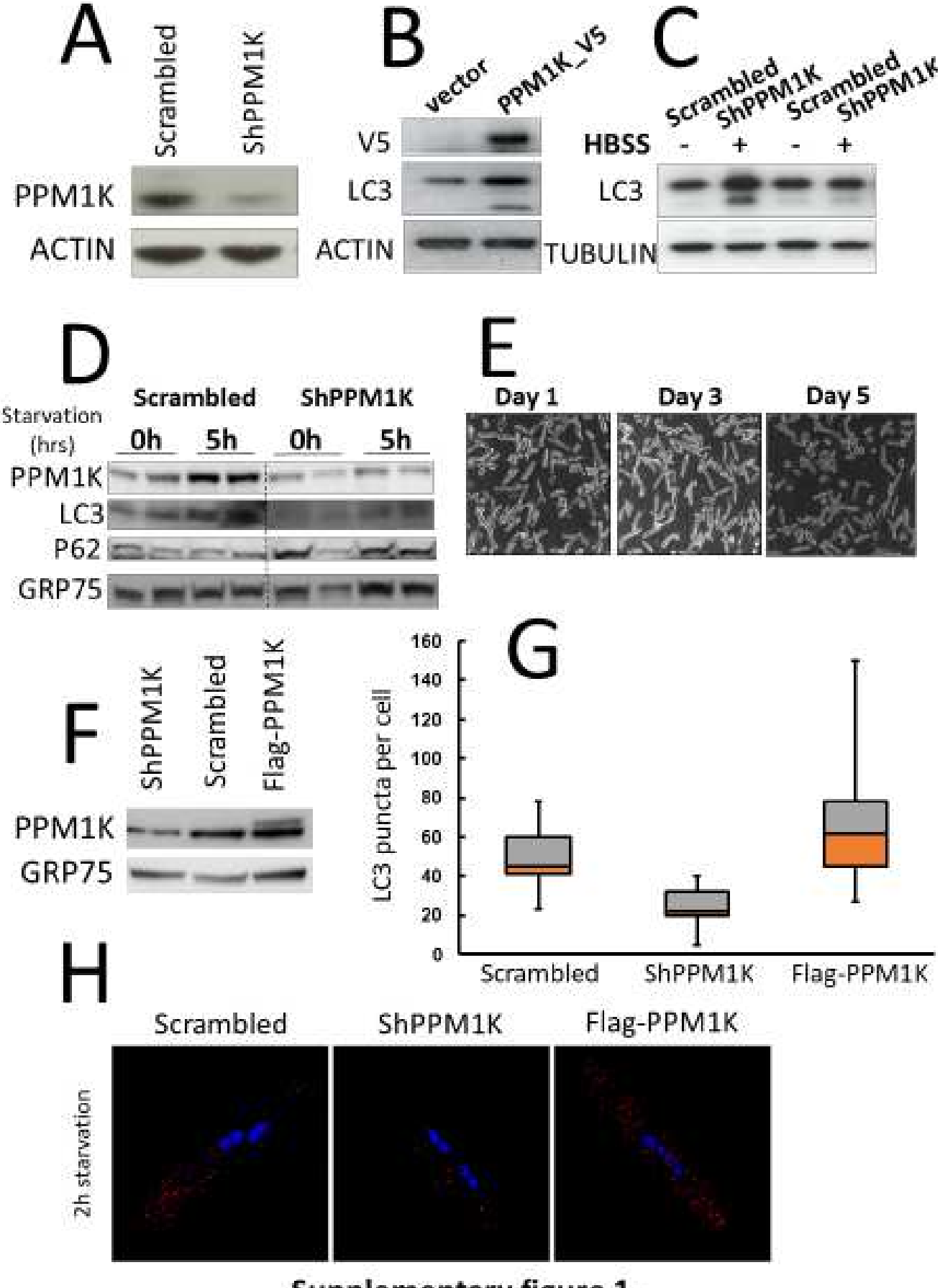
(G) Representative confocal images of adult mouse cardiomyocytes overexpressing or silencing PPM1K as indicated. Cells were starved for 2 hours and then fixed and stained for LC3.

Supplementary figure 2. PPM1K does not affect mitochondrial elongation and vitality upon starvation. Mitochondrial permeability transition pore opening is not affected by circulating metabolites of the BCAA catabolism.

(A) Morphometric analysis of mouse fibroblast mitochondria upon overexpression (left panel) or silencing (right panel) of PPM1K and starvation for the indicated time periods. Empty vector and shScrambled were used as controls respectively. Data represent mean ± SEM of 3 independent experiments (n=100 cells per condition).
(B) Line charts represent the vitality of mouse fibroblasts upon PPM1K silencing at the indicated starvation periods compared to control. Viability was determined by flow-cytometry. Data represent mean ± SEM of 3 independent experiments.

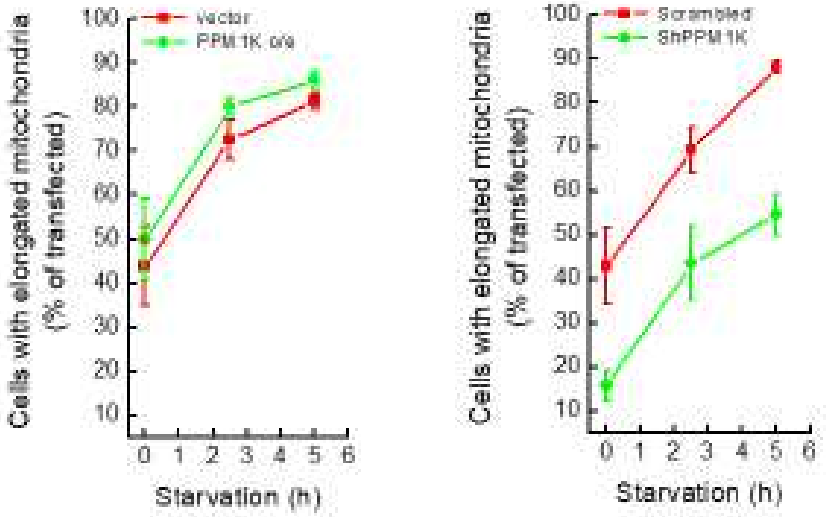
Supplementary Figure 3. PPM1K is degraded by the proteasome

(A) Mouse fibroblasts were treated with cycloheximide for the indicated time points to evaluate PPM1K half-life. Shown are Western blot panels to evaluate PPM1K levels. Cyclin B1 degradation levels were used as a control of cycloheximide function.

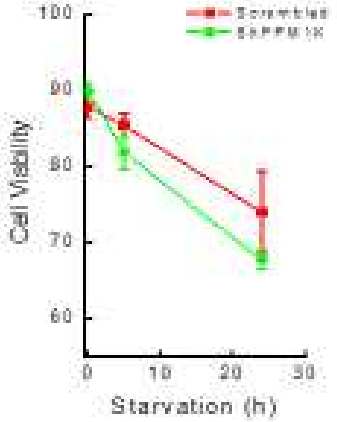


Supplementary figure 1.

A

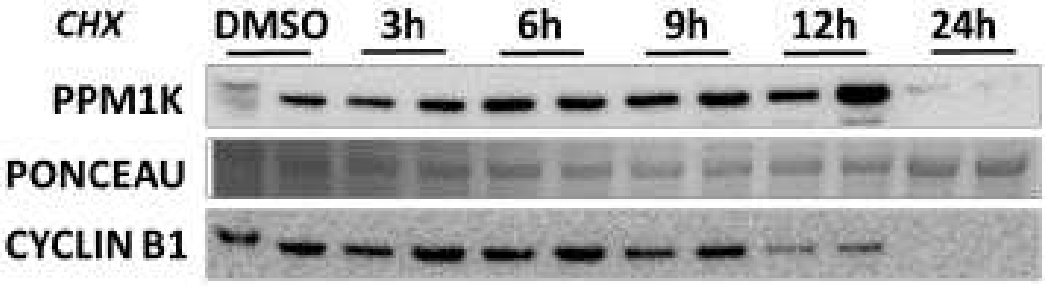


B



Supplementary figure 2.

A



Supplementary figure 3.

Table 1.

Metabolites identified by gas-chromatography mass spectrometry (GC-MS) steady state levels.

