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DISSERTATION

Titel der Dissertation

Analyses on the influence of AMPK on the activation of Nrf2 by Xanthohumol in MEF cells

Verfasserin

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"So it's been kind of a long road,

but it was a good journey altogether."

(Sidney Poitier)

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Abstract

The transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) plays a crucial role in cellular protection against stress and in fat metabolism. We tested whether the AMP-activated kinase (AMPK), a central regulator of energy metabolism, takes part in the Nrf2 dependent signalling. The natural compound Xanthohumol (XN) was used as a molecular tool for activating both Nrf2 and AMPK signalling in mouse embryonic fibroblasts (MEF). XN activates AMPK via the upstream kinase LKB1 by interfering with mitochondrial function and it activates Nrf2 via modulation of Keap1. The strictly Nrf2-dependent upregulation of the Heme oxygenase 1 (HO-1) was chosen as a molecular readout to compare the activation of Nrf2 by XN in WT and AMPK-/- MEF. In the AMPK-/- cells the upregulation of HO-1 was blunted both on the protein and mRNA level. Neither the total amount of Nrf2 nor the nuclear import of the protein markedly differed between WT and AMPK-/- cells. Multiple different factors may be involved in the AMPK/Nrf2/HO-1 signalling axis. We could rule out a major role for PKA, the MAPK pathway, GSK3ß, altered NADPH levels or fatty acid metabolism. The PI3K/Akt signalling was blunted in AMPK-/- cells and inhibiting PI3K impaired the upregulation of HO-1 by XN. Protein acetylation levels were generally higher in AMPK-/- cells and appeared to negatively influence the signalling: Treatment with deacetylase inhibitors further blunted, whereas a histone acetylase inhibitor increased the Nrf2/HO-1 response. The levels of p53, a negative regulator of Nrf2, were increased in AMPK-/- cells. Furthermore AMPK-/- cells showed an elevated level of ER stress which could be causally linked to their reduced Nrf2/HO-1 signal. Overall we revealed an enhancement of the Nrf2/HO-1 response by AMPK in which signals from Akt, protein acetylation events, p53 and/or ER stress seem to be involved.

Zusammenfassung

Der Transkriptionsfaktor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) spielt u.a. eine wichtige Rolle für den Schutz der Zelle vor oxidativem Stress und im Lipidmetabolismus. In dieser Arbeit wurde untersucht, ob die AMP-aktivierte Proteinkinase (AMPK), ein Schlüsselregulator des Energiehaushalts, an der Nrf2-Antwort beteiligt ist. Mit Hilfe des Naturstoffes Xanthohumol (XN) wurden Nrf2 und AMPK in embryonalen Maus-Fibroblasten (MEF) aktiviert. XN aktiviert AMPK über die Kinase LKB1, indem es die Mitochondrienfunktion beeinträchtigt, und führt zu einer Keap1-abhängigen Aktivierung von Nrf2. Die streng Nrf2-abhängige Hochregulierung des Proteins Hämoxygenase (HO-1) wurde als molekularer Indikator verwendet, um die Antwort auf die Behandlung mit XN in WT und AMPK-/- MEF zu vergleichen. In den AMPK-/- Zellen ist die Hochregulierung von HO-1 sowohl auf der Protein- als auch auf der mRNA Ebene beeinträchtigt. Weder die Gesamtmenge an Nrf2 noch der nukleäre Import des Proteins unterscheiden sich zwischen den WT und AMPK-/- Zellen. Zahlreiche unterschiedliche Faktoren könnten die positive Wirkung von AMPK auf das Nrf2/HO-1 Signal vermitteln. Wir konnten ausschließen, dass PKA, der MAPK Signalweg, GSK3ß, beeinträchtigte NADPH Level oder ein veränderter Fettmetabolismus eine tragende Rolle spielen. Eine Hemmung des PI3K/Akt Signalwegs beeinträchtigte die Hochregulierung von HO-1 durch XN und die aktivierende Phosphorylierung von Akt war in AMPK-/- Zellen reduziert. Außerdem war die Acetylierung von Proteinen in AMPK-/- Zellen generell höher; dementsprechend beeinträchtigten Deacetylase-Inhibitoren die Nrf2/HO-1 Antwort, während ein Acetyltransferase-Inhibitor sie positiv beeinflusste. Die Level von p53, einem negativen Regulator von Nrf2, waren in AMPK-/- Zellen erhöht. Zudem litten die AMPK-/- Zellen unter erhöhtem ER Stress, der mit dem reduzierten Nrf2/HO-1 Signal in Verbindung gebracht werden konnte. In Summa fanden wir, dass die AMPK einen positiven Effekt auf die Nrf2/HO-1 Antwort ausübt, in den Signale von Akt, Proteinacetylierung, p53 und/oder ER Stress einfließen.

A. Introduction

1. Nrf2

1.1 Nrf2 and cellular protection

Oxidative stress occurs in cells both as a by-product of mitochondrial respiration and in response to different external stimuli such as xenobiotics or drugs. Its hallmark is an increase in the level of reactive oxygen species (ROS) and electrophiles including the superoxide anion radical (O2-.), hydrogen peroxide (H_2O_2), and the hydroxyl radical (.OH) that is not met by an adequate response of the cellular antioxidant defence system. An excess of ROS is harmful to cells and associated with diseases such as cancer, diabetes, chronic inflammation and neurodegeneration. [1, 2] . A key player for the cellular defence against oxidative stress is the protein Nrf2 (Nuclear factor (erythroid-derived 2)-like 2), a ubiquitously expressed stress sensitive transcription factor of the basic leucine zipper family. It was first identified in 1994 as a transcriptional regulator of the β-globin gene [3], but was soon discovered to drive the expression of antioxidant proteins and phase II drug metabolizing enzymes in response to oxidative or electrophilic stress. The Nrf2 target genes have an antioxidant responsive element (ARE) in their promoter region that Nrf2 recognizes and binds as a heterodimer with a small musculoaponeurotic fibrosarcoma (Maf) protein. [4] Nrf2 influences both the basal and the inducible expression of its target genes. It protects the cell in different ways from redox, electrophilic and xenobiotic stress [5]: The Nrf2-regulated genes code for proteins with diverse cytoprotective functions. The glutamate-cysteine ligase (GCL) is responsible for the first synthesis step of the antioxidant glutathione (GSH), an important reducing agent. Several target proteins are specialized in the detoxification of specific substrates: The NAD(P)H quinone oxidoreductase 1 (NQO1) catalyses a two electron-reduction and detoxification of quinones, the superoxide dismutases (SODs) detoxify the superoxide anion via dismutation into hydrogen peroxide and oxygen and the epoxide hydrolases (EHs) hydrolyse epoxides. The multidrug resistance-associated proteins (MRPs) are involved in the transport of metabolites, mostly glutathione, glucuronide, and sulfate conjugates, out of the cells. [5] The Heme oxygenase-1 (HO-1), the one Nrf2 target gene in the focus of this study, is responsible for the rate-limiting degradation step of heme into biliverdin, carbon monoxide (CO) and free iron. Heme is a complex of iron and protoporphyrin that, as a part of functional hemeproteins, is essential for various processes including oxygen transport, respiration and drug detoxification. However, free heme is highly toxic to cells: It has haemolytic, pro-inflammatory and pro-oxidative effects. By generating ROS it damages lipids, proteins and DNA. The HO-1 is a highly

inducible protein that is upregulated by several transcription factors, including Nrf2, in response to different cellular stress conditions. The promoter contains an ARE for which Nrf2 competes with the transcriptional repressor Bach1. ROS and heme itself inhibit the binding of Bach1 to the ARE, thus allowing Nrf2 to bind and induce the HO-1 transcription. [6, 7] Collectively, the Nrf2-regulated enzymes scavenge the ROS, maintain a healthy redox environment and detoxify harmful xenobiotics.

1.2 Nrf2 and metabolism

Whereas in the beginning the Nrf2-research focused completely on the protective role against oxidative and xenobiotic stress, it is becoming increasingly clear that the transcription factor plays a broader role and also influences energy metabolism: It takes part in regulating different processes including fatty acid and lipid metabolism, the differentiation of adipocytes, insulin signalling, NADPH homeostasis and the function and biogenesis of mitochondria. [8]

1.2.1 Fat metabolism

a) Nrf2 and liver fat metabolisms

The regulation of lipid metabolism is an important part of the activity of Nrf2. Genetic activation of Nrf2 via disruption of the Nrf2 inhibitor Kelch-like ECH-associated protein 1 (Keap1) or pharmacologic activation by the small molecule CDDO-Im (1-(2-Cyano-3,12,28-trioxooleana-1,9(11)-dien-28-yl)-1H-imidazole) cause the largest changes in liver gene expression for genes related to lipid and fatty acid synthesis, that are mostly downregulated by the active Nrf2. [9] Accordingly the level of the corresponding proteins is lower in the liver of Nrf2-/- than WT mice [10] and Nrf2 protects mice against hepatic fat accumulation: An enhanced expression of Nrf2 delays the onset of a diet-induced fatty liver [11], while the deletion leads to a rapid onset and progression of the condition [12-14].

b) Nrf2 and high fat diet (HFD)-induced fat accumulation

Nrf2 also negatively influences the accumulation of fat in animals subjected to a high fat diet (HFD): Treatment with the Nrf2 activator CDDO-Im prevents the HFD dependent increases in body weight, adipose mass, and hepatic lipid accumulation in wild type, but not Nrf2-/- mice. [15] Upon feeding a HFD, in the liver of Nrf2-/- mice the mRNAs coding for transcriptional factors and genes involved in lipid synthesis have been shown to be higher than in WT animals [13, 14, 16], but at the same time several studies report that Nrf2-/- mice gain less weight than

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WT animals on a HFD. [14, 17, 18] One study reports conflicting data: It shows that the expression of genes related to lipid synthesis is lower in Nrf2-/- than WT mice on a HFD and that the Nrf2-/- animals gain more weight. [19] Overall, both the activation and the deletion of Nrf2 can protect against weight gain upon feeding of a HFD.

1.2.2 Adipogenesis

There are several studies about the role of Nrf2 in adipogenesis but the results are controversial: Nrf2 has been shown to both positively and negatively regulate adipogenesis. In one study it was found that Nrf2-/- mice have less adipose tissue and smaller adipocytes than WT animals. It was also found that deficiency of Nrf2 impairs adipogenesis in mouse embryonic fibroblast (MEF) cells, 3T3-L1 adipocytes and human subcutaneous preadipocytes, and that Nrf2 positively regulates Peroxisome proliferator-activated receptor γ (PPAR-γ), a master mediator of adipogenesis. [17] A follow up study further revealed that Nrf2 regulates CCAAT/enhancer-binding protein β (C/EBP-β), a transcription factor that works upstream of PPAR-γ. [20] A positive effect of Nrf2 on PPAR-γ has also been shown in airway epithelial cells [21]. Moreover Nrf2 has been shown to directly bind to and positively regulate the retinoid X receptor alpha (RXRα) gene. The nuclear receptor RXRα responds to retinoids and functions as an adipogenic transcription factor as a heterodimer with PPAR-γ. [22] However, this role of Nrf2 in promoting adipogenesis is in disagreement with a study where it was found that the adipocyte differentiation is faster in Nrf2-/- than WT MEFs. The effect was explained by the fact that Nrf2 induces the expression of the aryl hydrocarbon receptor (AHR), a transcription factor that negatively regulates adipogenesis: The AHR mRNA level was lower in the Nrf2-/- than WT cells, and treatment with the Nrf2 activator CDDO-Im could increase the AHR mRNA level only in the WT cells. [23] The negative influence of Nrf2 on adipogenesis is supported by a study reporting that both genetic and pharmacologic activation of Nrf2 with sulforaphane delay the adipogenesis in MEF cells and reduce the level of PPAR-γ. [24]

1.2.3 Insulin signalling

Activation of Nrf2 by different compounds has been shown to ameliorate insulin resistance in hepatocytes and in mice. [25-27] Both the genetic activation of Nrf2 via reduced levels of Keap1 and treatment with the Nrf2 activator CDDO-Im improve the insulin signalling in mice with leptin deficiency, a condition that causes obesity and diabetes. [28] However in the same mouse model the complete deletion of Keap1 impairs the insulin signalling. [24] Inducing Type

I diabetes with streptozotocin has a more dramatic effect on insulin signalling in Nrf2-/- than WT mice [29], while feeding a high fat diet affects the glucose tolerance more severely in WT than in Nrf2-/- mice. [18, 30] For the effect of Nrf2 on insulin signalling a similar picture emerges as for the effect of Nrf2 on high fat diet induced weight gain (see paragraph 1.2.1 about Nrf2 and fat metabolism): Depending on the study design, context, model system and chosen readout both the activation and the deletion of Nrf2 can show a protective effect.

1.2.4 NADPH homeostasis

Dihydronicotinamide-adenine dinucleotide phosphate (NADPH) is a reducing agent that is required both for biosynthetic processes and for regenerating the cellular pool of glutathione (GSH) and thus protecting against ROS induced stress. Liver gene transcription profiles of mice with different expression levels of Nrf2 - from no expression over normal levels to constitutively high levels - have revealed that higher Nrf2 activity correlates with higher NADPH levels. [31] The pentose phosphate pathway (PPP) is the main cellular source of NADPH. It generates NADPH by oxidizing glucose and is thus essential for anabolic processes and cellular proliferation. Several genes coding for important enzymes of the PPP are upregulated by Nrf2: The glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), transketolase (TKT) and transaldolase 1 (TALDO1) are all Nrf2-targets. [32] The PPP plays a crucial role for the Nrf2 dependent signalling. Activation of Nrf2 promotes both the uptake of glucose and its utilization via the PPP in MEF cells. Interfering with either the glucose supply or the PPP affects the Nrf2 dependent gene expression. [33] Through the induction of the PPP and NADPH synthesis Nrf2 favours cellular proliferation. In addition Nrf2 also contributes to anabolism and cellular proliferation by upregulating genes of the purine nucleotide synthesis and the utilization of glutamine. [32]

1.2.5 Mitochondrial function

Nrf2 positively influences the mitochondrial function. In the absence of Nrf2 the major aerobic source of ATP, the mitochondrial oxidative phosphorylation, is impaired and the cells thus rely more on glycolysis for ATP production. The loss of Nrf2 causes a lower mitochondrial membrane potential, lower ATP levels and a reduced mitochondrial respiration. It also leads to a reduced pool of NADH, the electron donor for the aerobic ATP production. [34] Nrf2 positively influences the efficiency of fatty acid oxidation, the process by which fatty acids are broken down to generate NADH and FADH2, both in MEF cells and in mitochondria isolated

from the heart and liver of mice. [35] In the lungs of pneumonia infected mice and in cardiomyocytes Nrf2 has been shown to promote mitochondrial biogenesis. [36, 37]

1.3 The functional domains of Nrf2

The Nrf2 protein comprises 7 functional domains, the Nrf2–ECH homology (Neh) domains 1–7. The basic CNC region and the leucine zipper structure necessary for binding the DNA and forming a dimer with small Maf proteins are contained within the Neh1 domain. [8] The Neh2 and Neh6 domains are involved in the proteasomal degradation of Nrf2 via two different mechanisms. The Neh2-dependent degradation is redox sensitive. Under homeostatic conditions the Neh2 domain is bound by Keap1, an adapter protein for Cul3 dependent ubiquitin ligases, which targets Nrf2 for degradation with a half-life of less than 10 minutes. Oxidative stress interferes with the Keap1-dependent degradation of Nrf2. In contrast the Neh6-dependent degradation is redox insensitive and instead responds to intracellular signalling events. Upon phosphorylation by the glycogen synthase kinase 3 (GSK3) the Neh6 domain recruits the ubiquitin ligase β-transducin repeat-containing protein (β-TrCP) that leads to the degradation of Nrf2 with a half-life of about 40 minutes. [38-40] Neh3, Neh4 and Neh5 are transactivation domains. The Neh3 domain increases transcription by recruiting the chromo-ATPase/helicase DNA binding protein (CHD) 6 [41]. Together the Neh4 and Neh5 domains can recruit different cofactors such as the cAMP response element-binding protein (CREB)-binding protein (CBP) and the nuclear cofactor RAC3/AIB1/SRC-3, leading to a strong transactivation of Nrf2. [42, 43] The CBP-interacting motif is within the Neh4 domain, while the Neh5 domain can recruit different cofactors and its role varies between different Nrf2-regulated genes. [44] The Neh7 domain negatively regulates the activity of Nrf2, as it mediates the binding of the transcription factor retinoid X receptor α (RXRα) that represses Nrf2 dependent signalling. [45]
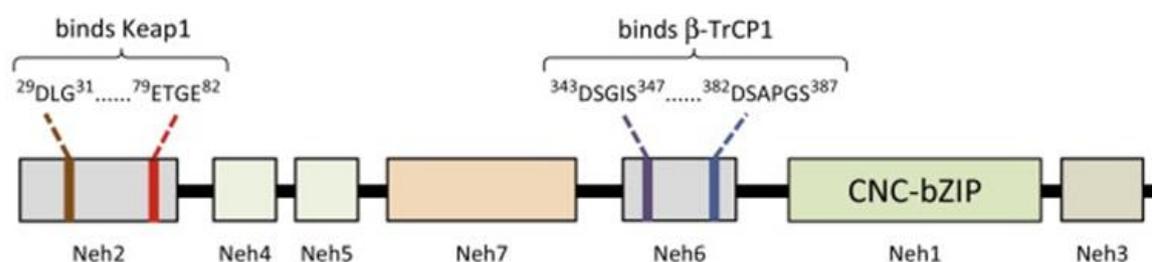
Figure 1: The functional domains of Nrf2

The Nrf2 protein comprises 7 functional domains, Neh1−7. Neh1 contains the basic CNC region and the leucine zipper structure. It binds to the DNA and forms a dimer with small Maf proteins. Neh2 and Neh6 mediate the proteasomal degradation of Nrf2, Neh2 by binding Keap1 and Neh6 by binding ß−TrCP. Neh3, Neh4 and Neh5 are transactivation domains. Neh3 recruits CHD6, Neh4 and 5 together can recruit CBP and RAC3/AIB1/SRC−3. Neh7 negatively regulates the activity of Nrf2 by binding the negative regulator RXRα. Figure adapted from [8].

1.4 Different factors that regulate the activity of Nrf2

Under basal conditions Nrf2 is located in the cytoplasm, where it is targeted for degradation via the ubiquitin-proteasome pathway. Upon the occurrence of oxidative stress or chemical activators Nrf2 is imported into the nucleus where it binds to promoters containing an ARE and drives the transcription of its target genes. The activity of Nrf2 is regulated at several different levels: The Nrf2 protein and mRNA levels are subject to a dynamic regulation. In addition different kinases and cellular pathways influence the nuclear/cytoplasmic localisation of Nrf2 via posttranslational modifications and several factors can regulate the transcriptional activity of Nrf2. [8]

1.4.1 Regulation of the Nrf2 protein and mRNA level

The Nrf2 protein level is regulated via different mechanisms. As mentioned under 1.3 Nrf2 can be targeted for proteasomal degradation via binding of the ubiquitine ligase adaptor Keap1 and the ubiquitin ligase β-TrCP. The ER (Endoplasmic reticulum)-associated E3 ubiquitin ligase Hrd1, the proteins CRIF1 (CR6-interacting factor 1), Siah2 (seven in absentia homolog 2) and RNF4 (RING finger protein 4) also lead to the ubiquitination and subsequent degradation of Nrf2. The autophagy adaptor p62 influences the Nrf2 protein level by sequestering Keap1, while p21 (Cip1/Waf1) and BRCA1 (Breast Cancer gene 1) both stabilize Nrf2 by binding to it and preventing the Keap1-dependent degradation. In addition the Nrf2 protein level is also influenced by miRNAs that target the Nrf2 and Keap1 mRNAs and by epigenetic modifications of the Nrf2 promoter that influence the transcription of the Nrf2 gene. [8, 46]

a) Keap1 dependent regulation

Under basal conditions Nrf2 is constantly targeted for proteasomal degradation via Keap1: The protein directly binds to the N-terminal Neh2 domain of Nrf2 and acts as an adaptor for the Cul3-based E3 ligase that promotes the ubiquitination and subsequent degradation of Nrf2. [47] One molecule of Nrf2 engages a Keap1 dimer. Nrf2 has a high-affinity ETGE and a low-affinity DLG site for binding Keap1. One Keap1 of the dimer binds to the ETGE and the other to the DLG site. Keap1 contains several cysteine residues that are sensitive to the oxidative environment, with Cys257, Cys273, Cys288 and Cys297 being the most reactive ones. [48] Cys273 and Cys288 have been shown to be essential for the repressive function of Keap1, while Cys151 is important for the de-repression of Nrf2. Modification of these motifs by oxidative stress or electrophilic compounds leads to conformational changes of Keap1 and the stabilization of Nrf2. [49, 50] There are several proposed mechanisms for how electrophiles interfere with the Keap1 dependent degradation of Nrf2. In the first model, which has already been contradicted by several studies, Keap1 entirely dissociates from Nrf2. The mechanism was refined into the 'hinge and latch model' in which electrophiles alter the conformation of the Keap1 dimer by modifying the reactive cysteine residues of Keap1, leading to the dissociation of Keap1 from the DLG but not from the ETGE motif (see Fig 2). A third possibility is that the electrophiles promote the dissociation of the ubiquitin ligase from Keap1. [51]
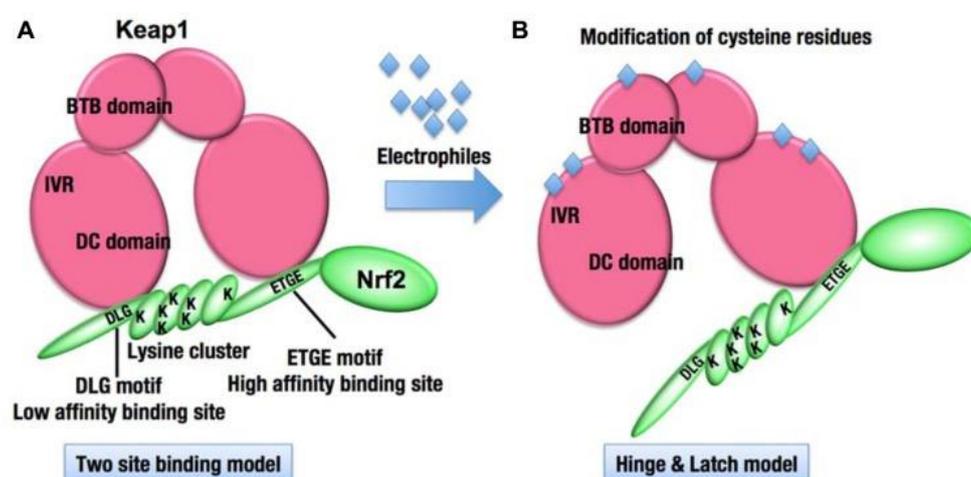
Figure 2: The 'hinge and latch model' of Keap1－Nrf2 binding

a）Keap1 binds Nrf2 as a dimer．One Keap1 monomer binds to the low affinity DLG motif while the other binds to the high affinity ETGE motif．b）Keap1 contains several redox sensitive cysteine residues．Oxidative stress or electrophiles modify the cysteine residues，leading to conformational changes of Keap1 that promote the dissociation from the DLG，but not the ETGE motif．Figure adapted from ［52］．

Not only oxidative stress influences the degradation of Nrf2 via Keap1: The protein p62, also called sequestosome 1 (SQSTM1), is an adaptor for autophagy that influences the amount of Keap1 available for binding Nrf2. It directly interacts both with ubiquitinated proteins and the autophagic machinery: p62 promotes the formation of protein aggregates that are degraded by autophagy. [53] It has a direct binding site for Keap1, which it sequesters into these aggregates thus activating Nrf2. [54] Phosphorylation of p62 by mTORC1 greatly increases the binding affinity of p62 for Keap1. [55] P62 itself is in turn positively regulated by Nrf2: The gene contains an ARE in the promoter region. [56] The proteins p21 (Cip1/Waf1) and BRCA1 stabilize Nrf2 by competing with Keap1 for the Nrf2 binding sites and thus interfering with the Keap1-mediated ubiquitination. [57, 58]

b) Keap1 independent regulation

Apart from the mostly redox sensitive Keap1-dependent proteasomal degradation, Nrf2 is also degraded in a redox independent way via the E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP). There are two Nrf2 binding sites for β-TrCP in mouse Nrf2, DSGIS and DSAPGS. The binding of β-TrCP to the DSGIS site, but not to the DSAPGS site, is increased by the GSK3 dependent phosphorylation of a serine cluster in the Neh6 domain of Nrf2. [39, 40] Keap1 and ß-TrCP are the best-studied regulators of the stability of Nrf2, but a few more proteins have been also been reported to promote the degradation of Nrf2. The ER-associated E3 ubiquitin ligase Hrd1 is upregulated in response to ER stress. Hrd1 directly interacts with the Neh4 and Neh5 domains of Nrf2 promoting a Keap1 and β-TrCP independent degradation. [46] Furthermore the ubiquitination of Nrf2 can also be promoted by CR6-interacting factor 1 (CRIF1) that interacts both with the N-terminal Neh2 and the C-terminal Neh3 domains, by seven in absentia homolog 2 (Siah2) that degrades Nrf2 during hypoxia in a Neh2-independent manner and by the SUMO-specific RING finger protein 4 (RNF4) that degrades the

polysumoylated Nrf2 within the nucleus. [8] The Nrf2 protein level is not only regulated via proteasomal degradation: It is also subject to regulation by miRNAs that bind to the Nrf2 mRNA and downregulate the translation of Nrf2. Several microRNAs have been shown to negatively regulate Nrf2 including miR-153, miR-27a, miR-142-5p, miR-144, miR-28 and miR-34a. MicroRNAs that target the Keap1 mRNA, such as miR-200a, can positively influence the level of Nrf2: Expression of miR-200a enhances the Nrf2 activation, whereas silencing it increases the level of Keap1 and thus diminishes that of Nrf2. [59] Additionally the Nrf2 protein level is also influenced by the transcriptional regulation of the Nrf2 gene via epigenetic modifications. Both in human and mice prostate cancer cells the transcription of Nrf2 is suppressed epigenetically by the methylation of CpG sites within the promoter. [60, 61] Two different Nrf2 activators, curcumin and sulforaphane, and a γ-tocopherol-rich mixture of tocopherols have been shown to increase the Nrf2 target gene expression by reducing the CpG methylation of the Nrf2 promoter. [62-64]

1.4.2 Regulation of Nrf2 via phosphorylation and intracellular signalling

A number of different kinases and cellular pathways have been shown to influence the activity of Nrf2. Protein kinase C (PKC) and the PI3K/Akt/GSK3 pathway play a major role, but CK2 (casein kinase 2), the Src subfamily kinases, the MAP (Mitogen-activated protein) kinases, PKA (Protein kinase A) and PERK (Protein kinase RNA-like endoplasmic reticulum kinase), a kinase that is activated by ER stress, also have an impact on the activity of Nrf2. Recurring themes common to these regulators are cellular growth and proliferation: PKC, PI3K, CK2 and the MAPKs are all positively regulating these processes and positively influence the activity of Nrf2, while GSK3 negatively regulates them and the activity of Nrf2.

a) PKC

The PKC family has a broad action spectrum. There are eight different PKC isozymes that are highly related but exhibit different functions. The kinases respond to different growth factors and contribute to diverse processes, including proliferation, differentiation, migration, apoptosis and carcinogenesis. [65] The PKC-δ isozyme has been shown to positively regulate Nrf2 by phosphorylating it on Ser-40, a residue that lies within the Keap1-binding Neh2 domain of Nrf2, thus promoting the release of Nrf2 from Keap1. [66-68] The PKCε isoform negatively regulates Nrf2: By phosphorylating Keap1 it increases the degradation of Nrf2. [69]

b) PI3K/Akt/GSK3 and the Src kinases

The PI3K/Akt pathway is activated by different growth factors and hormones, including insulin. They bind to receptors on the cell surface that activate the Phosphoinositide 3-kinase (PI3K), initiating a signalling cascade which in turn leads to the activation of Akt, also known as Protein kinase B (PKB). The PI3K pathway has a positive influence on Nrf2 signalling: Inhibiting the PI3K decreases both the nuclear translocation of Nrf2 and the induction of Nrf2 target proteins [70, 71], while for a great number of Nrf2-activating compounds it has been shown that the PI3K/Akt pathway takes part in the activation. Akt promotes cellular growth and survival. For a full activation it needs two phosphorylations: One by the 3'-phosphoinositide dependent kinase-1 (PDK1) on Thr308, in its kinase domain, and one by the mTOR Complex 2 (mTORC2) on Ser473, in its C-terminus. Akt promotes proliferation, transcription and protein synthesis by activating mTORC1 via TSC2 (Tuberous Sclerosis Complex 2). At the same time it increases the uptake of glucose by upregulating the transcription of the glucose transporter GLUT1 and promoting the translocation of GLUT4 to the plasma membrane. [72] Akt negatively regulates GSK3, a kinase that promotes the degradation of Nrf2. GSK3 was first discovered to inhibit the synthesis of glycogen, the storage form of glucose. In addition it also inhibits cell division and proliferation via the beta-catenin/Wnt pathway by phosphorylating and destabilizing ß-catenin, and it has both pro- and anti-apoptotic functions. There are two distinct isoforms, GSK3α and GSK3β. Akt inhibits both isoforms by phosphorylating GSK3α at Ser21 and GSK3β at Ser9. [73] The GSK3ß isoform promotes a Keap1 independent degradation of Nrf2. As already mentioned above under 1.4.1, the GSK3ß generates a recognition site for the E3 ligase β-TrCP by phosphorylating a group of Ser residues in the Neh6 domain of Nrf2. [40, 74] In addition GSK3ß has also been shown to promote the nuclear export of Nrf2 via a Fyn kinase-dependent phosphorylation of Nrf2 at Tyr568. [75] Fyn belongs to the Src subfamily of kinases that has three more members (Src, Yes, and Fgr). They can all phosphorylate Nrf2 on Tyr568 and promote its nuclear export, ubiquitination, and degradation. [76]

c) CK2

The ubiquitous CK2 is expressed with higher levels in proliferating tissues and is necessary for cell cycle progression and viability. [77] It regulates Nrf2 on two levels: CK2 both mediates a constitutive basal phosphorylation of Nrf2 and a dynamic phosphorylation in the Neh4 and Neh5 transactivation domains that promotes the nuclear localisation of Nrf2. [78-80]