Metabolite	Characteristics
2-oxoglutaric acid	Keto acid of glutamate deamination, Krebs cycle intermediate.
3-hydroxyglutaric acid	Dicarboxylic acid.
Alanine	Non essential aminoacid. Role in protein biosynthesis.
Asparagine	Non essential aminoacid. Role in protein biosynthesis.
Aspartic acid	Non essential aminoacid. Role in protein biosynthesis.
Beta-alanine	Non essential aminoacid. Precursor of carnitine.
Citric acid	Weak organic acid. Krebs cycle intermediate.
Cysteine	Non essential aminoacid. Role in protein biosynthesis.
Fumaric acid	Dicarboxylic acid, Krebs cycle intermediate.
gamma-Aminobutyric acid	Inhibitory neurotransmitter.
Glutamic acid	Non-essential aminoacid. Role in protein biosynthesis
Glutamine	Conditionally essential aminoacid. Role in protein biosynthesis.
Glycerol	Polyol compound found in all triglycerids.
Glycine	Non-essential aminoacid. Role in protein biosynthesis.
Histidine	Essential aminoacid. Role in protein biosynthesis.
Hypotaurine	Sulfinic acid. Endogenous neurotransmitter.
Isoleucine	Branched chain aminoacid.
Ketoisocaproic acid	Leucine ketoacid.
Lactic acid	Alpha-hydroxy-acid.
Leucine	Essential, ketogenic Branched chain aminoacid.
Lysine	Essential ketogenic aminoacid. Role in protein biosynthesis.
Malic acid	Dicarboxylic acid, Krebs cycle intermediate.
Methionine	Essential aminoacid. Substrate for cysteine, taurine, glutathione and SAM-e.
Ornithine	Non-proteinogenic aminoacid. Role in urea cycle.
Phenylalanine	Essential aminoacid. Precursor for tyrosine.
Pyruvic acid	a-keto acid derived from glucose. Supplies energy in Krebs cycle.
Serine	Non-essential aminoacid. Role in protein biosynthesis.
Succinic acid	Dicarboxylic acid, Krebs cycle intermediate.
Taurine	Sulfonic acid, major constituent of bile.
Threonine	Essential aminoacid. Role in protein biosynthesis.
Tryptophan	Essential aromatic aminoacid. Role in protein biosynthesis.
Tyrosine	Non-essential aminoacid. Role in protein biosynthesis.
Valine	Essential, Branched chain aminoacid.

Genetic inactivation in the heart of USP8, a deubiquitinase hyperactive in Cushing's syndrome adenomas, causes mitochondrial dysfunction and cardiomyopathy.

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Summary

Activating mutations in the *USP8* gene, coding for ubiquitin-specific protease 8, a deubiquitinase involved in endocytic trafficking and mitophagy, can cause Cushing's syndrome. Usp8 inhibitors are therefore scrutinized to treat Cushing's pituitary adenomas. However, because heart function requires mitophagy, it is unclear if Usp8 inhibitors could be detrimental for the already failing hearts of Cushing's patients. Here we show that acute *Usp8* genetic ablation in the mouse heart impairs mitochondrial function and autophagic clearance. Myocardial *Usp8* deletion in adult mice resulted in cardiomyopathy associated with the accumulation of damaged and dysfunctional mitochondria. Mechanistically, we found that USP8 interacted with, and stabilized PINK1 that senses dysfunctional mitochondria and activates Parkin dependent mitophagy. Consequently, in cardiomyocytes and cells lacking USP8, PINK1 was not stabilized upon mitochondrial dysfunction, mitophagy was not activated in response to mitochondrial depolarization and chemical mitochondrial uncouplers led to cell death. Our data not only shed light on the mechanisms of mitophagy regulation, but also recommend caution in investigative anti Usp8 therapy for Cushing's syndrome.

Introduction

Cushing's disease is a severe metabolic disorder caused by ACTH-secreting corticotrophin adenomas that can be potentially fatal if untreated, because of the many metabolic clinical features (central obesity, hypertension, cardiovascular disease and metabolic syndrome) (Kamenický et al., 2014; Nieman, 2015). While transsphenoidal surgery remains the first line of treatment, it is rarely resolutive and most patients relapse, because of the intrinsic difficulty in removing all the tumor even by experienced neurosurgeons using neuronavigation, emphasizing the need to develop novel therapeutic approaches for Cushing's disease (Ciric et al., 2012; Hofmann et al., 2008). Years of research into Cushing's disease genetics led to the identification of several potential drivers for the pituitary adenomas (PAs) that characterize the disease: they include reduced expression of Brg1, of histone deacetylase 2, of the cell cycle inhibitor $p27^{Kip1}$ (Bilodeau et al., 2006), as well as hyperactive EGFR signaling due to EGFR overexpression (LeRiche et al., 1996). Irrespective of the driver, more than 60% of ACTH-secreting corticotrophin adenomas carry several *USP8* variants that induce ACTH overproduction via deregulation of EGFR signaling, because they fail to bind 14-3-3 proteins and display an elevated deubiquitinating activity toward EGFR (Ballmann et al., 2018). Therefore, anti EGFR therapy with gefitinib, or USP8 inhibitors have been suggested as a promising therapeutic strategy for Cushing's disease cases with *USP8* mutations, especially for patients with residual or recurrent adenomas (Ma et al., 2015).

USP8 was originally identified as a key deubiquitinase (DUB) involved in the control of endocytic trafficking of EGFR (Mizuno et al., 2005; Niendorf et al., 2007). More recently, USP8 emerged as a hit with unique features in an unbiased screen for deubiquitinase

enzymes involved in the control of mitophagy, the selective removal of mitochondria by autophagy. The best studied mechanism of mitophagy is the PINK1-Parkin pathway. Upon mitochondrial damage the PTEN induced kinase 1 (PINK1), that is normally imported into the intermembrane space of mitochondria, is cleaved by the rhomboid protease PARL and stabilized on the outer mitochondrial membrane where it phosphorylates Ubiquitin to recruit a Ubiquitin ligase, Parkin, on mitochondria. Parkin then ubiquitinates a handful of proteins on the surface of mitochondria leading to the recruitment of the autophagic machinery for the degradation of the entire organelle. As expected, deubiquitination of these mitochondrial substrates targeted by Parkin antagonizes mitophagy: the DUBs USP30, USP35 and USP15 can indeed block mitophagy by counteracting Parkin activity, their ablation leading to enhanced mitophagy (Bingol and Sheng, 2016; Cornelissen et al., 2014; Wang et al., 2015). On the contrary, USP8 is the only DUB found to stimulate mitophagy. USP8 selectively remove K6-linked ubiquitin chains from Parkin, antagonizing its autoubiquitination and enabling its translocation to mitochondria. Indeed, USP8 knockdown leads to impaired Parkin translocation and delayed mitophagy (Durcan et al., 2014).

Mitophagy (like macroautophagy) is key for function of the heart, an organ crucially affected by Cushing's syndrome. Indeed, mechanisms that regulate mitochondrial health must insure a complete mitochondrial turnover on average every 17 days, to renew the pool of these organelles essential not only for energy conversion, but also for calcium storage and metabolic remodeling. Loss of PINK1 and Parkin function studies helped to understand the importance of this process in the heart. Parkin deficient mice have normal cardiac function, even though mitochondria are morphologically disorganized and some

of them dysfunctional (Song et al., 2015). The normal cardiac function is thought to be a consequence of the activation of compensatory mitophagic processes. Conversely, deletion of PINK1 in mice causes more severe cardiac effects, including increased oxidative stress and cardiac hypertrophy from 2 months of age (Siddall et al., 2013). If USP8 is essential for mitophagy also in the heart and whether USP8 inhibition for example in the context of a targeted therapy for Cushing's syndrome PA1 can adversely affects heart function is unclear. To address this issue, we generated an inducible model of Usp8 cardiomyocyte deletion. We show that Usp8 deletion does leads to accumulation of dysfunctional mitochondria because of impaired Pink1 stabilization on mitochondria. This leads to cardiomyopathy, raising questions on the safety of anti Usp8 therapies for Cushing's PAs.

Results

Mitochondrial proteins are reduced in an inducible USP8 cardiac knock-out mouse model.

To determine the role of USP8 in the heart we generated a mouse model of cardiomyocyte-specific *Usp8* ablation. To overcome potential compensatory effects of constitutive gene deletion, we decided to generate an inducible model where we could delete Usp8 in adult mouse life. This would also mimic the condition of Usp8 inhibition in a potential therapeutic setting. To this end, $Usp8^{flox/flox}$ mice were crossed with the MerCreMer mice, expressing the Cre recombinase under the tamoxifen inducible-Myh6 promoter, expressed only in cardiac myocytes, generating a $Usp8^{CKO}$ mouse (Suppl. Figure 1A). Acute *Usp8* ablation was induced by two different protocols of tamoxifen injections: in one we deleted the gene during neonatal life, by injecting the drug at P3-P5 and in the second we injected tamoxifen to one-month old adult mice (Figure 1A).

We first characterized the phenotype of young $Usp8^{CKO}$ mice. Acute *Usp8* ablation resulted in an almost complete reduction in heart USP8 protein one week after the first tamoxifen injection (Figure 1B and 1C). Because in other systems lack of *Usp8* impairs mitophagy, we wanted to verify if the same held true in the heart. However, when we measured mitochondrial mass in the hearts of these $Usp8^{CKO}$ mice, we surprisingly found that levels of several mitochondrial proteins were instead decreased (Figure 1D and 1E). Because decreased mitochondrial mass is can be regarded as an indicator of reduced biogenesis, or, especially in terminally differentiated tissues like the heart, of increased mitophagy, we turned our attention to bona fide markers of autophagy activation. LC3-II

levels were slightly lower in knockout mice, suggesting that autophagy is reduced, or that its flux is increased and the LC3II cargo is heavily degraded (Figure 1F and 1G).