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d) The MAP kinases

The MAPK pathway is activated downstream of different ligand bound growth factor receptors. These receptors initiate highly conserved kinase cascades that result in the activation of the specific MAP kinases. The three main MAPK families are the ERKs (extracellular signal regulated kinases), the JNKs (Jun amino terminal kinases), and the p38/SAPKs (stress activated protein kinases). The different MAP kinases mediate specific cellular responses. ERK1 and ERK2 regulate cell proliferation and cell cycle progression, p38 is critical for the immune and inflammatory responses, JNK1 and JNK2 are involved in the control of cell proliferation and the apoptotic response. [81] The MAPKs phosphorylate Nrf2 at multiple sites, though only with a minor positive influence on the transcriptional activity of Nrf2. [82] In HepG2 cells inhibiting ERK and p38 reduces the binding of Nrf2 to and subsequent transcription of the glutamylcysteine synthetase (GCS) subunit genes. However mutations in the conserved MAPK phosphorylation sites of Nrf2 do not affect its activity, suggesting an indirect effect of the MAPKs on Nrf2. [83, 84] ERK and JNK have been shown to promote the transactivation of Nrf2 while p38 negatively influenced it. [85]

e) PKA

The protein kinase A (PKA) is activated by the second messenger cAMP and regulates different processes, including sugar and lipid metabolism. It positively influences Nrf2 signalling in mouse and human hepatocytes. [86] PKA can phosphorylate and thus inactivate a negative regulator of Nrf2, GSK3. [87] It has also been shown to activate SIRT1, a deacetylase that negatively regulates the transcriptional activity of Nrf2. [88]

f) ER stress and PERK

Nrf2 promotes cellular survival under ER stress conditions at least in part by raising the cellular glutathione levels. [89] The orthologue of Nrf2 in *C. elegans*, SKN-1, is required for the transcriptional response to ER stress, that is overlapping yet distinct from the response to oxidative stress. Moreover, the response of SKN-1 to oxidative stress is dependent on a functioning ER signalling. [90] The kinase PERK activates Nrf2 under conditions of ER stress. PERK is activated when unfolded proteins accumulate in the endoplasmic reticulum and in response it inhibits translation and causes a cell cycle arrest. It directly phosphorylates Nrf2 and promotes the release from Keap1 and subsequent nuclear import. [91] However ER stress

can also negatively regulate Nrf2 by promoting the proteasomal degradation of Nrf2 via the ubiquitin ligase Hrd1. [46]
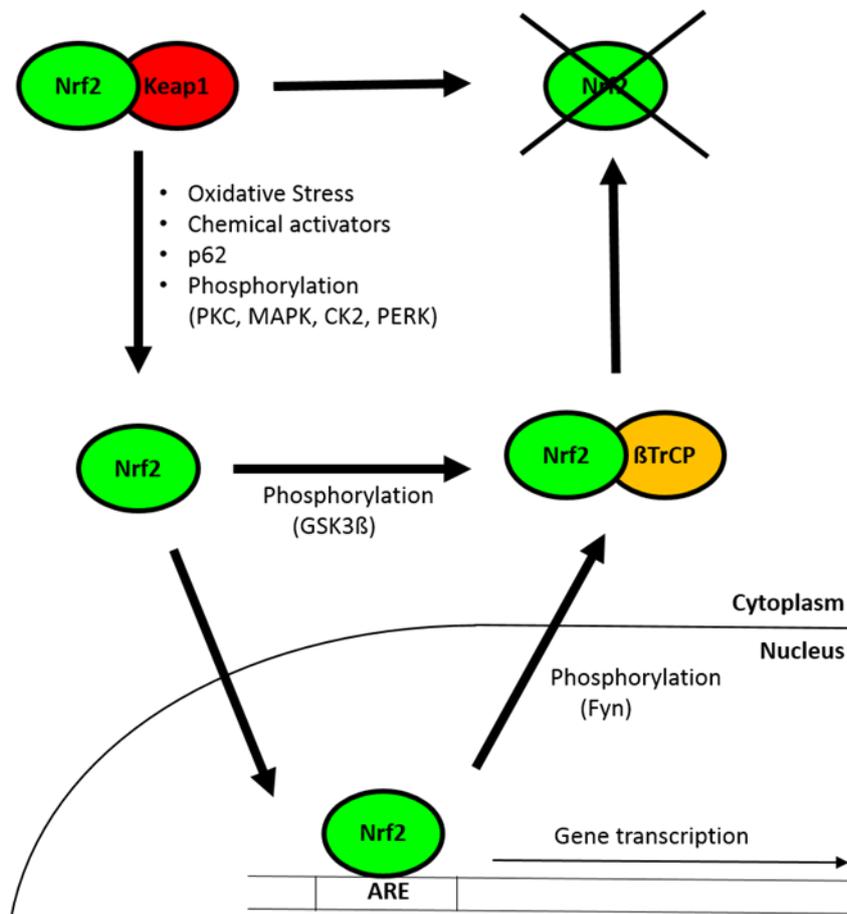
Figure 3: The main players regulating the stability and localization of Nrf2

Under basal conditions Keap1 binds to Nrf2 and targets it for proteasomal degradation. Oxidative stress, chemical activators and phosphorylation of Nrf2 by different kinases interfere with the Keap1-dependent degradation. The protein p62 activates Nrf2 by binding to Keap1 and targeting it for autophagic degradation. When not bound to Keap1, Nrf2 is free to migrate into the nucleus where it binds to ARE-containing promoter regions and drives gene transcription. Phosphorylation of Nrf2 by GSK3ß promotes the binding of ßTrCP that targets Nrf2 for a Keap1-independent proteasomal degradation. The Fyn kinase, which is positively regulated by GSK3ß, phosphorylates Nrf2 in the nucleus thus promoting the nuclear exclusion and subsequent degradation of Nrf2 via ßTrCP.

1.4.3 Regulation of the transcriptional activity of Nrf2

Several proteins negatively influence the Nrf2 dependent signalling by interfering with the promoter binding and transcriptional activity of Nrf2: This is the case for p53, Bach1 and RXRα. P53 is activated and upregulated by oxidative stress. Low levels of p53 promote cellular survival and activate Nrf2, while high levels of p53 induce apoptosis and reduce the activity of Nrf2. [92] p53 binds to ARE-containing promoters and physically interferes with the Nrf2 dependent gene transcription. [93, 94] Also the transcriptional repressor Bach1 antagonizes Nrf2 by competing for the binding to the ARE. [45, 95] Redox stress and free heme inhibit the DNA-binding activity of Bach1 by modifying its cysteine residues and thus allow Nrf2 to bind [96, 97], while antioxidants promote a temporary nuclear exclusion of Bach1 by promoting its phosphorylation at Tyrosine 486. [98] RXRα (retinoic X receptor alpha) binds both to the Neh7 domain of Nrf2 and to the ARE and negatively regulates the transcriptional activity of Nrf2. The histone acetyltransferase (HAT) p300/CBP strongly increases the transactivation activity of Nrf2 by binding to the Neh4 and Neh5 transcription activation domains of Nrf2. [42] Modification of Nrf2 by acetylation influences the transcriptional activity of Nrf2: p300/CBP directly acetylates Nrf2 and thus increases its promoter-specific DNA binding [82, 99], while the NAD+ dependent deacetylase sirtuin 1 (SIRT1) decreases both the acetylation of Nrf2 and the Nrf2-dependent gene transcription. [99] The effect of other histone deacetylases on the activity of Nrf2 is less clear: In astrocytes a decreased histone acetylation reduces the Nrf2 dependent signalling that is restored by the HDAC inhibitor Trichostatin A (TSA). [100] Also in RAW 264.7 cells treatment with TSA activates Nrf2 [101], but in bronchial epithelial cells treatment with TSA or knockdown of HDAC2 reduce the stability and activity of Nrf2. [102]

1.5 Natural compounds that activate Nrf2

There is a great number of naturally occurring compounds that activate Nrf2. They are chemically quite diverse. What most of them have in common is their ability to modify the thiol residues of the Nrf2-repressor Keap1. The human Keap1 has 27 cysteine residues. Site-directed mutagenesis of the Keap1 cysteine residues has shown that Cys151, Cys273 and Cys288 are particularly important for regulating the activity of Nrf2. [50] A big group of Nrf2 activators are Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups) that can be found in various classes of phytochemicals, including flavonoids, coumarins, chalcones, terpenoids, curcuminoids, cinnamic acid derivatives, and thiophenes. The Michael acceptors undergo reversible alkylating reactions with different Keap1 sensor

thiols, especially Cys151: For example this is the case for xanthohumol, isoliquiritigenin and 10-shogaol. [103] Nrf2 activators that covalently bond with Keap1 include oxidizable diphenols and quinones, isothiocyanates (ITCs), dithiolethiones, diallyl sulphides and polyenes. [104] The isothiocyanate sulphoraphane stabilizes Nrf2 by forming thionoacyl adducts with Keap1. [105] The ability of flavonoids to activate the Nrf2-dependent gene expression directly correlates with their intrinsic potential to generate oxidative stress, suggesting that they indirectly activate Nrf2 by generating stress. [106] Natural compounds can also activate Nrf2 by activating other cellular pathways that in turn positively influence the activity of Nrf2 or negatively influence that of Keap1 via phosphorylation events: PKC, the MAPK pathway and the PI3K pathway have all been shown to contribute to the activation of Nrf2. The PKC dependent phosphorylation of Nrf2 on Ser40 contributes to the stabilization and nuclear translocation of Nrf2 by the antioxidant tertiary butylhydroquinone (tBHQ). [67] The antioxidant-dependent nuclear export of Keap1 requires its phosphorylation at Tyr85. [107] Phenolic antioxidants and isothiocyanates promote the Nrf2 dependent gene transcription by activating the MAPK pathway [108] and the PI3K pathway also positively regulates Nrf2. Accordingly several natural compounds activate both Nrf2 and PI3K, to name a few epigallocatechin gallate, geniposide, capsaicin and carnosol. [109-112]

2. AMPK

2.1 AMPK and the regulation of energy balance

The AMP-activated protein kinase (AMPK) is an enzyme that reacts to changes in the AMP/ATP ratio. It is composed of a catalytic α subunit and the two regulatory β and γ subunits. When there are high AMP and low ATP levels AMP binds to the γ subunit and favours the activating phosphorylation of AMPK at Thr172. LKB1 is the main upstream kinase that mediates the AMP-dependent activation, while the calcium dependent CAMKKß activates AMPK in response to intracellular calcium increases. AMPK promotes processes that generate ATP and limits those that consume it, thus acting as a cellular switch that turns off energy consuming anabolic processes in favour of catabolic energy generating ones (see Fig. 4). [113]
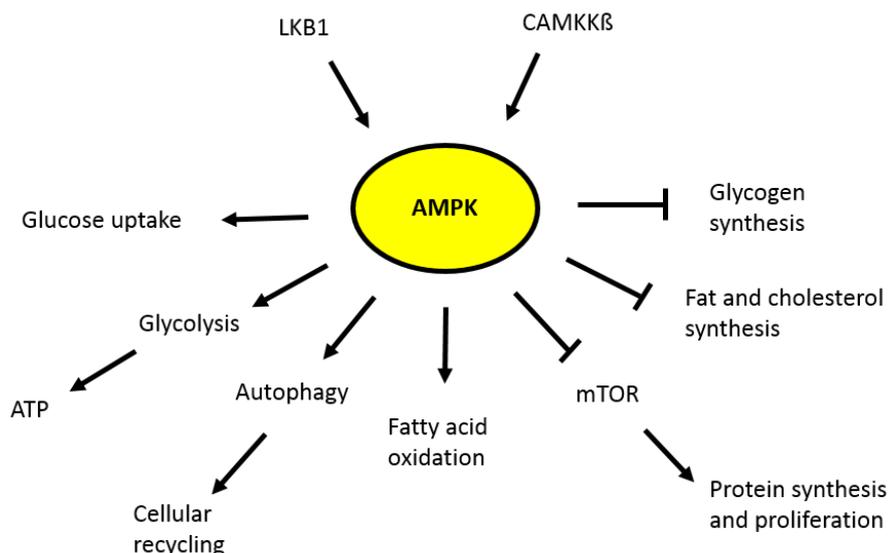
Figure 4: An overview of AMPK signalling

LKB1 and CAMKKß are the two major kinases that activate AMPK via phosphorylation at Thr172. Upon activation AMPK negatively regulates anabolic processes such as the synthesis of glycogen, fat and cholesterol. It inhibits protein synthesis and proliferation by inhibiting mTORC1. At the same time it promotes glucose uptake and the generation of ATP via glycolysis, it promotes autophagy to recycle cellular resources and it induces the breakdown of fatty acids via ß-oxidation.

When the cellular ATP levels are low AMPK restores energy homoeostasis by influencing several different metabolic processes. It negatively regulates the synthesis of fatty acids, cholesterol and glycogen and inhibits cellular growth and proliferation. At the same time it favours the uptake of glucose, cellular recycling via autophagy, fatty acid oxidation and glycolysis. Below follows a brief description of how AMPK regulates these different processes.

a) Cellular growth and proliferation

AMPK negatively regulates cellular growth and proliferation by inhibiting mTORC1. The mTOR (mechanistic target of rapamycin) is a kinase that is activated by high nutrient levels and regulates cellular growth. There are two distinct mTOR multi-protein complexes. The nutrient-sensitive mTOR complex 1 (mTORC1) is inhibited by rapamycin. It responds to insulin, growth factors and amino acids by activating cell growth, proliferation, protein synthesis and anabolism. The mTORC2 is neither sensitive to nutrients, nor is it inhibited by rapamycin. AMPK negatively regulates mTORC1 by phosphorylating its upstream regulator TSC2 (Tuberous Sclerosis Complex 2) on Ser1387. This leads to TSC2 inactivating the small Ras-like

GTPase Rheb, a protein that directly activates mTORC1. In addition AMPK also inhibits the kinase activity of mTORC1 by phosphorylating one of its components, raptor, on Ser722 and Ser792. [114]

b) Fatty acid metabolism

AMPK negatively regulates the synthesis of fatty acid while it increases their oxidation. It does so by inhibiting the acetyl-CoA carboxylase (ACC). There are two ACC isoforms, ACC1 and ACC2, and both of them contribute to fatty acid synthesis by catalysing the carboxylation of acetyl-CoA to malonyl-CoA, but ACC1 is the essential one. AMPK inhibits the enzymes by phosphorylating the ACC1 at Ser79 and the ACC2 at Ser221. In addition AMPK also favours oxidative metabolism by modulating the activity of transcription factors. Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α promotes a shift to oxidative metabolism and stimulates mitochondrial biogenesis. AMPK promotes a post-translational activation of PGC-1α by phosphorylating it on Thr1722 and Ser538. [115]

c) Autophagy

Together with mTOR AMPK regulates the recycling of cellular resources via autophagy: The protein Ulk1 (Unc-51 Like Autophagy Activating Kinase 1) is necessary for the initiation of autophagy. When cellular glucose levels are low AMPK activates Ulk1 through phosphorylation at Ser317 and Ser777. When nutrients levels are high mTOR prevents the interaction between Ulk1 and AMPK by phosphorylating Ulk1 at Ser757. [116]

d) Glucose metabolism

AMPK promotes the cellular uptake of glucose: By phosphorylating TBC1D1 (TBC1 domain family member 1) it stimulates the translocation of the GLUT4 transporter to the plasma membrane. [117] At the same time it inhibits the synthesis of glycogen in the liver by phosphorylating the glycogen synthase [118] and it increases the rate of the ATP producing glycolysis. [119, 120]

2.2 AMPK and Nrf2

When reading the literature, it strikes that an overlapping set of cellular processes and molecules is either susceptible to or influencing the activity of both Nrf2 and AMPK, indicating that the two proteins operate in a common field of action. Both are involved in regulating the cellular fat metabolism and both crosstalk with the PI3K pathway, ER stress, autophagy and p53. Below follows a brief overview of how these different cellular processes and players are connected with AMPK and Nrf2 (also see Fig. 5).

a) Energy metabolism

Both AMPK and Nrf2 inhibit fatty acid synthesis, Nrf2 by negatively regulating lipogenic genes [9] and AMPK by inhibiting ACC [121]. They also both raise the cellular NADPH levels: Nrf2 by regulating the expression of enzymes of the pentose phosphate pathway [31] and AMPK by decreasing fatty acid synthesis in favour of oxidation [122].

b) The PI3K/Akt/GSK3 pathway

The PI3K/Akt crosstalks both with AMPK and Nrf2. It positively regulates Nrf2 and negatively regulates AMPK: Inhibiting the Akt signalling reduces the activity of Nrf2, while upon activation by insulin Akt inhibits AMPK. [123, 124] Akt also inhibits GSK3 that in turn has been shown to inhibit the catabolic action of AMPK and to promote the degradation of Nrf2. [40, 125]

c) ER stress

AMPK attenuates ER stress [126], a condition that has been shown to both positively and negatively influence the activity of Nrf2: ER stress can activate Nrf2 by activating PERK that phosphorylates Nrf2 and thus promotes the dissociation from the inhibitor Keap1 [91], but ER stress can also promote a Hrd1-dependent degradation of Nrf2 [46].

d) Autophagy

AMPK directly activates autophagy and at the same time it inhibits the inhibitor of autophagy mTORC1 [116]. The autophagy adaptor p62 activates Nrf2 by sequestering Keap1 into protein aggregates that it targets for autophagic degradation. Phosphorylation of p62 by mTORC1 increases the binding affinity of p62 for Keap1 [55].

e) p53

In response to metabolic stress AMPK activates p53 and thus induces a cell cycle arrest [127]. Activation of p53 can suppress the Nrf2 dependent gene transcription [93].

Moreover, a great number of natural compounds is known to lead to both Nrf2 and AMPK activation, to name just a few berberine [128], epigallocatechin gallate [129], alpha-lipoic acid [130] and resveratrol [131]. Activated AMPK and activated Nrf2 share many positive effects: For instance, both protect against inflammation, diabetes and obesity and both promote longevity. [132-134] This suggests that the two proteins might be interconnected signalling hubs in the cell. A few recent studies have addressed this issue and shown that the AMPK positively influences both the activity and target gene expression of Nrf2. Treatment with the AMPK inhibitor compound c impairs both the accumulation of Nrf2 and the Nrf2 dependent induction of HO-1 induced by different activators in cell culture studies. [135-137] Silencing of the AMPK abolishes the Nrf2-dependent upregulation of HO-1 by the AMPK activator AICAR, while the expression of a constitutively active AMPK mimics the effect of AICAR on the HO-1 expression. [138] In human lung epithelial cells exposed to cigarette smoke extracts, treatment with AICAR restores a normal Nrf2 signalling. [139] In addition, transfection with a dominant negative AMPK prevents both the nuclear accumulation of Nrf2 and the upregulation of the target proteins HO-1 and NQO1 in berberine treated cells. Transfection with a dominant negative Nrf2 does not affect the phosphorylation and activity of AMPK, indicating that the AMPK operates upstream of Nrf2. [140] The molecular details underlying the interaction of AMPK and Nrf2 signalling so far remained undisclosed.
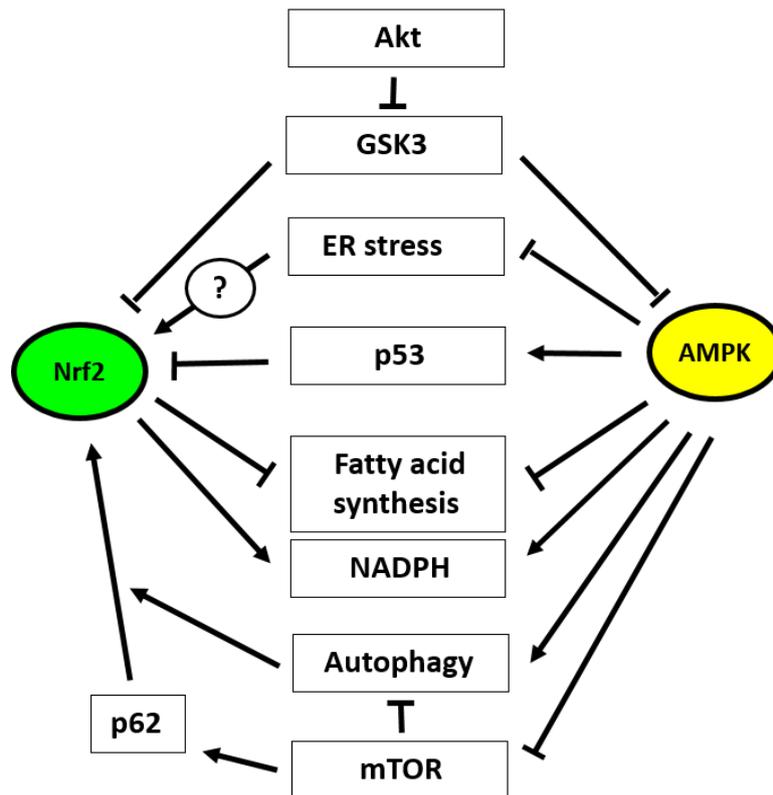
Figure 5: Factors and processes that crosstalk both with AMPK and Nrf2

The **GSK3** is inhibited by **Akt** and in turn it inhibits both Nrf2 and AMPK. The AMPK attenuates **ER stress** that can both activate Nrf2 via PERK and promote its degradation via Hrd1. In response to stress the AMPK activates **p53** that can prevent the transcription of Nrf2 dependent genes. Both AMPK and Nrf2 inhibit **fatty acid synthesis** and raise the cellular **NADPH** levels. AMPK promotes autophagy and inhibits **mTOR** that negatively regulates autophagy. Phosphorylation of the autophagy adaptor **p62** by mTOR activates Nrf2: It increases the binding affinity of p62 for the Nrf2 inhibitor Keap1 that is then degraded via autophagy.

3. Xanthohumol

3.1 Background information

Xanthohumol (XN) is a prenylated chalcone that is mainly found in in the female inflorescences of the hop plant (humulus lupulus), where it is the most abundant prenylflavonoid. The principal dietary source is beer, to which it gives bitterness and flavour. [141] A brewing experiment has shown that only 22-30% of the hops xanthohumol can be found in the beer, mostly isomerized into isoxanthohumol. [142] Accordingly most commercial beers contain less than 0.1 mg/L of xanthohumol. But pale malt brews contain up to 3 mg/L XN when the

'XAN' technology is applied and the addition of roasted malt allows to achieve dark beers with concentrations above 10 mg/L. [143] Rat feeding experiments have shown that around 89% of xanthohumol is recovered unchanged from the feces, indicating that mostly it is not metabolized. [144] The pharmacokinetics were found to be similar for rats and humans with a dose dependent bioavailability. In humans 20, 60, and 180 mg doses of XN were administered and the maximum XN concentrations (C(max)) in the blood were 33 ± 7 mg/L, 48 ± 11 mg/L, and 120 ± 24 mg/L respectively, while the areas under the curve (AUC) were 92 ± 68 h × µg/L, 323 ± 160 h × µg/L, and 863 ± 388 h × µg/L. Peak concentrations were found 1 hour and 4-5 hours after the ingestion, with a mean half-life of 20 h for the 60 and 18 h for the 180 mg dose. [145, 146]
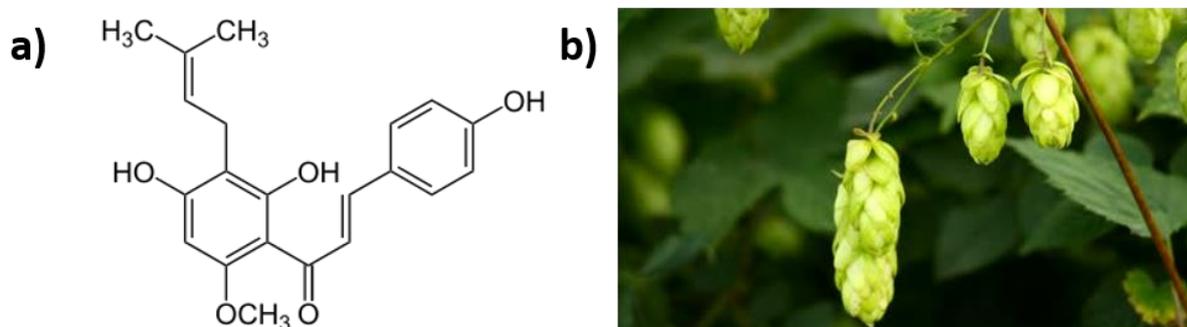
Figure 6: Xanthohumol

a）The chemical structure Xanthohumol，a prenylated chalcone．b）XN is found in the female inflorescences of the hop plant（humulus lupulus），where it is the most abundant prenylflavonoid．Picture source：［147］

3.2 Positive effects on health

A number of positive effects have been documented for Xanthohumol. [148] The broad action spectrum includes anti-inflammatory, anti-oxidative, anti-proliferative and pro-apoptotic activities. Out of a combination of these positive effects treatment with XN plays a protective and ameliorating role in several complex disease conditions including cancer, diabetes, atherosclerosis and chronic liver diseases such as steatohepatitis. [149-152] XN also has anti-infective properties: It protects against bacteria, viruses, fungi and malarial protozoa. [153] Furthermore XN influences energy metabolism. It inhibits the differentiation of preadipocytes, and induces apoptosis in mature adipocytes. [154] In mice it protects against obesity and

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lowers the blood glucose. [155, 156] The metabolic action of XN has been shown to correlate with the activation of AMPK in the liver of mice, [157] while XN has been shown in different cell lines to protect against oxidative stress via activation of Nrf2 [158-160] and it reduces inflammation and angiogenesis by inhibiting the activity of cyclooxygenase-1, cyclooxygenase-2 and NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells). [149, 161] The antioxidant properties of XN appear to be closely connected to its anti-inflammatory action: In microglial BV2 cells XN reduces the production of inflammatory mediators while at the same time upregulating HO-1 via Nrf2-ARE signalling. [162] In a hepatic ischemia/reperfusion model XN is both antioxidant and reduces inflammation. [163] Also in diabetic mice it decreases inflammation and oxidative stress. [151] The protective action of XN appears to be concentration dependent: In breast cancer cells a low concentration (0.001μM) reduces oxidative stress while a high concentration (5 μM) worsens it. [164] This could be due to the generation of ROS in the mitochondria: In different cell lines it has been shown that treatment with low micromolar concentrations of XN led to uncoupled respiration, while higher concentrations caused a ROS-dependent inhibition of the respiration. [165] Through the generation of ROS XN exerts an antiproliferative activity and triggers apoptosis in different cancer cell lines. [166, 167]

4. Aim of the work

It is well known from the existing literature that Nrf2 plays a crucial role for protecting the cells against oxidative and electrophilic stress. In addition it also regulates metabolic processes, such as fatty acid synthesis, mitochondrial function, insulin signalling and NADPH homeostasis. However it has not been investigated yet whether major metabolic signalling pathways crosstalk with and thus take part in regulating the Nrf2-dependent signalling. The AMPK is key player for regulating the cellular energy metabolism. It adapts the cellular energy household to the cellular ATP levels. Several natural compounds with similar bioactivities have been shown to activate both Nrf2 and AMPK, suggesting that there might be a link between the two proteins, and in a few recent studies AMPK has been shown to positively influence the activity and target gene expression of Nrf2. The aim of this work was to investigate whether the AMPK crosstalks with the Nrf2-dependent signalling, and if so, to study how it influences Nrf2 to ultimately gain further insights into whether cellular metabolism influences the Nrf2-dependent stress response. To do so, we used the natural compound Xanthohumol as a chemical tool for studying the potential crosstalk: It has been shown to activate both Nrf2 and AMPK. As a molecular readout for the activation of Nrf2 the levels of the highly inducible Nrf2-regulated protein HO-1 were detected. The effect of AMPK on the Nrf2-/HO-1 axis was assessed by comparing the effect of treatment with XN on the upregulation of HO-1 in WT and AMPK-/- MEF (mouse embryonic fibroblast) cells.

B. Materials and Methods

1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) or Carl Roth (Karlsruhe, Germany).

2. Cell culture

2.1 Employed cell lines

a) MEF cells

The Nrf2-/- , Keap1-/- and WT mouse embryonic fibroblasts were kindly provided from Thomas Kensler (University of Pittsburgh, USA), the AMPK-/- and WT from Benoit Viollet (Institute Cochin INSERM, Paris, France) and the LKB1-/- and WT from Reuben J. Shaw (Salk Institute, La Jolla, California, USA).

b) CHO cells

The Chinese hamster ovary (CHO-K1 clone) cells were obtained from ATCC (Manassas, VA) and stably transfected with the expression vector pGL4.22[luc2CP/Puro] (Promega, Madison, WI) carrying the luciferase gene from Photinus pyralis behind the ARE of the murine GSTA1 gene and a puromycin resistance. The cells were co-transfected with the expression vector pEGF-N1 (ClonTech, Mountian View, CA) carrying the gene for the enhanced green fluorescent protein that they also stably integrated. The GFP signal was used as an internal control to normalize the luciferase signal.

2.2 General cell culture procedures

The MEF and CHO/ARE-Luc cells were both grown as adherent monolayer cultures. For general cultivation they were kept on 75 cm² cell culture flasks (Sarstedt, Nümbrecht, Germany) in complete growth medium and stored in humidified incubators (37 °C, 5 % CO2). The cells were routinely examined under the microscope to evaluate the health and growth and regularly tested and found to be negative for mycoplasma contaminations. Upon reaching 80% of confluence the cells were subcultured, generally twice a week. To detach them from the wall of the flask they were washed once with PBS, incubated for 3 minutes at 37°C with trypsin-EDTA and then rinsed off the wall with complete growth medium. After collection through centrifugation the cells were resuspended in fresh complete growth medium and counted with the aid of a ViCell (Beckman Coulter, Brea, CA). An appropriate amount of cells was then

re-seeded in new flasks and/or in cell culture vessels for the experiments. 6 well, 12 well, 24 well or 96 well plates and 6- and 10-cm diameter cell culture dishes from Greiner Bio-One were employed. Cells were used for experiments upon reaching 80% of confluence. To avoid the Xanthohumol being absorbed onto the serum, for all experiments carried out in MEF cells the complete growth medium was replaced with a treatment medium containing only 2% heat inactivated fetal calf serum one hour prior to the treatment, and the incubation was carried out in this medium. Unless otherwise stated, all compounds were dissolved in DMSO. The final concentration of DMSO did not exceed 0.2%.

2.3 Freezing and thawing

a) Freezing

The cells were passaged as described above. After the counting they were collected by centrifugation and then resuspended in the freezing medium with a concentration of 1.000.000 cells/ml. 1 ml of the suspension was pipetted into each cryovial. The cryovials were immediately placed on ice, then kept at -20°C for 1 hour followed by one day at -80°C and long term storage in liquid nitrogen.

b) Thawing

The cryovial containing the cells was removed from the liquid nitrogen and placed at room temperature. Just before the content had completely thawed it was poured into 10 ml of pre-warmed complete growth medium. The cells were collected by centrifugation (5 minutes at 200 g), gently resuspended in 15 ml of fresh growth medium and transferred to a cell culture flask. On the following day the medium was changed.

2.4 General materials, media and buffers

a) General materials

General material	Specifications	Provider
DMEM (Dulbecco's Modified Eagle Medium)	4.5 g/L glucose, w/o L-glutamine, w/o phenolred	Lonza, Basel, Switzerland
Penicillin/streptomycin mixture	10.000 U/ml potassium penicillin, 10.000 µg/ml streptomycin sulphate	Lonza, Basel, Switzerland
L-glutamine	200 mM	Lonza, Basel, Switzerland

FBS (Fetal bovine serum)	Heat inactivated for 30 minutes at 56°C, sterile-filtered (0.22 μm)	Gibco/Life Technologies, Carlsbad, CA
T/E (Trypsin/EDTA in PBS)	Trypsin 0.05 %, EDTA 0.02 %, sterile-filtered (0.22 μm)	Cambrex Bio Science, Verviers, Belgium

b) Cell culture media

Cell type	Medium	Composition
CHO/ARE-Luc	Complete growth medium	DMEM
		10% FBS
		2 mM L-glutamine
		100 U/ml penicillin
		100 μg/ml streptomycin-sulphate
		4 μg/ml puromycin
MEF	Complete growth medium	DMEM
		10% FBS
		2 mM L-glutamine
MEF	Treatment medium	DMEM
		2% FBS
		2 mM L-glutamine
CHO, MEF	Freezing medium	Complete growth medium
		10% FBS
		10% DMSO

c) Buffer

Buffer	Specifications	Composition
PBS (Phosphate-buffered saline)	In double distilled water, pH 7.4, autoclaved	NaCl 123 mM
		Na_2HPO_4 10 mM
		KH_2PO_4 3.2 mM

2.5 Employed inhibitors and activators

Unless otherwise stated the inhibitors and activators were dissolved in DMSO to obtain a concentrated stock solution (generally 1000x) that was stored at -20°C. The Nrf2 activator CDDO-Im was a kind gift from Michael Sporn (Dartmouth Medical School, Hanover, NH, USA). The NADPH regenerating system was purchased from Xenotech (Lenexa, Kansas, USA) and used according to the instructions of the manufacturer.