USP8 ablation impairs heart structure and function.

Intrigued by the loss of mitochondrial mass we recorded as early as 1 month following *Usp8* deletion, we wondered if this resulted in any pathological heart phenotype. Pups treated with tamoxifen did not show any major developmental phenotype (not shown), but when we inspected their hematoxylin eosin stained hearts by histological examination we noticed right ventricular wall thinning in most knockout mice (Figure 2A). Indeed, Masson's trichrome staining of cardiac sections identified extensive fibrotic lesions in specific areas of knockout mice, suggesting that lack of Usp8 induces a fibrotic response also during development (Fig. 2B). These histological changes were accompanied by altered mitochondrial ultrastructure, as shown by electron microscopy that highlighted the accumulation of smaller, less electrondense mitochondria (Fig. 2C) whose perimeter resulted significantly reduced at a morphometric analysis (Fig. 2D). Thus, neonatal heart Usp8 deletion impairs mitochondrial abundance, morphology and leads to right ventricular wall thinning with extensive fibrosis.

Because we wanted to perform functional analyses on fully developed hearts, we resorted to the second model of inducible Usp8 deletion we devised, where we characterized the consequences of acute *Usp8* ablation in adult (>1m old) mice. One month the first tamoxifen injection, heart USP8 protein levels were reduced (Figure 3A and quantification in 3B). Immunoblotting for a variety of mitochondrial proteins confirmed the decrease in mitochondrial mass also when Usp8 was deleted in adult life (Figure 3C and quantification

in 3D). Because the mitochondrial DNA encoded mitochondrial cytochrome oxidase I (MTCOI) protein was among the reduced ones, we excluded the possibility that the reduced mitochondrial protein levels was due to an impairment in nuclear DNA translation or in import of mitochondrial proteins encoded by the nuclear DNA. While these data seem in accordance with a role for Usp8 in maintenance of mitochondrial mass in the heart, further experiments are required to understand if upon USP8 deletion this mitochondrial mass loss is caused by increased autophagy and mitophagy or by reduced mitochondrial biogenesis.

We then analyzed by EM 3 months old adult *Usp8*^{CKO} (i.e., 2 months after tamoxifen injection). We found a whole array of cardiomyocyte defects, ranging from altered mitochondrial morphology and distribution to loss of myofibril integrity. Of note, *Usp8*^{CKO} hearts harbored huge “empty” mitochondria that we could not find in their control tamoxifen injected wild type littermates (Figure 4A). These ultrastructural alterations were accompanied by profound functional mitochondrial defects: when we measured by Clarke electrode the oxygen consumption rate and the respiratory control ratio (RCR) of heart mitochondria isolated from 3-month old mice, *Usp8*^{CKO} mitochondria appear uncoupled (Figure 4B) and indeed their RCR was almost halved (Figure 4C). Not surprisingly, noninvasive echocardiography of 3 months old *Usp8*^{CKO} and control littermates revealed reduced wall motility (Figure 4D) and impaired cardiac function, as determined by the reduced fractional shortening (Figure 4E). Altogether, these data indicate that neonatal or adult deletion of Usp8 in the heart is detrimental for mitochondrial morphology and function, and leads to cardiac dysfunction.

USP8 ablation leads to impaired mitophagy through defective PINK1 stabilization on mitochondria.

To address the mechanism of the altered phenotype of USP8 knockout hearts we explored the potential involvement of USP8 in mitochondrial turnover. We first prepared primary fibroblasts from *Usp8*$^{flox/flox}$. E12 embryos that we could then infect with Adenoviruses carrying an empty insert (EV) or Cre recombinase (Cre) to acutely delete Usp8 (Fig. supplementary 2).

We first inspected mitochondrial morphology in these cells 2 days after adenoviral infection. While we did not observe any change in unstressed cells, the fragmentation caused by CCCP treatment was abolished when cells were infected with the Cre adenovirus (Figure 5A), suggesting that lack of USP8 might impair mitophagy. Indeed, when we turned to investigate the major molecular players in the mitophagic process, we found that the expected PINK1 stabilization induced by mitochondrial depolarization did not occur in cells lacking USP8 (Figure 5B). This was not rescued when we overexpressed Parkin, suggesting that the USP8 mediated stabilization of PINK1 is independent of Parkin and happens upstream of the mitochondrial translocation of the latter. To understand further whether lack of Usp8 impaired mitophagy, we checked levels of TOM20 that is among the first proteins to be degraded following mitophagy induction. In Usp8 deficient cells, TOM20 was not degraded upon CCCP induced mitophagy. The effect was specific, because we could completely rescue it, together with PINK1 stabilization, by the genetic reintroduction of USP8 in the knockout cells (Figure 5C). The failure to actively engage mitophagy in Usp8 deficient cells was mirrored by their

enhanced sensitivity to CCCP-induced cell death, that was fully rescued by Usp8 genetic reintroduction (Figure 5D).

Finally, we wanted to understand how lack of Usp8 could lead to failure to stabilize Pink1 and hence to activate mitophagy. One possibility is that Usp8 works as Pink1 DUB: indeed, while in one model Pink1 is degraded intramitochondrially, in another one it is ubiquitinated (Murata et al, 2013) and degraded by the proteasome (Li et al, 2017). If Usp8 was the Pink1 DUB, necessary to stabilize it, it shall at least interact, in conditions of proteasome inhibition, with its substrate. Indeed, in a pull down experiment, we could find a fraction of Pink1 interacting with Usp8 (Figure 5E), offering an initial indication that Usp8 might interact with Pink1 and stabilize it upon mitophagy induction.

Discussion

In this study, we present several novel findings that bring new insights into mitochondrial quality control. Fist we identified USP8 as a novel regulator of cardiac health through the control of mitochondrial health. Our data also indicate that USP8 ablation lead to mitochondrial impairment. Finally, we confirmed that USP8 is essential for the induction of PINK1-Parkin mediated mitophagy through the stabilization of PINK1 on the mitochondrial surface upon depolarization. Thus, these findings suggest that the pathology of Usp8 cardiac knockout mice might be mediated by impaired mitochondrial quality control and accumulation of dysfunctional mitochondria, confirming their importance in cardiac function.

Previous reports showed that there are alternative compensatory mechanisms that activate mitophagy independently from PINK1 using the constitutive PINK1 knockout mouse. However, in our model ablation of USP8 is acute and led us understand the importance of the PINK1-Parkin mediated mitophagy in the heart at baseline levels.

One of the first things we observed was the decrease in the mitochondrial mass of knockout mice hearts. While we were expecting aberrant mitophagy, electron microscopy revealed in many areas of the myocardium the presence of swollen, low electron dense mitochondria, which would explain the decreased mitochondrial mass. This led us hypothesize that there is rather an impairment in mitochondrial turnover in these hearts. In order to understand the role of USP8 in mitochondrial turnover and clearance we moved to a simpler model, primary embryonic fibroblasts from floxed mice, transduced with cre adenovirus. In order to get a quick glance at mitochondrial clearance, we

pharmacologically induced mitochondrial depolarization and investigated mitochondrial morphology upon cre induction. The fact that USP8 knockout mitochondria do not fragment as the wild type one means they can't undergo mitophagy. It is yet to be clarified whether there is an impairment in Drp1 recruitment or in other steps of fission rather than in mitophagy itself. In the present study we focus on PINK1 stabilization upon mitochondrial depolarization, which is completely absent from USP8 knockout cells. USP8 is a deubiquitinating enzyme already known to be necessary for proper mitophagy, but the mechanism that has been proposed is different from the one we found, since it has been published that USP8 deubiquitinates Parkin and is needed for its translocation on mitochondria. What we found however is an interaction between USP8 and PINK1, and our theory is that USP8 deubiquitinates and stabilizes PINK1 on the outer mitochondrial membrane in order to signal for mitophagy induction. USP8 is already known to protect other proteins from proteasomal degradation (Sun et al. 2018), and the fact that PINK1 seems to be degraded as well by the proteasome encourages us to sustain our hypothesis. Upon mitophagy stimulation many proteins are ubiquitinated and the proteasome is strongly activated, thus PINK1 needs to be constantly protected from a possible degradation in order to maintain mitophagy on. The importance of mitochondrial turnover in cellular life and death was confirmed by vitality assays.

Going back to the cardiac phenotype we observe, we believe that absence of USP8 causes impaired mitochondrial quality control in myocytes and this has severe consequences for the heart. including accumulation of damaged mitochondria and loss of myocytes. However, additional mechanisms (like the capacity of mitochondria to undergo fission must be taken in account) and future studies need to focus on identifying

these mechanisms, as well as how to exploit our findings for the contribution in novel therapeutic approaches to treat cardiac disease.

Materials and methods

Mouse models

For inducible cardiac ablation of Usp8, $Usp8^{flox/flox}$ mice (Niendorf et al., 2007) were crossed with $MerCreMer^{+/-}$ mice (Jackson' s Laboratory) and injected intraperitoneally with tamoxifen. Neonatal mice were injected for three days with 50ng of tamoxifen per day dissolved in corn oil, adult mice were injected for five days with 1mg of tamoxifen per day dissolved in corn oil. All breedings were performed in a BL6 genetic background. All animal experiments were performed according to the approved ethical protocols.

Immunoblotting

For SDS-PAGE experiments, proteins were separated on NOVEX polyacrilamide precasted gels (Invitrogen), transferred onto PVDF membranes (BioRad) and probed using the indicated antibodies and isotype matched HRP-conjugated secondary antibodies. The following primary antibodies were employed at 1:1000 dilution: rabbit anti-USP8 (Abcam); rabbit anti-TUBULIN (Santa Cruz Biotechnology); rabbit anti-GRP75 (Santa Cruz Biotechnology); rabbit anti TOM20 (Santa Cruz Biotechnology); mouse anti-ATP5A (Mitosciences-Abcam) rabbit anti-LC3/MAP1LC3A (Novus Biologicals); rabbit anti HSP60 (Santa Cruz Biotechnology); mouse anti-CypD (Calbiochem); mouse anti-ACTIN (Abcam); mouse anti-OXPHOS (Abcam); rabbit anti-PINK1 (Novus Biologicals); rabbit anti-HA (Cell Signaling); mouse anti-MYC-tag (Thermo Fisher Scientific). The bands were visualized by enhanced chemiluminescence ECL kit (Pierce). Densitometry was performed using ImageJ gel measure tool.

Immunoprecipitation

After DNA transfection, cells were washed with ice-cold PBS. Whole-cell lysates were prepared in Tris-lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, and protease inhibitors). Cell lysates (2mg per sample) were incubated overnight at 4^{o} C with 0.5 lg of the indicated antibodies. Samples were incubated with protein A-sepharose beads for 2 hr at 4^{o}C. Beads were washed three times in lysis buffer. Samples were eluted with sample buffer, resolved by SDS-PAGE, and transferred to PVDF membranes.

Real time quantitative PCR

RNA from organs and cells was isolated by TRIZOL-chloroform extraction. RT-PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, 4368814). qRT-PCR analyses were carried out on retrotranscribed cDNAs with QuantStudio5 (Applied Biosystems). Expression levels are given relative to Gapdh. Primers used were: Usp8 FW:5'GAAGCGCTCCTACTCCTCAC3' and Usp8RV:5'CATGGTGGCTTGTTTTCCCG3', GapdhFW: 5'TGCACCACCAACTGCTTAGC3' and GapdhRV: 5'GGCATGGACTGTGGTCATGAG3'.

Cell culture and transfection

Mouse embryonic fibroblasts we extracted from skin of Usp8$^{flox/flox}$ mouse embryos at E12 and maintained in DMEM, 10%FBS, 1% penicillin/streptomycin.

For *in vitro* experiments, Usp8 ablation was achieve by transducing $Usp8^{flox/flox}$ cells with adenoviral vectors carrying Cre recombinase; transduction with adenoviral empty vector was used as control.

For Usp8 overexpression, cells were transduced with retroviral USP8-HA-FLAG (Addgene) vector or empty control vector. Retroviral particles were produced in PlatE packaging cell line (CellBiolabs) using polyethylammine (PEI) in a 3:1 PEI:DNA ratio. PlatE supernatant, containing viral particles, was collected 48 hours after transfection and filtered.

For PINK expression, cells were transduced with MYC-tagged PINK vector using Lipofectamine 2000 (Thermofisher) according to manufacturer's instruction.

Vitality of $Usp8^{flox/flox}$, $Usp8^{ko}$ and $Usp8^{ko+Usp\_HA}$ cells was assayed by FACS using annexin PI staining kit (eBioscience).

When indicated cells were treated 50nM cccp for the indicated time and 10 μM MG132 for 1hour.

TMRM (Thermofisher) was used to observe mitochondrial morphology in $Usp8^{flox/flox}$ and $Usp8^{ko}$cells (250nM).

Histology

For histology hearts were excised and perfused with PBS prior to overnight fixation in formalin at 4°C. The next day the hearts were embedded in paraffin and cut at microtome. For H&E protocol sections were deparaffinized in xylene for 10 minutes at RT and then gradually rehydrated. Samples were then incubated for 2 minutes in hematoxylin solution and then in eosin solution for 1 minute. Slices were subsequently dehydrated and

mounted in xylene mounting medium. For fibrotic tissue stain Masson's trichrome kit (Polysciences) was used according to manufacturer's instructions.

Transmission electron microscopy

Tissue was cut, immediately immersed in fixative (half strength Karnovsky) and kept at 4^{o}C overnight. The next day it was washed in sodium cacodylate 0.1M, pH7.4 and sent for processing. EM was performed as described (Scorrano et al., 2002). Thin sections were imaged on a Tecnai-20 electron microscope (Philips-FEI).

Echocardiography

Echocardiography was performed in adult (3months old) Usp8$^{flox/flox}$ (n = 3) and age- and sex-matched littermate Usp8^{Cko} (n = 3) using a Vevo 2100 (Visual Sonics, Toronto) system equipped with a 30-MHz transducer. Anesthesia was induced with 3% isoflurane, maintained with 1.5% isoflurane during constant monitoring of temperature, respiration rate, and ECG.

Mitochondrial assays

Mitochondria from the indicated cell lines were isolated as described (Frezza, Cipolat, & Scorrano, 2007). For mitochondrial respiration rate, freshly isolated mitochondria were resuspended in experimental buffer supplemented with 5 mM glutamate/2.5 mM malate. Oxygen consumption levels of isolated cardiac mitochondria from 3-mo-old mice, measured by Clark-type oxygen electrode and presented as respiratory control ratio (RCR; State-III/State-IV) levels.

Statistical analysis

Experiments were displayed graphically (bar or line charts) as mean and SEM (Standard Error of the Mean). Values were tested for significance by the one-sample T-test. Results were considered significant when *p< 0.05, ** p< 0.01.

Acknowledgements

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Figure legends

Figure 1. Mitochondrial proteins are reduced in an inducible USP8 cardiac knock-out mouse model at P20.

(A) Schematic representation of the in vivo experimental setup. $Usp8^{flox/flox}$ mice were crossed with $Usp8^{flox/flox}$ $MerCreMer^{-/+}$ mice. Tamoxifen was administered at P3,4,5 in order to obtain the $Usp8^{cko}$ mice to study at young age, or at one month of age for five days to study the adult phenotype at both genotypes.

(B) Specificity of USP8 ablation in the heart at P20 mice injected at P3, 4, 5. Protein levels of USP8 in total heart lysate extracted were measured using Western blot analysis Image is representative of 2 $Usp8^{flox/flox}$ and 2 $Usp8^{Cko}$.

(C) USP8 was ablated in the heart of P20 mice injected at P3, 4, 5. Bar graph shows the quantification of PPM1K protein levels of $Usp8^{flox/flox}$ and $Usp8^{Cko}$ (n = 6 $Usp8^{flox/flox}$ and 6 $Usp8^{Cko}$ mice). Data represent mean ± SEM of n=*3* mice for each group. Statistical analysis was performed with T-test. P-value: *<0.05, **<0.01.

(D) Mice were treated with tamoxifen at P2,3,4 and sacrificed at P20. Mitochondrial mass of total heart lysates was measured using Western blot analysis (n = 3 $Usp8^{flox/flox}$ and 3 $Usp8^{Cko}$ mice).

(E) Bar graphs show the densitometric analysis quantification of mitochondrial mass of $Usp8^{flox/flox}$ and $Usp8^{Cko}$ mice at P20. Data represent mean ± SEM of n=*3* mice for each group. Statistical analysis was performed with T-test. P-value: *<0.05, **<0.01.

(F) Western blot analysis for measurement of LC3 protein levels of total heart lysate extracted by $Usp8^{flox/flox}$ and $Usp8^{Cko}$ mice at P20.

(G) Quantification of the relative levels of LC3 protein upon densitometric analysis of $Usp8^{flox/flox}$ and $Usp8^{Cko}$ mice at P20 (n = 3 $Usp8^{flox/flox}$ and 3 $Usp8^{Cko}$ mice).

Figure 2. USP8 ablation causes impaired cardiac structure in mice of young age.

(A) Representative pictures of whole hearts and the corresponding histology by Hematoxylin & Eosin staining of cardiac sections of 20 days old mice (n=3 mice per genotype). LV: Left ventricle. RV: Right ventricle.

(B) Representative pictures of cardiac regions after Masson' s trichrome staining of cardiac sections of 20 days old mice (n=3 mice per genotype). Fibrotic areas with collagen deposition are stained in blue. Nuclei in dark red. Cytoplasm in red.

(C) Representative pictures of electron microscopic analysis of cardiac tissue of the indicated genotypes at P20.

(D) Bar graph represents the normalized mitochondrial perimeter. Morphometric analysis was performed with ImageJ. n=3 mice per genotype, 5 pictures per mouse. Statistical analysis was performed with T-test. P-value: *<0.05.

Figure 3. Mitochondrial proteins are reduced in an inducible USP8 cardiac knock-out mouse model at 3 months of age.