Substance	Use	Company
Antimycin A	Mitochondrial respiration inhibitor (complex III)	Seahorse Bioscience, Copenhagen, Denmark
C646	p300/CBP inhibitor	Sigma-Aldrich, St. Louis, MO
C-75	FAS inhibitor	Cayman Chemical, Ann Arbor, MI
CHIR 99021	GSK3 inhibitor	Santa Cruz Biotechnology, Dallas, Texas
Chloroquine	Autophagy inhibitor	Sigma-Aldrich, St. Louis, MO
Compound c	AMPK inhibitor	Sigma-Aldrich, St. Louis, MO
DMSO	Solvent control	Sigma-Aldrich, St. Louis, MO
Etomoxir	CPT1 inhibitor	Merck Millipore, Billerica, MA
EX-527	SIRT1 inhibitor	Santa Cruz Biotechnology, Dallas, Texas
FCCP	Mitochondrial uncoupler	Seahorse Bioscience, Copenhagen, Denmark
Gö 6983	PKC inhibitor	Santa Cruz Biotechnology, Dallas, Texas
KT 5720	PKA inhibitor	Santa Cruz Biotechnology, Dallas, Texas
Oligomycin	ATP synthase inhibitor	Seahorse Bioscience, Copenhagen, Denmark
PD98059	ERK inhibitor	Tocris, Ellisville, MO
Phenylbutyrate	ER stress alleviator	Sigma-Aldrich, St. Louis, MO
Rapamycin	mTORC1 inhibitor	Cell Signaling Technologies, Danvers, MA
Rotenone	Mitochondrial respiration inhibitor (complex I)	Seahorse Bioscience, Copenhagen, Denmark
Rottlerin	PKC inhibitor	Santa Cruz Biotechnology, Dallas, Texas
Rp-8-Br-cAMPS	PKA inhibitor	Santa Cruz Biotechnology, Dallas, Texas
SB202190	p38 inhibitor	Tocris, Ellisville, MO
SB203580	p38 inhibitor	Tocris, Ellisville, MO
SP600125	JNK inhibitor	Tocris, Ellisville, MO
Thapsigargin	ER stress inducer (SERCA inhibitor)	Sigma-Aldrich
Trichostatin A	HDAC inhibitor	Cell Signaling Technologies, Danvers, MA
TOFA	ACC inhibitor	Cayman Chemical, Ann Arbor, MI
Tunicamycin	ER stress inducer (N-glycosylation inhibitor)	Sigma-Aldrich, St. Louis, MO
U0126	ERK inhibitor	Tocris, Ellisville, MO
Wortmannin	PI3K inhibitor	Santa Cruz Biotechnology, Dallas, Texas
Xanthohumol	Nrf2 activator	Sigma-Aldrich, St. Louis, MO
YS-49	Akt activator	Sigma-Aldrich, St. Louis, MO

3. Stable transfection of CHO cells

The CHO cells were co-transfected with the expression vector pGL4.22[luc2CP/Puro] (Promega, Madison, WI) carrying the ARE-Luciferase construct and the puromycin resistance and with the pEGF-N1 vector (ClonTech, Mountian View, CA) carrying the eGFP gene. The transfection was performed with the FuGENE® HD Transfection Reagent (Promega) following the instructions of the manufacturer. To select for the transfected cells beginning from the day after the transfection the cells were kept for 2 weeks in medium containing 4µg/ml of puromycin. This concentration had been previously found to be lethal for cells that do not carry a puromycin resistance. Every two days the medium was replaced. After the two weeks the cells were trypsinized and diluted to a concentration of 10 cells/ml. This solution was pipetted into a 96 well plate (100µl/ well) and under the microscope the wells containing a single cell were identified. The populations arising from the single cells were transferred to a 24 well plate and then to cell culture flasks. They were then tested both for the luciferase activity and for GFP fluorescence. The two clonal cell lines with the strongest signal, named C4 and D5, were used for testing the compounds.

4. Luciferase reporter assay

The luciferase reporter assay was used to assess the effect of different compounds on the Nrf2-dependent gene transcription. CHO cells that had been stably transfected with a plasmid carrying an ARE-driven luciferase gene and a plasmid carrying the EGFP (enhanced green fluorescent protein) gene were treated with the compounds and then the amount of luciferase was quantified via a luciferase assay and normalised to the GFP signal.

4.1 Treatment conditions

The CHO/ARE-Luc cells were seeded in a 96 well plate (60.000 cells/well) in complete growth medium and allowed to attach for 4 hours at 37 °C. Then they were incubated for 20 hours with the test compounds and the solvent control DMSO. The compound CDDO-Im (100 nM) was always included as a positive control to ensure that the assay was working correctly. Each compound was tested in quadruplicates. Following the incubation the cells were washed twice with PBS (phosphate-buffered saline), the buffer was completely removed and the plate was stored at −80 °C.

4.2 Luminescence and fluorescence detection

The luciferase is an enzyme that catalyses the oxidation of the substrate luciferin into oxyluciferin under the emission of light, using ATP as a co-substrate:

Luciferin + ATP + $O_2 \rightarrow$ oxyluciferin + CO_2 + PP_i + AMP + light

The plate was taken from -80°C and 50 µL of lysis buffer were added to each well. To ensure a thorough disruption of the cells the plate was subjected to vigorous shaking for 10 minutes and then 40 µL of the lysates were transferred to a black 96-well plate. The measurement was done with a Tecan GeniosPro (Tecan Group, Maennedorf, Switzerland) microplate reader with the XFluor GeniosPro software (Version: V 4.63). The instrument injected the ATP and the substrate luciferin directly into the wells and then measured first the fluorescence and then the luminescence for each well. The luminescence values were normalized to the EGFP-derived fluorescence values to account for differences in cell number. The normalized luminescence values of each tested compound were then divided by the value of the solvent control to determine the fold activation of the ARE-dependent gene transcription. Each compound was tested in a minimum of three independent experiments.

4.3 Settings of the microplate reader

EGFP-derived fluorescence measurement
Measurement mode: Fluorescence
Excitation wavelength: 485 nm
Emission wavelength: 520 nm
Gain: Optimal
Number of reads: 1
Integration time: 1000 µs
Lag time: 0 µs
Mirror selection: 40 ms

Luminescence measurements
Measurement mode: Luminescence
Integration time (manual): 2000 ms
Attenuation: None
Time between move and integration: 50 ms
Well kinetic number: 1
Well kinetic interval (minimal): 2020 ms

Injector settings

Injector A volume: 50 µl
Injector A speed: 200 µl/s
Injector B volume: 50 µl
Injector B speed: 200 µl/s
Injection mode: Standard

4.4 Buffers and solutions

Buffer/Solution	Composition
Lysis buffer	Reporter Lysis Buffer #E3971 (Promega, Madison, WI)
	2mM DTT
	270 µM Coenzyme A
Luciferin solution	10mM Luciferin
	1 M Tricine pH 7,8
ATP solution	20mM Tricine pH 7,8
	21,5 mM $MgCl_2$
	3,7 mM ATP

5. Protein detection by Western Blotting

Different proteins were immunodetected via western blotting. The proteins were extracted from the cells with an appropriate lysis buffer. Then they were separated according to their molecular weight via gel electrophoresis and transferred with electricity from the gel onto a PVDF membrane. The membrane was first incubated with a primary antibody binding to the protein of interest and then with a secondary antibody recognizing the primary antibody and coupled to the reporter enzyme HPR (horseradish peroxidase). When supplied with the appropriate substrate, the HPR generates a quantifiable reaction product. The amount of this product stands in proportion with the amount of the detected protein.

5.1 Whole cell protein extracts

The cells were seeded in 6-well culture plates and once they had reached 80% confluence treated with the compounds and the solvent control DMSO. After the desired treatment the cells were washed once with ice cold PBS and then lysed for 10 minutes on ice with 100 µl RIPA (Radioimmunoprecipitation assay) buffer. The lysates were scraped together, transferred to 1.5 ml microcentrifuge tubes and sonicated for 8 seconds, always keeping them on ice. After spinning down the cellular debris at 10.000 g for 10 minutes, the supernatants were transferred to fresh tubes and subjected to protein determination according to Bradford.

For short term storage (up to a week) the lysates were kept at -20°C, for long term storage at -80°C.

Buffer	Specifications	Composition
RIPA buffer	Add prior to use: 40 : 1000 cOmplete (Roche, Basel, Switzerland), 10 : 1000 PMSF (100 mM), 5 : 1000 NaF (200 mM), 5 : 1000 NaVO$_3$ (200 mM)	50 mM Tris/HCl pH 7,4
		500 mM NaCl
		1% Igepal CA 630
		0,5% Na-Deoxycholate
		0,1% SDS
		0,05% NaN$_3$

5.2 Nuclear and cytosolic protein extracts

The cells were seeded in 6- or 10-cm cell culture dishes and once they had reached 80% confluence treated with the compounds and the solvent control DMSO as desired. After the treatment the cells were washed once with ice cold PBS. Then 100 μl of Buffer 1 were added to the cells, the lysate was scraped together and transferred to a 1.5 ml microcentrifuge tube. The lysate was kept on ice for 15 minutes during which it was subjected to vigorous vortexing every 3 minutes. After centrifugation at 10.000 g for 5 minutes, the supernatant containing the cytosolic fraction was collected. The pellet containing the nuclear fraction was resuspended in 200 μl of Buffer 1 and the tube was centrifuged at 10.000 g for 5 minutes. The supernatant was completely removed and the pellet resuspended in 20 μl of Buffer 2, followed by 15 minutes incubation on ice with vigorous vortexing every 3 minutes. After centrifugation at 10.000 g for 5 minutes, the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and mixed well with 20μl of Buffer 3, thus obtaining the nuclear fraction. For short term storage (up to a week) the lysates were kept at -20°C, for long term storage at -80°C

Buffer	Specifications	Composition
Buffer 1	Add prior to use: 1% NP40 (Igepal) , 1 mM DTT and 0.5 mM PMSF, 40 : 1000 cOmplete (Roche, Basel, Switzerland)	10 mM HEPES pH 7.5
		0.1 mM EDTA
		0.1 mM EGTA
		10 mM KCl

Buffer 2	Add prior to use: 1 mM DTT, 0.5 mM PMSF, 40 : 1000 cOmplete	20 mM HEPES pH 7.5
		1 mM EDTA
		0.1 mM EGTA
		420 mM NaCl
Buffer 3	Add prior to use: 1% NP 40 , 1 mM DTT , 40 : 1000 cOmplete and 0.5 mM PMSF	20 mM HEPES pH 7.5
		1mM EDTA
		0.1 mM EGTA
		100 mM KCl
		20 % glycerol

5.3 Protein quantification

The protein concentration was determined according to the Bradford method. [168] 5 μl of the protein extract were used to prepare a 1:10 or 1:20 dilution with double distilled water. 10 μl of the dilution of each sample and a series of BSA standards (2.5 - 25 μg/ml final BSA concentration) were pipetted in triplicates into a 96 well plate and mixed with 190 μl of Bradford reagent (a 1:5 dilution of Roti®-Quant (Carl Roth, Karlsruhe, Germany) in double distilled water). The plate was left to rest for 5 minutes and then the absorbance at 595 nm was determined with a Sunrise microplate reader (Tecan Group, Maennedorf, Switzerland). The absorbance values of the BSA standards were used to generate a standard curve based on which the protein concentrations of the extracts were calculated.

5.4 Gel electrophoresis

For each sample equal amounts of protein (20-30 μg for whole cell extracts, 40- 50 μg for nuclear proteins) were mixed with the sample buffer, heat inactivated for 5 minutes at 95°C and then directly loaded onto polyacrylamide gels of an appropriate percentage (7.5 to 12 % depending on the size of the protein of interest). The proteins were resolved by SDS-PAGE at 25 mA per gel for about 90 minutes. The polyacrylamide (PAA) gels were prepared with the Mini-PROTEAN™ 3 Cell System (Bio-Rad Laboratories, Hercules, CA) using a 30 % solution of PAA (Rotiphorese® Gel 30, Carl Roth, Karlsruhe, Germany).

Buffer	Specifications	Composition
3x sample buffer	Add 10 mM DTT prior to use	Tris-HCl 187.5 mM pH 6.8
		SDS 0.2 M
		glycerol 30 %
		bromphenol blu 0.2 mM
10x Running buffer		Tris-base 248 mM
		glycine 1.9 M
		SDS 35 mM

Gel	Specifications	Composition
Stacking gel	Add 0.1% APS and 0.2 % TEMED to initiate polymerization	PAA 5 %
		Tris-HCl 125 mM pH 6.8
		SDS 0.1 %
Resolving gel	Add 0.05% APS and 0.1% TEMED to initiate polymerization	PAA 7.5 - 12 %
		Tris-HCl 375 mM pH 8.8
		SDS 0.1 %

5.5 Protein blotting

The PVDF membranes (Immuno-blot™; Bio-Rad Laboratories) were activated in methanol for 10 seconds and then equilibrated in blotting buffer for one minute. The Mini Trans-Blot™ Electrophoretic Transfer Cell System (Bio-Rad Laboratories) was used to transfer the proteins from the gels to the membranes at 100 V for 85-110 minutes, depending on the size of the proteins. After the transfer the membranes were blocked for 1 hour at room temperature with 5% non-fat dry milk in TBS-T and then washed three times for 5 minutes with TBS-T before proceeding with the immunodetection.

Buffer	Composition
Blotting buffer	Tris-base 25 mM
	Glycine 200 mM
	Methanol 20%
10x TBS-T pH 8.0	Tris-base 248 mM
	NaCl 1.9 M
	Tween-20 1 %

5.6 Immunodetection

The membranes were probed overnight at 4°C with the primary antibodies. After washing them three times for 5 minutes with TBS-T they were incubated for 1-2 hours at room temperature with the HRP-conjugated secondary antibodies. Again the membranes were washed three times for 5 minutes with TBS-T and then incubated with the ECL (enhanced chemiluminescence) solution. The chemiluminescent protein-antibody complexes were visualized with the Fuji LAS 3000 CCD camera (Fujifilm, Tokyo, Japan) and quantified with the AIDA software (Raytest GmbH, Straubenhardt, Germany).

Solution	Specifications	Composition	
ECL-solution	Add luminol, p-coumaric acid and H_2O_2 immediately prior to use	Tris-base (100 mM, pH 8.5) 5 ml	
		Luminol (0.25 M in DMSO) 25 µl	
		p-coumaric acid (90 mM in DMSO) 11 µl	
		H_2O_2 30 % 3 µl	

5.7 List of antibodies

The antibodies were used according to the instructions of the manufacturers. Generally the antibodies were diluted in TBST-T containing 5% of BSA. An exception is the secondary anti-rabbit antibody that was diluted in TBST-T containing 5% of non-fat dry milk. For the Nrf2 antibody the shown bands were localized just above the 100 kDa marker, while Nrf2 has a predicted molecular weight of 68 kDa. However it has been shown that the biologically relevant species of Nrf2 have a molecular weight that ranges from 95 to 110 kDa depending on the percentage of the gel. [169]

a) Primary antibodies

Antibody	Company	Catalog #
ACC	Cell Signaling Technologies, Danvers, MA	3662
pACC (Ser79)	Cell Signaling Technologies, Danvers, MA	3661
Acetylated-Lysine	Cell Signaling Technologies, Danvers, MA	9814
Actin	MP Biomedicals, Santa Ana, CA	69100
Akt	Cell Signaling Technologies, Danvers, MA	9272
p-Akt (Ser473)	Cell Signaling Technologies, Danvers, MA	9271

AMPKα	Cell Signaling Technologies, Danvers, MA	2532
p-AMPKα (Thr172)	Cell Signaling Technologies, Danvers, MA	2535
BiP	Cell Signaling Technologies, Danvers, MA	3183
ß-Catenin	Cell Signaling Technologies, Danvers, MA	9563
p-GSK3 (Ser9)	Cell Signaling Technologies, Danvers, MA	9323
HO-1	Enzo Life Sciences, Farmingdale, NY	ADI-OSA-150
Lamin B1	Cell Signaling Technologies, Danvers, MA	9087
LKB1	Cell Signaling Technologies, Danvers, MA	3047
Nrf2 (C-20)	Santa Cruz Biotechnology, Dallas, Texas	sc-722
p53	Cell Signaling Technologies, Danvers, MA	9282
p-p53 (Ser15)	Cell Signaling Technologies, Danvers, MA	9284
p-PKC substrates	Cell Signaling Technologies, Danvers, MA	2261
Raptor	Cell Signaling Technologies, Danvers, MA	2280
p-Raptor (Ser792)	Cell Signaling Technologies, Danvers, MA	2083
α/β-Tubulin	Cell Signaling Technologies, Danvers, MA	2148

b) Secondary antibodies

Antibody	Company	Catalog #
Anti-mouse IgG, HRP-linked	MP Biomedicals, Santa Ana, CA	55563
Anti-rabbit IgG, HRP-linked	Cell Signaling Technologies, Danvers, MA	7074

6. RNA detection

The amount of RNA coding for a given protein of interest can be detected via real-time quantitative PCR (RTqPCR). The total RNA is extracted from the cells and transcribed into cDNA (complementary DNA). Then the cDNA of the target of interest is amplified in a PCR reaction using the total cDNA as template and primers that are specific for the target gene. During elongation the DNA double-strand-specific SYBR Green I dye binds to the amplified PCR products. At the end of each elongation cycle the total amount of double stranded DNA is monitored by detecting the SYBR Green I fluorescence.

6.1 RNA extraction

The cells were seeded in 6-cm cell culture dishes and treated at 80% confluence. The total RNA was extracted following the instructions of the peqGOLD Total RNA Kit (S-Line) (PEQLAB, Erlangen, Germany). To avoid DNA contaminations an on-column DNase digestion was performed with the peqGOLD DNase I Digest Kit (PEQLAB). The RNA concentration was

measured and the purity assessed with the Nanodrop spectrophotometer (Peqlab, Erlangen, Germany). The RNA was stored at -80°C.