(A) Specificity of USP8 ablation in the heart at 3-month-old mice injected at one month. Protein levels of USP8 in total heart lysate extracted were measured using Western blot analysis. Image is representative of 2 $Usp8^{flox/flox}$ and 3 $Usp8^{Cko}$.

(B) Quantification of USP8 mRNA and protein levels of the indicated genotypes of 3-month-old mice injected with tamoxifen at 1 month of age. Data represent mean ± SEM of

n=*3* mice for each genotype. Statistical analysis was performed with T-test. P-value: *<0.05.

(C) Mice were treated with tamoxifen at one month of age and sacrificed after two months. Mitochondrial mass of total heart lysates was measured using Western blot analysis of different mitochondrial proteins (n = 3 $Usp8^{flox/flox}$ and 3 $Usp8^{Cko}$ mice).

(D) Bar graphs show the densitometric analysis quantification of mitochondrial mass of $Usp8^{flox/flox}$ and $Usp8^{Cko}$ of 3-month-old mice. Data represent mean ± SEM of n=*3* mice for each group. Statistical analysis was performed with T-test.

Figure 4. USP8 ablation causes impaired cardiac structure and function in adult mice.

(A) Representative pictures of electron microscopic analysis of the myocardium of adult mice (n=3 mice per genotype) of the indicated genotypes. Mice were treated at 1 month of age with tamoxifen and sacrificed at 3 months of age.

(B) Traces of oxygen utilization levels of isolated cardiac mitochondria from 3-mo-old mice of the indicated genotypes, measured by Clark-type oxygen electrode. Stage III: Addition of ADP completes the necessary substrates needed for operation of the electron transport chain (ETC); State IV: addition of oligomycin; return to basal rate of respiration**.** State V: addition of FCCP; uncoupling of the ETC to ATP synthesis.

(C) Graph shows oxygen consumption levels of isolated cardiac mitochondria of mice of the indicated genotypes presented as respiratory control ratio (RCR; State-III/State-IV) levels. Cardiac mitochondrial respiration traces of the indicated genotypes in presence of 5mM Glutamate (N=1 per genotype).

(D) Representative figure of short axis M-Mode (Motion over time) imaging of mouse hearts of the indicated genotype.

(E) Fractional shortening percentage (FS%) graphs from cardiac function analysis by 2D-directed M-mode echocardiography in 3-month old mice (n shown in Figure). Measurements were done on the parasternal short axis in M-Mode.

Figure 5. USP8 ablation leads to impaired mitophagy through defective PINK1 stabilization on mitochondria.

(A) Representative pictures of TMRM mitochondrial staining of $USP8^{flox/flox}$ and $USP8^{ko}$ primary MEFs at basal conditions and upon cccp-mediated mitochondrial depolarization. Ablation of Usp8 was induced by adenoviral transduction of GFP-cre adenovirus (GFP-empty adenovirus was used as control).

(B) Primary $Usp8^{flox/flox}$ MEFs were transduced with GFP-cre adenovirus or GFP-empty adenovirus. 24 hours upon transduction $USP8^{flox/flox}$ and $USP8^{ko}$ cells were transfected with Parkin or empty vector. After 24 hours hey were treated with cccp for 24 or 48 hours to induce mitophagy. Levels of PINK1 were assessed by Western Blot analysis of total protein extracts.

(C) Primary $Usp8^{flox/flox}$ MEFs were transduced with GFP-cre adenovirus or GFP-empty adenovirus. 24 hours upon transduction $USP8^{flox/flox}$ and $USP8^{ko}$ cells were infected with USP8-HA or empty retrovirus or empty vector. 24 hours later they were treated with cccp for 24 hours Western Blot analysis of PINK1, TOM20 and HA is shown.

(D) Line chart shows the vitality of $USP8^{flox/flox}$, $USP8^{ko}$ and $USP8^{ko}$+USP8_HA primary embryonic fibroblasts upon cccp treatment for 24 and 48h.

(E) Immunoprecipitation (IP) assay shows interaction between USP8 and PINK1. MEFs were transfected with Usp8_HA and PINK1_MYC in primary embryonic fibroblasts and treated either with cccp 24 hours before harvesting or with MG132 for one hour before harvesting. Total cell lysates were immunoprecipitated with a rabbit anti-HA antibody and analyzed by Western blotting using a mouse anti-MYC antibody.

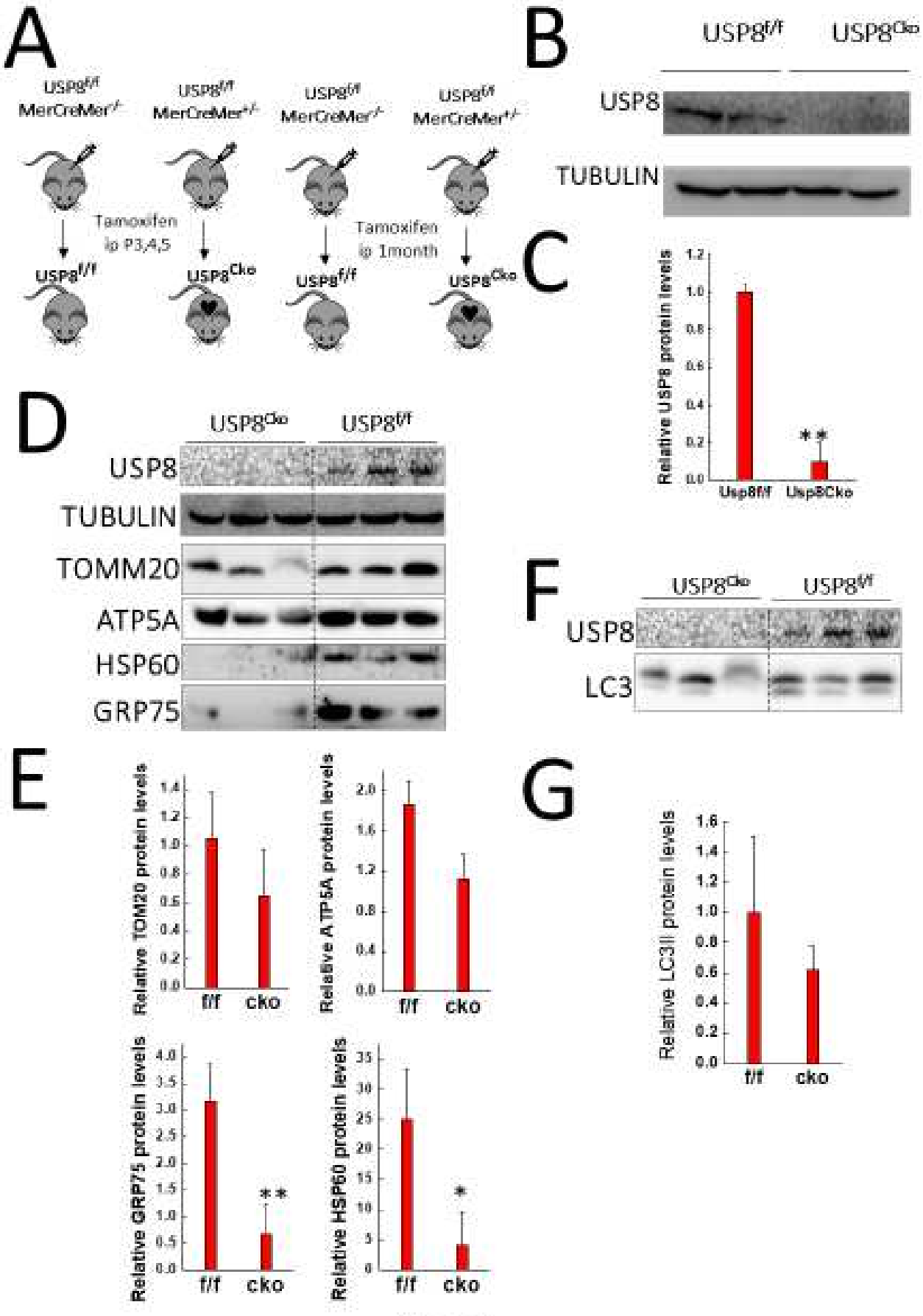
A
USP8f/f MerCreMer-/-
USP8f/f MerCreMer+/-
USP8f/f MerCreMer-/-
USP8f/f MerCreMer+/-
Tamoxifen @ P3,4,5
Tamoxifen @ 1month
USP8f/f
USP8Cko
USP8f/f
USP8Cko
B
USP8f/f
USP8Cko
USP8
TUBULIN
C
Relative USP8 protein levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Usp8f/f
Usp8Cko
D
USP8Cko
USP8f/f
USP8
TUBULIN
TOMM20
ATP5A
HSP60
GRP75
F
USP8Cko
USP8f/f
USP8
LC3
E
Relative TOM20 protein levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
f/f
cko
Relative ATP5A protein levels
2.0
1.6
1.2
0.8
0.4
0.0
f/f
cko
Relative GRP75 protein levels
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
f/f
cko
Relative HSP60 protein levels
35
30
25
20
15
10
5
0
*
f/f
cko
G
Relative LC3II protein levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
f/f
cko

Figure 1.