6.2 cDNA synthesis

The reverse transcription of the RNA into cDNA was performed according to the protocol of the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Darmstadt, Germany). For each sample 1 µg of RNA was used as a template for transcription. The different volumes were filled up with RNase free water to 10 µl, to which 10 µl of the reaction mix (10x RT Buffer 2.0 µl, 25x dNTP Mix (100 mM) 0.8 µl, 10x RT Random Primers 2.0 µl, MultiScribe Reverse Transcriptase 1.0 µl, RNase Inhibitor 1.0 µl, Nuclease-free H_2O 3.2 µl) were added. The following sequence was run in a PCR machine:

1. 10 minutes at 25°C
2. 120 minutes at 37°C
3. 5 seconds at 85°C
4. Cooling to 4°C

After the transcription, the samples were diluted with RNase free water to a concentration of 20 ng/µl and stored at -20°C.

6.3 qPCR

The gene of interest heme oxygenase-1 (HO-1) and the reference gene murine hypoxanthin-phosphoribosyl-transferase (HPRT) were amplified via qpCR. The specific primers were ordered from Qiagen (Venlo, Netherlands) and diluted following the instructions of the manufacturer (QuantiTect Primer Assays, Cat. No. QT00159915 forHO-1 and QT00166768 for HPRT). The qPCR was performed with the LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland) according to the protocol of the manufacturer and run on the LightCycler® 480 System (Roche). A mixture of 7.5 µl of cDNA template, the 10x concentrated primers and the 2x concentrated master mix was brought to a volume of 65 µl with RNase free water. The cDNA had a concentration of 20 ng/µl, based on the concentration of the RNA that was used for the reverse transcription and assuming a 100% successful reverse transcription. For all samples, three technical replicates of 21 µl were loaded onto a 96 well plate, which was then sealed with an adhesive optical film. A no-template control with water instead of cDNA was always included as a general control for contaminations. The following PCR program was run: Denaturation for 10 min at 95°C followed by 40 amplification cycles (30 s at 61°C for annealing

followed by 15 s at 72°C for elongation). The data were analysed using the Light Cycler LC480 Software (Roche Diagnostics): The melting curves of the amplified DNA were surveyed for the amplification of only one specific product and the relative expression level of the HO-1 m-RNA was determined with the $2^{-\Delta\Delta Ct}$ method.

7. Mitochondrial ROS detection

Mitochondrial ROS was detected by treating live cells with the fluorogenic MitoSOX Red (Life Technologies, Carlsbad, CA) reagent. It is targeted to the mitochondria and specifically oxidized only by superoxide and not by other ROS sources. Upon oxidation it produces red fluorescence. The cells were incubated for 15 minutes with 5 µM MitoSox Red and then treated with the vehicle DMSO, 5 µM XN or 10 µM of the positive control antimycin A for different periods of time. The red fluorescence was determined in the FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). An excitation wavelength of 488 nm and a FL2 emission filter (586/42 nm) were used. The mean fluorescence intensities were taken as a direct measure for the produced mitochondrial ROS.

8. Mitochondrial respiration measurements

The mitochondrial respiration was determined with the Seahorse 24XFe extracellular flux analyzer and the Wave software (Seahorse Bioscience, North Billerica, MA). The MEF cells were seeded with a cell density of 2.7 x 10^4 cells/well in collagen-coated 24-well cell cultures plates (XF24 cell culture microplates, Seahorse Biosciences). One hour before treatment the normal growth medium was replaced with serum free medium (DMEM without glucose, supplemented with 2 mM glutamine and 2 mM pyruvate, pH 7.35-7.40) and the cells were kept at 37°C and ambient CO_2. A complete mitochondrial profile was recorded by using the XF Cell Mito Stress Test Kit (Seahorse Biosciences) according to the instructions of the manufacturer (readout: oxygen consumption rate (OCR) in pmole O_2/min). The following inhibitor concentrations optimized for MEF were used: 2 µM of oligomycin A, 1.5 µM of carbonylcyanid-p-trifluoromethoxyphenylhydrazon (FCCP), 1 µM of rotenone A and 1 µM of antimycin A. To account for potential differences in cell number, following the measurement the biomass was quantified with a crystal violet (CV) staining and the values were normalized to the CV absorption. The oxygen consumption rate (OCR) for basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and residual oxygen consumption were determined as follows:

Basal respiration: ∆OCR without stressor minus OCR after addition of antimycin A and rotenone

Respiration coupled with ATP production: ∆(OCR (without stressor) - OCR (+oligomycin))

Maximal respiration: ∆(OCR (+FCCP) - OCR (+antimycin/rotenone)

Respiratory spare capacity: ∆(OCR maximal - OCR basal)

Proton leak: ∆(OCR (+oligomycin) - OCR (+ antimycin/rotenone))

Residual oxygen consumption (ROX): OCR after addition of antimycin/rotenone

9. Statistics

Unless otherwise stated all experiments were performed at least three times. The error bars in the pictures represent the SD (standard deviation) of the mean. Statistical significance was determined by using Student's t test or by one-way ANOVA followed by Bonferroni's post test. All statistical analyses were done with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). P-values <0.05 were considered as significant and are designated with * in the figures. (*P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

C. Results

1 Xanthohumol as a tool for studying the crosstalk between AMPK and Nrf2

1.1 Xanthohumol and Nrf2

1.1.1 Xanthohumol activates the Nrf2 dependent gene expression

Xanthohumol has been reported to activate Nrf2 in different cellular models [158, 162, 170]. To verify this activation a reporter assay for Nrf2 dependent gene transcription was performed. CHO cells that had been stably transfected with a plasmid carrying the Nrf2 promoter element ARE in front of the luciferase gene were treated with 2.5 and 5 µM XN. For both of the tested concentrations there was a clear increase of the luciferase signal that was higher for the 5 µM Xanthohumol, corresponding to an over 3 fold activation of the Nrf2 dependent gene transcription. (Fig 7)
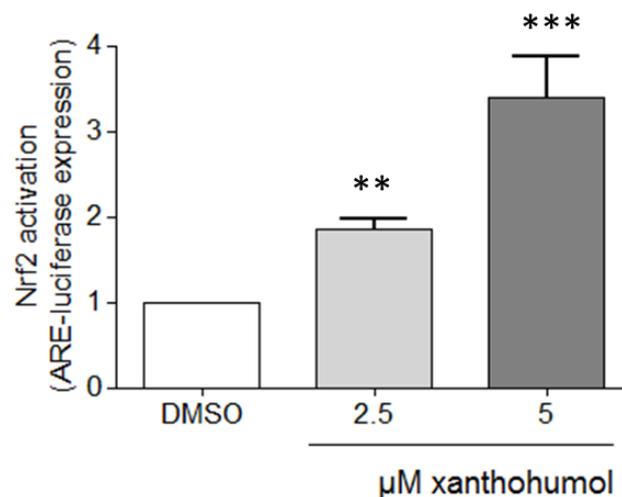
Figure 7: Activation of Nrf2 by Xanthohumol

CHO cells were stably co-transfected with an ARE-Luciferase reporter construct and GFP. The cells were treated with the solvent DMSO or XN (2.5 and 5 µM) for 18 hours. Then luciferase activity was determined and normalized to the GFP fluorescence. The Nrf2 activation is relative to the DMSO control. Results of three independent experiments are shown (mean + SD; ** $p < 0.01$, *** $p < 0.005$, ANOVA, Bonferroni). Data in part generated and provided by Elke Heiß.

1.1.2 Xanthohumol upregulates HO-1 in a Nrf2-dependent manner

HO-1 is a protein that is highly inducible in response to oxidative stress. The gene is a well described target of Nrf2 [171]. To explore to which extent the induction of HO-1 by Xanthohumol depends on Nrf2, WT and Nrf2-/- MEF cells were treated for different durations (1-24 hours) with 5 µM Xanthohumol, and the HO-1 protein amounts were determined via western blotting (Fig 8a). In the WT cells there was a visible upregulation of the protein already after 3 hours. The HO-1 level further increased until the 7 hours timepoint, while after 24 hours it had gone back down and was close to the basal level. In the Nrf2-/- cells there was no upregulation of the HO-1 protein for any of the analysed timepoints. To test if XN increases the protein level via increased transcription of the HO-1 gene we had a look at the effect of XN on the mRNA level of HO-1. In the WT cells we found a strong upregulation of HO-1 mRNA after 4 hours, while again for the Nrf2-/- cells there was no upregulation at all (Fig 8b). These results clearly show that Xanthohumol upregulates HO-1 both on the protein and the mRNA level, and that the induction is dependent on Nrf2, since it is completely gone in the absence of the transcription factor.
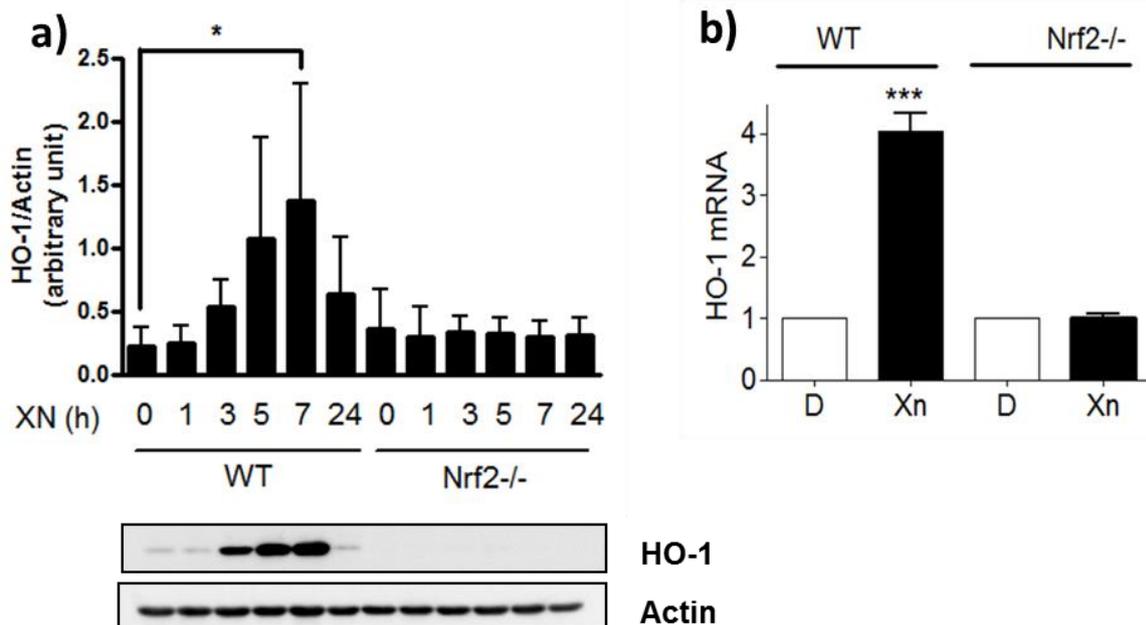
Figure 8: The influence of Nrf2 on HO−1 protein and mRNA induction by XN

a）WT and Nrf2−/− MEF cells were treated with XN（5 µM）for 0, 1, 3, 5, 7 and 24 hours before total cell lysates were subjected to immunoblot analyses for HO−1 protein and actin as a loading control. Representative blots and compiled densitometric analyses of four independent experiments are depicted（mean + SD; ∗

p<0.05; ANOVA, Bonferroni). b) WT and Nrf2-/- MEF cells were treated with vehicle or XN (5 µM) for 4 hours before the total mRNA was extracted. Levels of HO-1 mRNA were determined by RTqPCR analysis. (n=3; mean + SD; ∗∗∗ p<0.001; ANOVA, Bonferroni). RT-PCR data were generated by Johannes Baldinger.

1.1.3 Xanthohumol upregulates HO-1 in a Keap1-dependent manner

Keap1 regulates Nrf2 by targeting it for degradation. To test to which extent the activation of HO-1 by XN relies on Keap1, we treated WT and Keap1-/- MEF cells for 1-24 hours with 5 µM Xanthohumol and had a look at the induction of the protein. The upregulation of HO-1 that XN elicited in the WT was completely abolished in the Keap1-/- cells (Fig 9a). A look at the induction of HO-1 mRNA after 4 hours treatment with XN revealed that in Keap1-/- cells there is still an upregulation of HO-1 mRNA, but it is significantly lower than in the WT (Fig 9b). In the absence of Keap1 the induction of HO-1 is completely abolished on the protein level and largely diminished on the RNA level. The dynamic Keap1-Nrf2 interaction is necessary for the induction of HO-1 by XN.
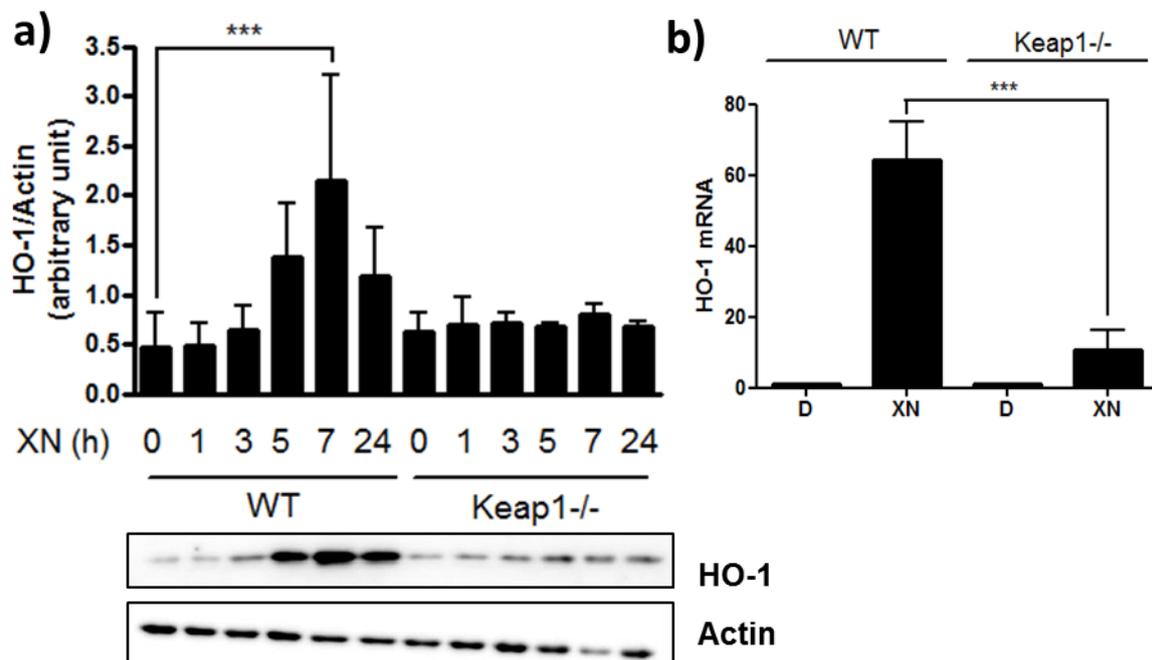

Figure 9: The influence of Keap1 on HO-1 protein and mRNA induction by XN

a) WT and Keap1-/- MEF cells were treated with XN (5 µM) for 0, 1, 3, 5, 7 and 24 hours before total cell lysates were subjected to immunoblot analyses for HO-1 protein and actin as a loading control. Representative blots and compiled densitometric analyses of five independent experiments are depicted (mean + SD; ∗∗∗ p<0.001; ANOVA, Bonferroni). b) WT and Keap1-/- MEF cells were treated

with vehicle or XN （5 μM） for 4 hours before the total mRNA was extracted. Levels of HO–1 mRNA were determined by RTqPCR analysis. （n=4; mean + SD; ✱✱✱ p<0.001; ANOVA, Bonferroni）.