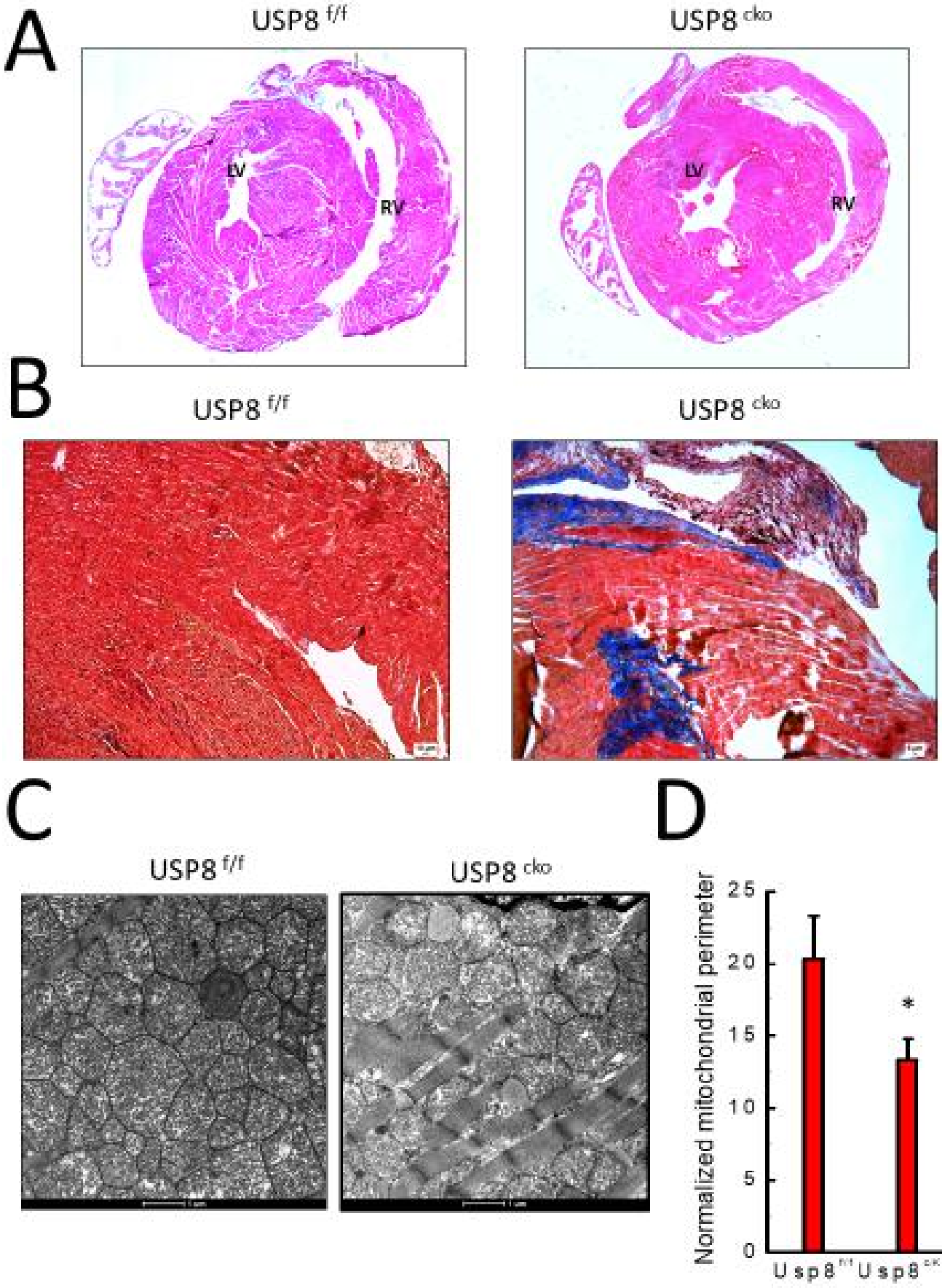
A
USP8 f/f
USP8 cko
LV
RV
LV
RV
B
USP8 f/f
USP8 cko
C
USP8 f/f
USP8 cko
D
Normalized mitochondrial perimeter
25
20
15
10
5
0
*
Usp8 f/f
Usp8 cko

Figure 2.

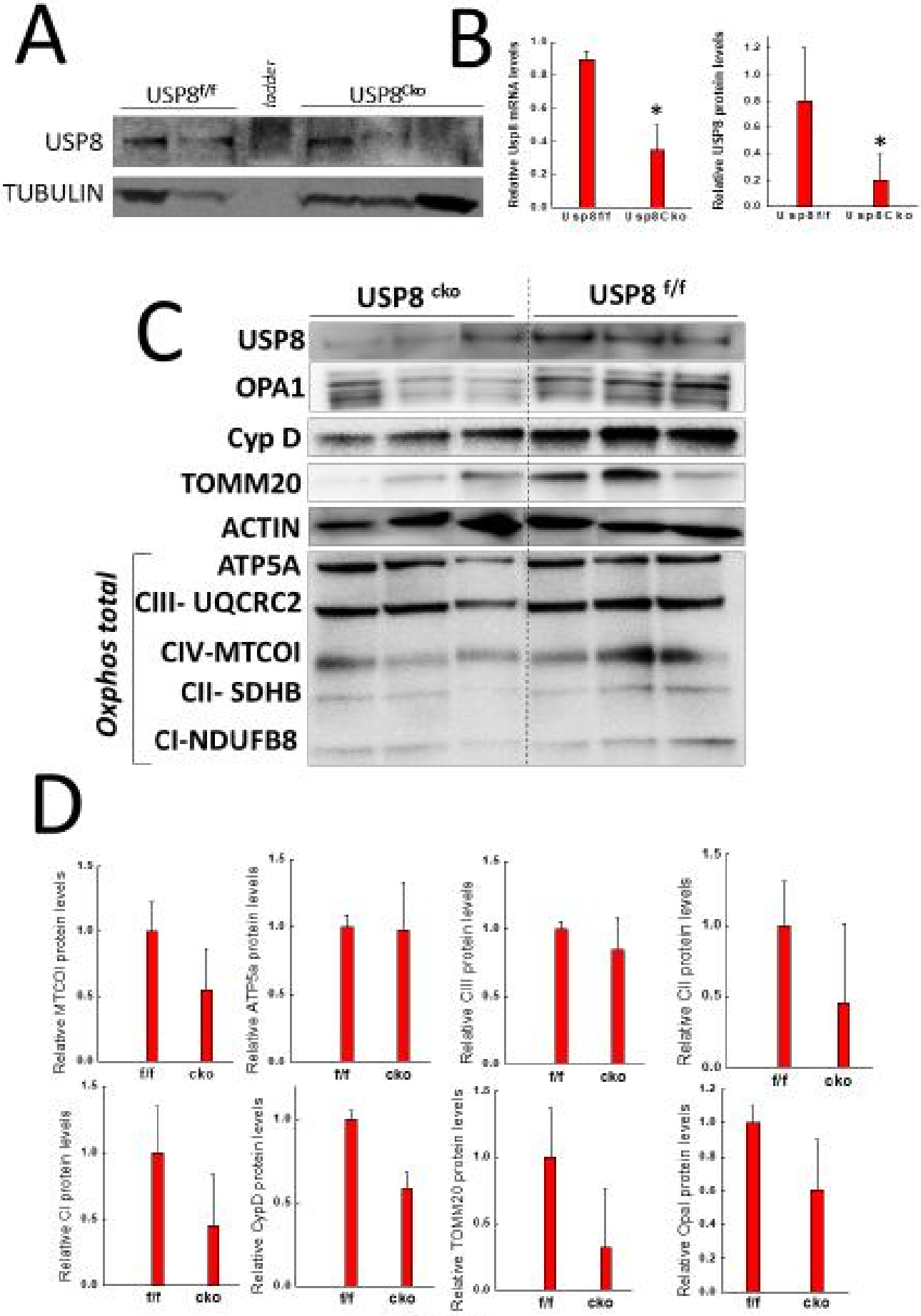
A
USP8f/f
ladder
USP8Cko
USP8
TUBULIN
B
Relative Usp8 mRNA levels
Usp8f/f
Usp8Cko
Relative USP8 protein levels
Usp8f/f
Usp8Cko
C
USP8 cko
USP8 f/f
USP8
OPA1
Cyp D
TOMM20
ACTIN
Oxphos total
ATP5A
CIII- UQCRC2
CIV-MTCOI
CII- SDHB
CI-NDUFB8
D
Relative MTCOI protein levels
Relative ATP5a protein levels
Relative CIII protein levels
Relative CII protein levels
Relative CI protein levels
Relative CypD protein levels
Relative TOMM20 protein levels
Relative Opa1 protein levels
f/f
cko

Figure 3.