1.2 Xanthohumol and AMPK

1.2.1 Xanthohumol activates AMPK in a LKB1 dependent manner

Xanthohumol has been previously described to activate AMPK [157]. To assess the activation of AMPK by XN in our cell model we had a look at the activating Thr172 phosphorylation of AMPK and at the AMPK-dependent Ser79 phosphorylation of ACC [172, 173]. Both were increased upon 30 minutes of treatment with 5 μM Xanthohumol, but not with vehicle alone (Fig 10), indicating that XN leads to AMPK activation also in our cell system. LKB1 is one major kinase upstream of AMPK [174, 175] and we wanted to know if it is involved in the activation of AMPK by XN. We found that in LKB1-/- cells both the AMPK and the ACC phosphorylations seen upon treatment with XN in the WT are abrogated (Fig 10), indicating that LKB1 is necessary for the activation of AMPK by XN.
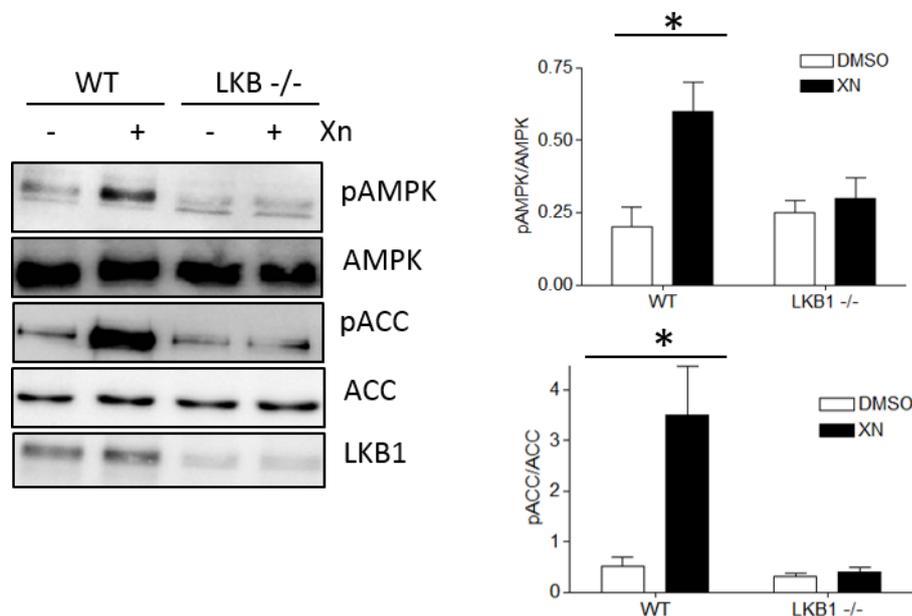

Figure 10: The influence of LKB1 on the activation of AMPK

WT and LKB1–/– cells were treated with vehicle or XN （5 μM） for 30 minutes before total cell lysates were subjected to immunoblot analyses for P–AMPK （Thr172）, AMPK, P–ACC （Ser79）, ACC, LKB1 and actin as a loading control. Representative

blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD; ∗ p<0.05; One way ANOVA with Bonferroni post test). Data were generated and provided by Elke Heiß.

1.2.2 Xanthohumol impairs respiration and increases ROS in the mitochondria

AMPK is activated by low cellular ATP levels. The mitochondria are the main cellular source of ATP, and XN has been reported to interfere with mitochondrial function by generating ROS and leading to mitochondrial uncoupling [165, 166]. The resulting lower ATP production is a possible explanation for the activation of AMPK. To test if XN interferes with mitochondrial function in our model an extracellular flux analyzer was used to measure the mitochondrial oxygen consumption rate (OCR). First the analyzer determines the basal respiration rate. Then ATP synthesis is inhibited by addition of oligomycin, revealing how much of the oxygen is used for ATP production and how much is due to a proton leak. Mitochondrial uncouplers, such as FCCP, reduce the coupling between oxygen consumption and ATP production by making the mitochondrial membrane potential collapse and triggering maximal uncontrolled oxygen consumption. Therefore, the addition of FCCP allows to determine the maximal respiratory capacity and the spare respiratory capacity (maximal-basal). Finally antimycin A and rotenone are added, two inhbitors that completely shut down the mitochondrial respiration, allowing to determine the residual oxygen consumption (ROX) that is non-mitochondrial. WT MEF cells pretreated with 5 µM XN showed a reduced basal respiration and oxygen consumption for ATP synthesis, as well as reduced maximal and spare respiratory capacity compared to only solvent treated cells (Fig 11a). XN appears to slightly inhibit the mitochondrial respiration. To test if XN is an uncoupler, cells were treated first with the inhibitor of ATP synthesis oligomycin and then with XN. If XN promotes uncoupling, it should be able to increase the respiration in oligomycin treated cells, as does FCCP. We found only a very slight and non-significant increase in respiration after treatment with XN and could thus exclude an uncoupling effect of XN (Fig 11b). As XN is not a mitochondrial uncoupler, the inhibitory effect on respiration could be due to an impaired mitochondrial electron transport and subsequent mitochondrial superoxide generation. To test this, MitoSox Red and flow cytometric analysis were used to measure the mitochondrial ROS generation. We found that treatment with 5 µM XN leads to an increase of the mitochondrial ROS level that is marked compared to the solvent control DMSO but not as strong as for the positive control antimycin A, a well known inhibitor of

complex III of the mitochondrial electron transport chain (Fig 11c). XN does not act as a mitochondrial uncoupler in our model, but it does inhibit mitochondrial respiration and generate mitochondrial ROS.

Overall, XN was confirmed as an activator of both Nrf2 and AMPK signalling in the MEF cells used in this study. XN promotes the induction of HO-1 in a strictly Nrf2- and largely Keap-1 dependent manner, while it activates AMPK via a mild mitochondrial dysfunction and LKB1 as upstream AMPK kinase.
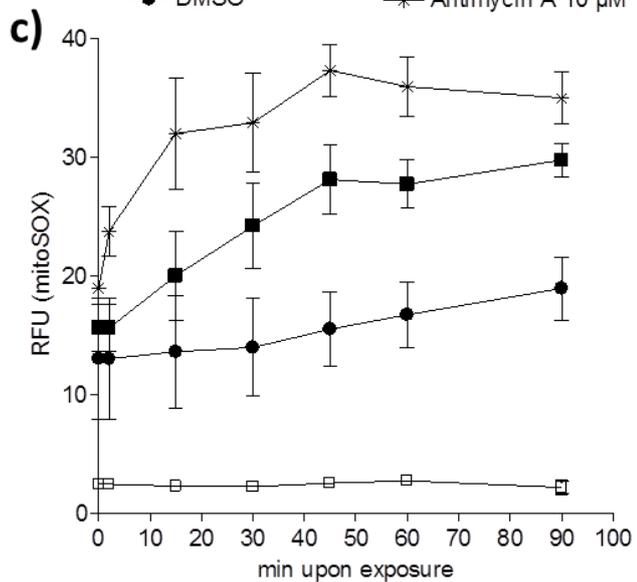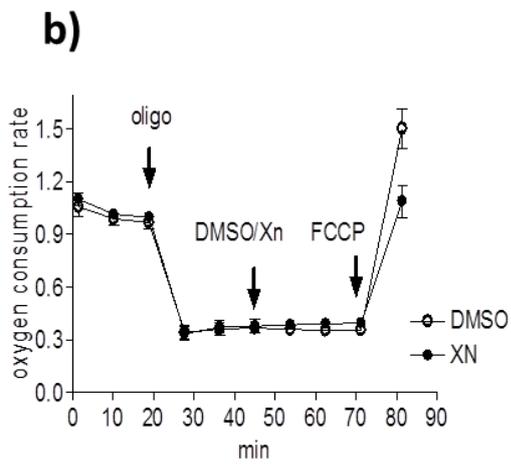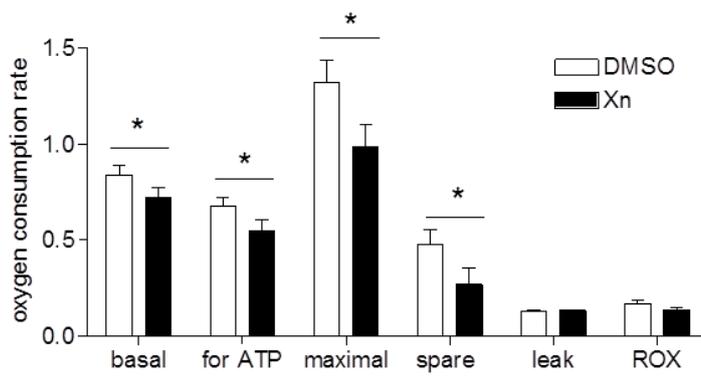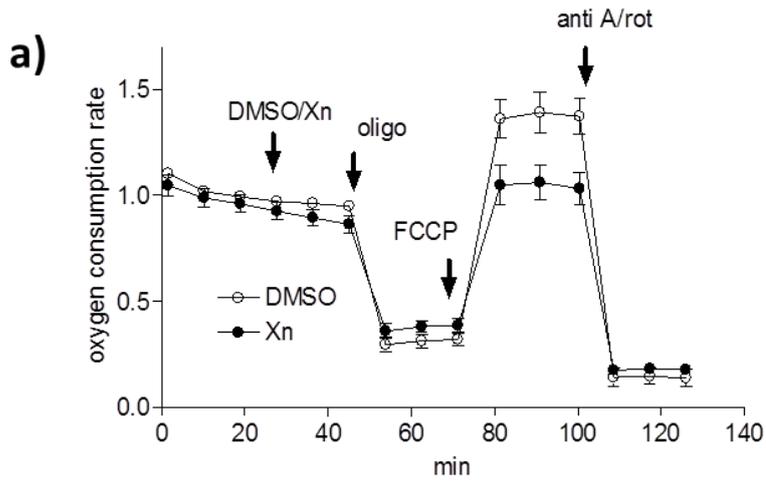
Figure 11: The effect of XN on mitochondrial function and ROS production

a）WT MEF cells were subjected to a mitochondrial stress test as described in the Methods section. Cells were successively exposed to vehicle or 5 µM XN, 2 µM oligo（mycin）, 1.5 µM FCCP and 1 µM each of antimycin A and rotenone（A+R）. The mean oxygen consumption rate（OCR）corrected for biomass of three independent experiments is depicted. The upper panel shows the total measured oxygen consumption rate（OCR）. The lower panel shows the basal, maximal and spare OCR and the part of the OCR used for ATP production, lost due to proton leak or corresponding to the non-mitochondrial residual oxygen consumption（ROX）.（mean + SD, * p< 0.05,（vs vehicle control）; ANOVA）. Data were in part generated and provided by Elke Heiß. b）In order to assess the uncoupling potential of XN the OCR of WT MEF cells was assessed upon successive addition of 2 µM oligo（mycin）, vehicle or 5 µM XN and 1.5 µM FCCP（positive control）. The OCR was corrected for biomass. Compiled data of three independent experiments are shown（mean + SD）. c）The mitochondrial ROS production in MEF cells was recorded over time after exposure to vehicle, 5 µM XN or 10 µM antimycin A（positive control）by using MitoSox Red and flow cytometric analysis. Compiled data of three independent experiments are depicted.（mean + SD）. Data were generated by Johannes Baldinger.

1.3 The influence of AMPK on Nrf2

1.3.1 The upregulation of HO-1 by XN is impaired in AMPK-/- cells

Both Nrf2 and AMPK play an important role for cellular energy metabolism, stress resistance and cytoprotection. To assess whether AMPK is directly involved in and influencing the Nrf2 dependent gene regulation, we examined the upregulation of HO-1 by XN as a molecular readout for the activation of Nrf2 in WT and isogenic AMPK-/- MEF counterparts. WT and AMPK-/- MEF cells were treated for different durations (1-24 hours) with 5 µM Xanthohumol, and the induction of the HO-1 protein was compared by protein expression analysis via western blotting (Fig 12a). Both in the WT and in the AMPK-/- cells we could see an upregulation of the HO-1 protein already after 3 hours. The protein levels further increased untill the 7 hours timepoint followed by a decrease back down to the basal level after 24 hours. However in the AMPK-/- cells the total protein levels were significantly lower than in the WT cells.
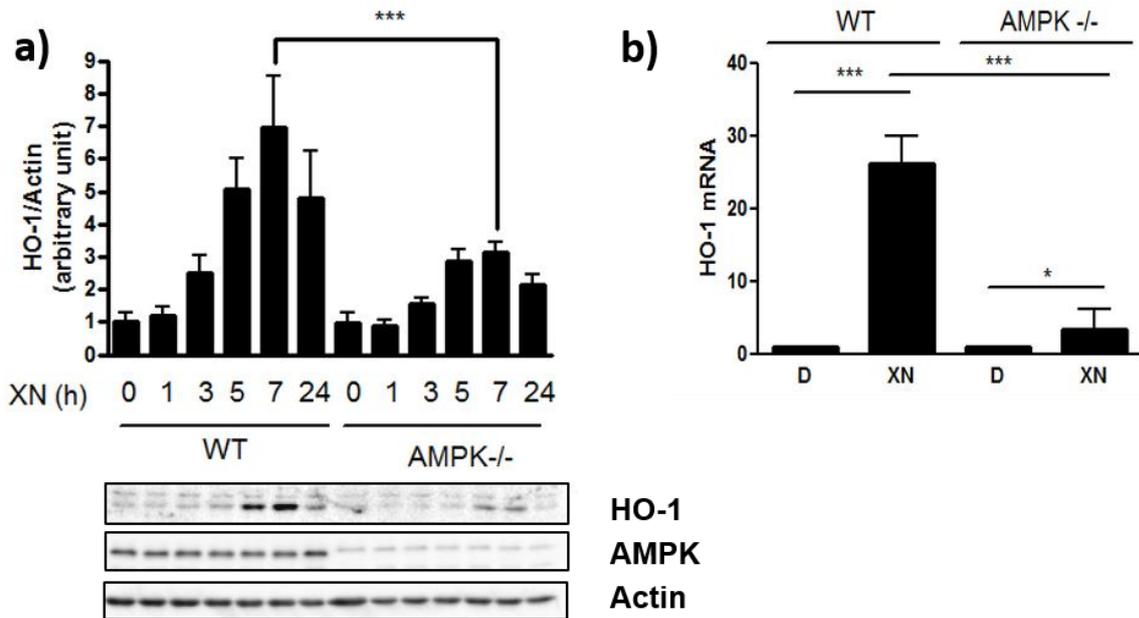
Figure 12: The influence of AMPK on HO-1 protein and mRNA induction by XN
a）WT and AMPK-/- MEF cells were treated with XN（5 µM）for 0, 1, 3, 5, 7 and 24 hours before total cell lysates were subjected to immunoblot analyses for HO-1 protein and actin as a loading control. Representative blots and compiled densitometric analyses of five independent experiments are depicted（mean + SD; ∗∗∗ $p<0.001$; ANOVA, Bonferroni）. b）WT and AMPK-/- MEF cells were treated with vehicle or XN（5 µM）for 4 hours before the total mRNA was extracted. Levels of HO-1 mRNA were determined by RTqPCR analysis.（n=4; mean + SD; ∗ $p<0.05$, ∗∗∗ $p<0.001$; ANOVA, Bonferroni）.

Detection of the HO-1 mRNA after 4 hours treatment with XN revealed that also on the mRNA level the response to XN is blunted: While there still is an induction of HO-1 in the AMPK-/- cells, it is much weaker than in the WT (Fig 12b). Thus, AMPK deficiency hampers the Nrf2-dependent HO-1 expression in XN-treated MEF cells already at the level of the mRNA.

1.3.2 Pharmacological inhibition of AMPK impairs the upregulation of HO-1 by XN
To confirm the positive effect of AMPK on the Nrf2 dependent upregulation of HO-1 the kinase was pharmacologically inhibited in WT MEF cells. Cells were co-treated for 5 hours with compound C, a well described inhibitor of AMPK [5], and Xanthohumol. Compound C alone showed no effect on the HO-1 protein level, but co-treatment with compound C and Xanthohumol largely prevented the Xanthohumol dependent HO-1 upregulation (Fig 13a).