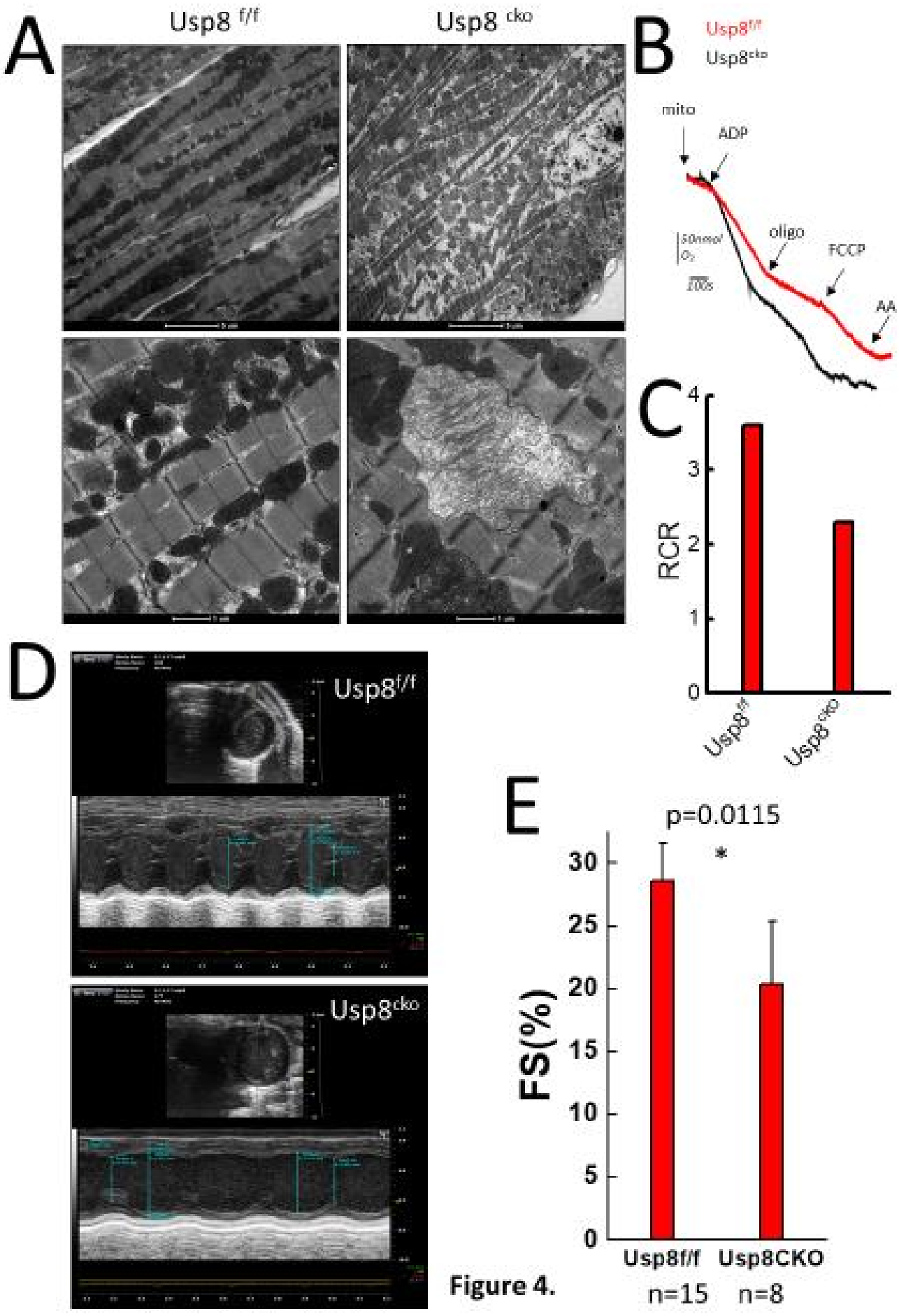
A
Usp8 f/f
Usp8 cko
B
Usp8f/f
Usp8cko
mito
ADP
oligo
FCCP
AA
50nmol/
O2
200s
C
RCR
4
3
2
1
0
Usp8f/f
Usp8cko
D
Usp8f/f
Usp8cko
E
p=0.0115
*
FS(%)
30
25
20
15
10
5
0
Usp8f/f
Usp8CKO
n=15
n=8

Figure 4.

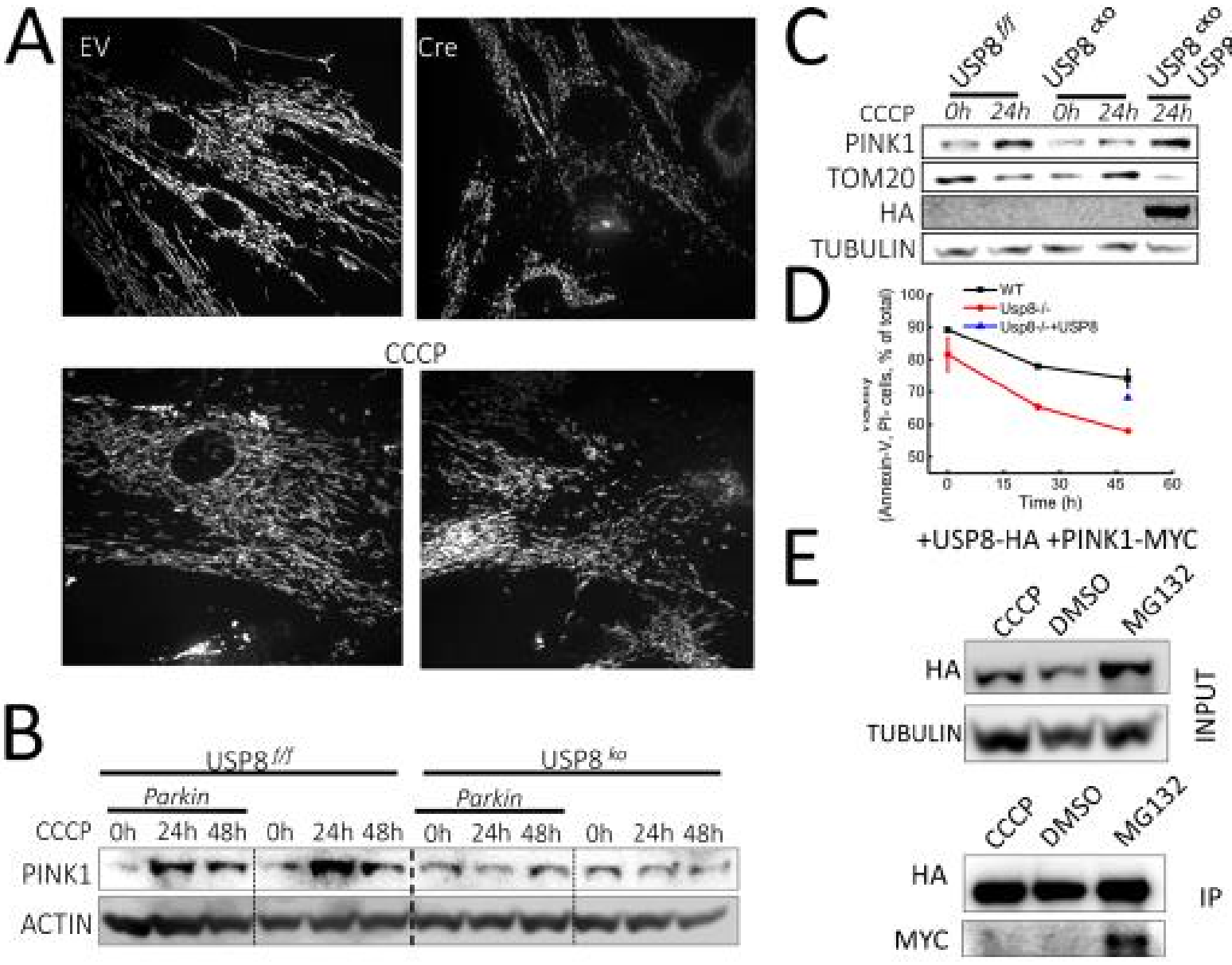
A
EV
Cre
CCCP
B
USP8 f/f
USP8 ko
Parkin
Parkin
CCCP 0h 24h 48h 0h 24h 48h 0h 24h 48h 0h 24h 48h
PINK1
ACTIN
C
USP8 f/f
USP8 cko
USP8 cko USP8HA
CCCP 0h 24h 0h 24h 24h
PINK1
TOM20
HA
TUBULIN
D
WT
Usp8-/-
Usp8-/-+USP8
(Annexin-V, PI- cells, % of total)
Time (h)
E
+USP8-HA +PINK1-MYC
CCCP DMSO MG132
HA
TUBULIN
INPUT
CCCP DMSO MG132
HA
MYC
IP

Figure 5.

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Supplementary online material

Supplementary Figure legends.

Supplementary Figure 1.

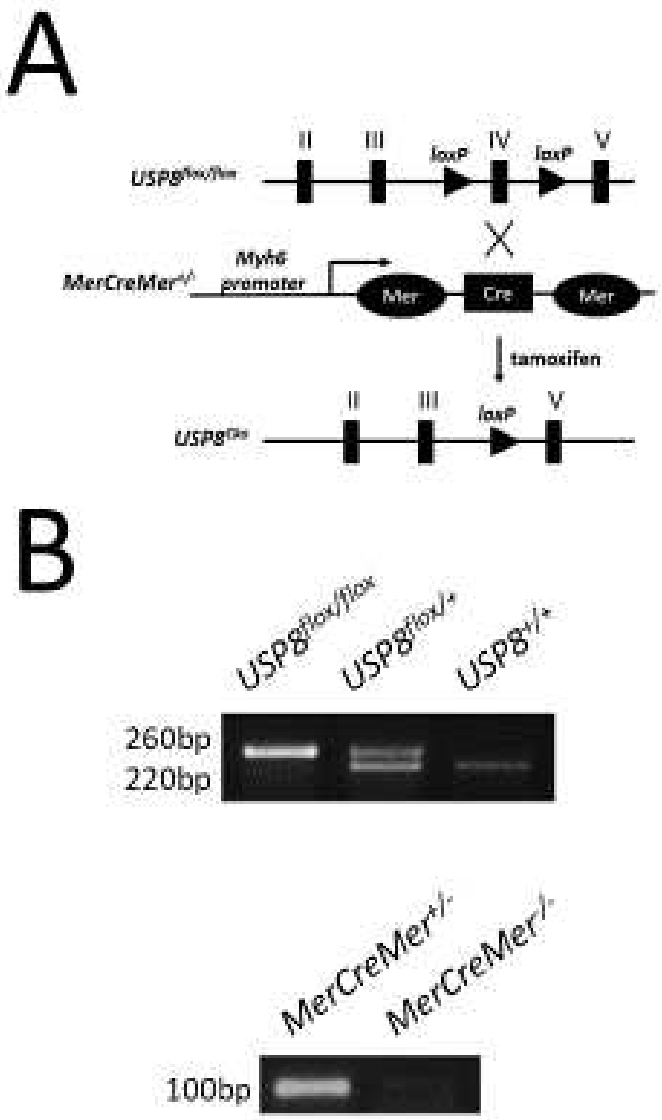
(A) Schematic representation of the generation of USP8^{Cko} mice: USP8flox/flox mice were crossed with the MerCreMer mice, expressing a tamoxifen inducible cre recombinase under the Myh6 promoter (a-myosin heavy chain, cardiomyocyte specific), therefore leading the generation of a specific cardiomyocyte knockout mouse.

(B) The genotype of Usp8$^{flox/flox}$ littermates was verified using PCR gene amplification. Homozygous WT Usp8$^{+/+}$ mice are characterized with a single band at 220bp, homozygous Usp8$^{flox/flox}$ mice with a single band at 260bp and heterozygous Usp8$^{flox/+}$ mice present two bands: one at 220bp and one at 260bp.

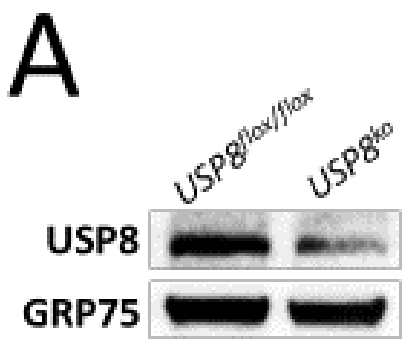
(C) Cre expression was detected by amplification of a single band at 100bp. Combination of the two PCR analysis gives the correct genotype of the animal.

Supplementary figure 2.

(A) Usp8$^{flox/flox}$ MEFs were transduced with cre-GFP adenovirus to acutely delete Usp8 or empty-GFP as control. The representative Western Blot shows the successful decrease in USP8 protein levels.



Supplementary Figure 1.



Supplementary Figure 2.

General conclusions

The general aim of this thesis was to understand the relationship between mitochondria and autophagy, with a focus on heart biology. Autophagy is of extreme importance for cardiac function. Animal models of impaired autophagy display cardiac phenotypes at basal levels as well as when stressed. In this work we dissected molecularly two different facets of mitochondrial biology related to autophagy: BCAA-catabolism mediated regulation of mTORC1, and the role of the deubiquitinating enzyme USP8, involved in EGFR signaling and mitophagy, in heart mitochondrial function and more generally in heart function.

In our first approach we sought to better understand the metabolic regulation of autophagy by aminoacids. For this purpose, we used an appealing model, based on the study of the relationship between PPM1k a key phosphatase controlling the rate limiting step of BCAA catabolism, and macroautophagy. PPM1K is a phosphatase located in the mitochondrial matrix (Lu *et al.*, 2009); yet, interestingly enough, its yeast orthologue Aup1p is needed for mitochondrial autophagy, a process regulated by cytosolic inputs (Tal *et al.*, 2007). Thus, we asked if PPM1K affects the autophagic process. Our work indicates that PPM1K is necessary for efficient starvation induced autophagy in the human and murine cellular models we used. Among them we also adult murine cardiomyocytes, where autophagy has in the heart. In addition, the finding that PPM1K modulates autophagy paves the way to understanding the cardiac defects and heart failure phenotype of mice lacking PPM1K (Sun *et al.*, 2011).

We addressed the mechanism by which mitochondrial metabolism affects autophagy. Our data point to mTORC1 hyperactivation in the absence of PPM1K. To better understand this mechanism, we turned to a metabolomic approach to evaluate if mTORCI hyperactivation upon PPM1K silencing was linked to a modulation of BCAA catabolism. GC-MS analysis revealed that in PPM1K silenced cells, the main TCA cycle intermediates were less abundant, and that aKIC and leucine levels were increased. These results were expected, vis-à-vis the fact that leucine catabolism feeds the TCA cycle. Moreover, in PPM1K silenced cells, we found a significant increase in the aminoacid methionine that was recently found to activate mTORCI eventually leading to autophagic inhibition. (Gu *et al.*, 2017). How starving cells supplemented in Leucine accumulate the essential AA Methionine is still unclear and deserves further investigation.

The role of mitochondrial BCAA metabolism in the control of autophagy prompt us to study it in the context of cardiovascular disease, since genetic disorders due to mutations on the BCAA catabolic pathway result in cardiac defects. (Bhan e Brody, 2001; Prada *et al.*, 2011). We therefore hypothesize that *in vivo* induction of autophagy in models with impaired BCAA catabolism such as the PPM1K knockout mouse, could result in an amelioration of their cardiac phenotypes. If we succeed in this approach we believe that it could lead to useful approaches for ameliorating the outcome of these BCAA genetic diseases.

In a complementary approach we studied USP8, a deubiquitinating enzyme whose activating mutations cause corticotrophic adenomas of the pituitary gland in Cushing's syndrome (Reincke *et al.*, 2015). Since Usp8 inhibitors have been posited as useful therapeutic agents to contrast the Cushing's syndrome pituitary adenomas, we sought

to investigate the impact of USP8 inhibition in the heart, which is heavily affected in Cushing's syndrome patients (Berlin *et al.*, 2010a; Durcan, Tang, P‚Äö√†√∂¬¨¨©Russe*, et al.*, 2014). Indeed, if Usp8 inhibition failed to prove safe for heart function, our results would have called for a cautionary approach towards the use of these drugs in the management of Cushing's syndrome patients. In addition, impaired mitochondrial turnover leads to cardiac defects and USP8 is important for mitophagy. We therefore studied the role of USP8 in the heart by taking advantage of an inducible cardiac knockout mouse model studied both at neonatal stage as well as during adulthood. USP8 deletion caused ultrastructural damage of the cardiac tissue and swollen mitochondria accumulation. In line with this, mice lacking USP8 displayed impaired cardiac function. To better understand the role of USP8 in mitochondrial health we turned to a cellular model of USP8 deletion. In USP8 deficient primary MEFs we could not observe the expected mitochondrial fragmentation induced by the mitophagy inducer CCCP, suggesting a possible impairment in mitophagy. Indeed, USP8 proved necessary for PINK1 stabilization upon mitochondrial depolarization and USP8 ablation blocks at the very first steps the PINK1 mediated autophagy, that has been showed to be important for proper cardiac function (Siddall *et al.*, 2013). We postulate that USP8 might be the endogenous deubiquitinating enzyme of PINK1, preventing it from proteasomal degradation (Liu *et al.*, 2017) when translocated on the outer mitochondrial membrane. This hypothesis is corroborated by the finding that PIMK1 and USP8 physically interact; however, we need still to prove the effect of USP8 on PINK1 ubiquitination. Finally, because USP8 deletion in MEFs leads to increased cell death in response to mitophagy

inducers, we hypothesize that USP8-dependent PINK1 stabilization is necessary for proper mitochondrial clearance in the heart, a key feature for proper cardiac function.

In addition to the novel mechanistic insights in the broadly studied PINK1-mediated autophagy, our findings confirm the importance of this pathway in the heart. Because our studies highlight the importance of USP8 for proper cardiac function, USP8 inhibitors, suggested to be useful for their antiproliferative effects on corticotroph tumor cells, shall be used with extreme caution in Cushing's disease patients that display several features of heart function impairment (Ma, Z. Y. *et al.*, 2015)**.**

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