The same effect could be seen on the mRNA level: Co-treatment with compound C dramatically reduced the XN dependent HO-1 mRNA accumulation (Fig 13b). Both the stable genetic and the transient pharmacologic inactivation of AMPK show the same effect: They reduce the Nrf2 dependent upregulation of HO-1 on the protein and on the mRNA level.
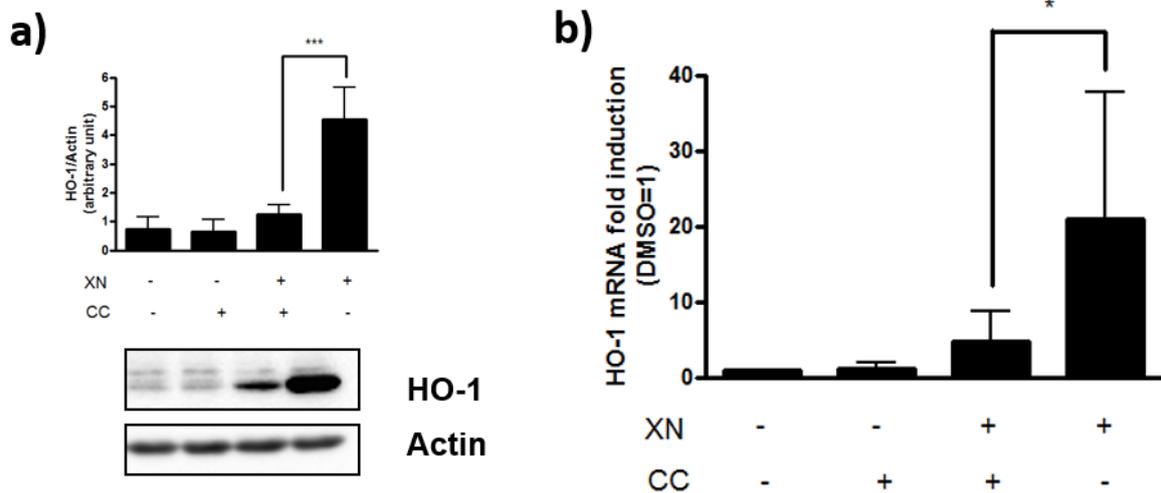
Figure 13: The influence of pharmacologic inhibition of AMPK on HO-1 protein and mRNA induction by XN

a）WT cells were treated with vehicle, XN （5 μM） and compound C （10μM） for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as a loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted （mean + SD; *** $p<0.001$; ANOVA, Bonferroni）. b）WT MEF cells were treated with vehicle, XN （5 μM） and compound C （10μM） for 4 hours before the total mRNA was extracted. Levels of HO-1 mRNA were determined by RTqPCR analysis. （n=3; mean + SD; * $p<0.05$; ANOVA, Bonferroni）.

1.3.3 The upregulation of HO-1 by XN is impaired in LKB1-/- cells

The kinase LKB1 is necessary for the activation of AMPK by XN. For further corroboration of the positive impact of AMPK on the Nrf2/HO-1 signalling axis we compared the XN-induced HO-1 expression in WT and LKB1-/- MEF. A timecourse (1-24 hours) of the HO-1 protein induction by 5 μM Xanthohumol in WT and LKB1-/- cells revealed that the upregulation of HO-1 is blunted in LKB1-/- cells (Fig 14), just as in the AMPK-/- cells. XN activates the AMPK via LKB1, and in the absence of either LKB1 or AMPK the Nrf2 dependent HO-1 upregulation is

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blunted. This indicates that the activation of AMPK by LKB1 plays a positive role for Nrf2 dependent signaling. We also treated WT MEF cells with an inhibitor of CAMKKß, a kinase that activates AMPK in response to intracellular calcium increases. Inhibition of the CAMKKß with STO-609 only had a minor effect on the expression of HO-1 by XN that was not signicant (data not shown), further corroborating that LKB1 mediates the XN dependent activation of AMPK.
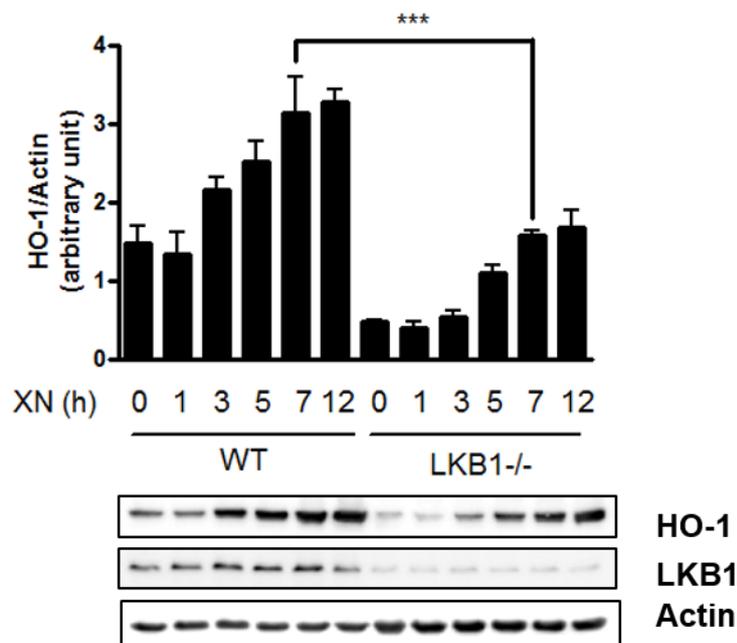
Figure 14: The influence of LKB1 on HO-1 protein and mRNA induction by XN
WT and LKB1-/- cells were treated with XN（5 µM）for 0, 1, 3, 5, 7 and 12 hours before total cell lysates were subjected to immunoblot analyses for HO-1, LKB1 and actin as a loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD; ∗∗∗ p<0.001; ANOVA, Bonferroni）. Data were generated with the help of Barbara Mayerhofer.

1.4 Molecular mechanisms underlying the AMPK/Nrf2 crosstalk

1.4.1 AMPK does not influence the XN dependent nuclear accumulation of Nrf2

The canonical activation of Nrf2 begins with the stabilization of the protein due to reduced degradation via Keap1 and its concurrent migration into the nucleus. In the absence of AMPK the Nrf2/HO-1 response to XN is blunted, and this could be due to lower total Nrf2 levels or an impaired nuclear accumulation of the protein. We had a look at the total Nrf2 protein levels after 4 hours treatment with 5 µM XN in WT and AMPK-/- cells. Treatment with XN led to a

significant increase of the Nrf2 protein levels that was comparable for both cell lines (Fig 15a). The accumulation of Nrf2 is not affected in the absence of AMPK. A time-course study of the nuclear Nrf2 protein levels upon treatment with 5 µM XN (1-8 hours) revealed that the nuclear amount of Nrf2 is increased already after one hour and further increases until the 8 hours time point, both in the WT and AMPK-/- cells (Fig 15b). The basal Nrf2 levels and the nuclear accumulation were slightly higher in the AMPK-/- than the WT cells but the difference was not significant. The XN-dependent stabilisation and nuclear import of Nrf2 are both not impaired in AMPK-/- cells. It appears that the active AMPK does not influence the total levels or nuclear accumulation of Nrf2.
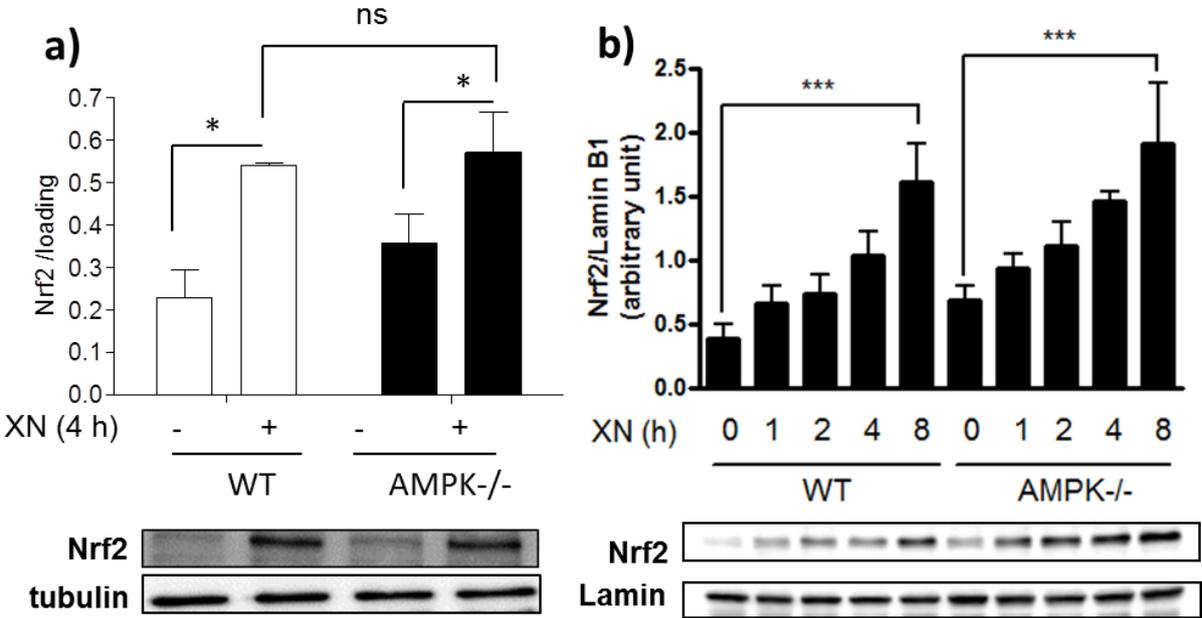
Figure 15: Total levels and nuclear accumulation of Nrf2 upon treatment with XN

a）WT and AMPK-/- cells were treated with XN（5 µM）for 4 hours before total cell lysates were subjected to immunoblot analyses for Nrf2 and tubulin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD; ∗ $p<0.05$; ANOVA, Bonferroni）. Data generated and provided by Elke Heiß. b）WT and AMPK-/- cells were treated with XN（5 µM）for 0, 1, 2, 4, 8 and 24 hours before nuclear cell lysates were subjected to immunoblot analyses for Nrf2 and Lamin B1 as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD;∗∗∗ $p<0.001$; ANOVA, Bonferroni）.

1.4.2 Factors that could be involved in the AMPK-Nrf2 crosstalk: A brief overview

AMPK boosts the XN-triggered and Nrf2-dependent expression of HO-1 at the mRNA level without increasing the total protein level or the static nuclear accumulation of Nrf2. Several additional factors could influence the strength of the Nrf2 signal and thus potentially mediate the positive effect of AMPK on Nrf2/HO1 signalling. We focused on the following:

1) Posttranslational modification of Nrf2: AMPK crosstalks with different cellular pathways that could influence the activity of Nrf2 [176]. Several kinases and kinase signalling pathways have been reported to modulate the activity of the transcription factor via phosphorylation, most notably PKC, the MAPK/ERK pathway, the PI3K/Akt pathway and GSK3ß. PKA can also influence the activity of Nrf2. [86, 177] Furthermore Nrf2 is subject to regulation by acetylation: Both the histone acetyltransferase p300/CBP and the deacetylase SIRT1 have been shown to interact with the protein [82, 99]. The role of these factors in the observed AMPK/ Nrf2 crosstalk was examined, mainly by the use of well-established pharmacological inhibitors and activators.

2) p53 levels: p53 has been shown to negatively influence the expression of Nrf2 target genes by competing with Nrf2 for the binding to the ARE sequence of their promoters [92, 93]. The deletion of AMPK could lead to an increase in the protein level or activity of p53 [178] and we investigated whether this is the case.

3) Autophagy: Both AMPK and Nrf2 crosstalk with the mTOR signalling [179, 180], a cellular pathway that is tightly linked to cellular proliferation and autophagy. Furthermore the autophagy adaptor p62 activates Nrf2 by degrading the inhibitor Keap1 in an mTOR dependent way. [55] Out of these reasons the mTOR/autophagy axis was tested for involvement in the positive effect of AMPK on Nrf2.

4) Fat Metabolism and NADPH homeostasis: AMPK and Nrf2 both negatively regulate fat synthesis, AMPK by inhibiting the enzyme ACC and Nrf2 on the gene expression level. [121, 181] They also both raise the cellular levels of the reducing agent NADPH: AMPK by inducing fatty acid oxidation and Nrf2 by upregulating genes of the pentose phosphate pathway (PPP) [31, 122]. Inhibition of the NADPH production via the PPP affects the Nrf2-dependent gene expression. [33] We tested if the regulation of fatty acid synthesis and oxidation or the cellular NADPH levels play a role for the AMPK/Nrf2 crosstalk.

5) <u>Endoplasmic reticulum stress</u>: ER stress activates the unfolded protein response and has been shown to both activate Nrf2 via PERK [89] and down regulate it via Hrd1. [46] Activation of AMPK attenuates ER stress while deletion of it causes ER stress. [126, 182] We tested whether the AMPK boosts the Nrf2 dependent signalling by attenuating ER stress.

2. Study of the crosstalk between AMPK and Nrf2

2.2 The effect of different kinases and kinase pathways on the AMPK/Nrf2/HO-1 signalling

2.2.1 Protein kinase C

Protein kinase C has been described as a positive regulator of Nrf2 that influences the activity of the transcription factor through phosphorylation at Ser40 [66-68, 183]. If PKC is involved in the positive effect of AMPK on Nrf2 signalling then inhibition of the PKC should reduce the HO-1 expression in the WT MEF. We studied the effect of the PKC inhibitors rottlerin and GÖ 6983 on the XN dependent induction of the HO-1 protein in WT and AMPK-/- cells. Both inhibitors reduced the phosphorylation of PKC substrates (Fig 16a) demonstrating the functionality of the inhibitors at the employed concentrations. Rottlerin increased the HO-1 protein level both in the WT and the AMPK-/- cells already in the absence of XN, and it boosted the XN-dependent HO-1 expression. This effect can be explained by the inhibitor being a mitochondrial uncoupler that generates mitochondrial ROS and thus activates Nrf2 [184]. Treatment with the inhibitor GÖ 6983 slightly but not significantly reduced the upregulation of HO-1 by XN (Fig 16b). Based on the divergent results obtained with the two PKC inhibitors and the fact that the response to each inhibitor is comparable in the WT and AMPK-/- MEF we conclude: Inhibition of the PKC only plays a minor role for the upregulation of HO-1 by XN under the tested conditions. The PKC is not responsible for the positive effect of AMPK on Nrf2 signalling.
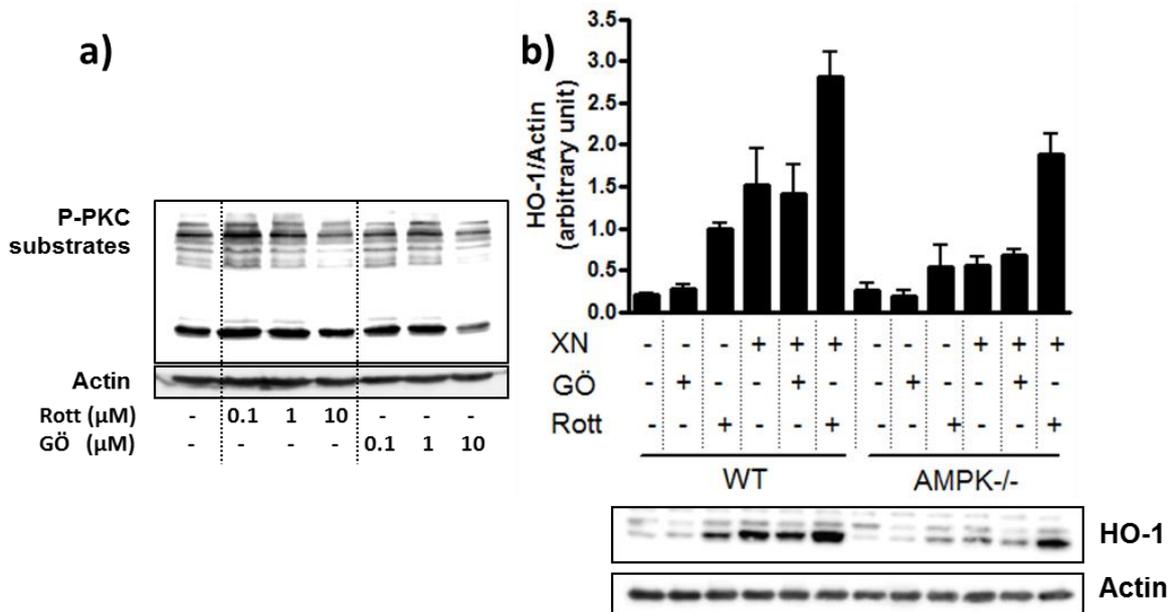
Figure 16: Influence of PKC on the activation of HO−1 by XN
a）WT cells were treated with vehicle, rottlerin（0.1, 1 and 10 μM）and Gö 6983 （0.1, 1 and 10 μM）for 5 hours before total cell lysates were subjected to immunoblot analyses for phospho−（Ser）PKC substrates and actin as a loading control. b）WT and AMPK−/− cells were treated with vehicle, XN（5 μM）, rottlerin（10 μM）and Gö 6983（10 μM）for 5 hours before total cell lysates were subjected to immunoblot analyses for HO−1 and actin as a loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD）.

2.2.2 The MAPK/ERK pathway

The mitogen activated protein kinases (MAPKs) can phosphorylate Nrf2 at multiple sites and thus positively affect the activity of Nrf2. P38, ERK and JNK are all members of the MAPK family that have been shown to be involved in the activation of Nrf2 by different compounds [82]. We treated the WT and AMPK-/- cells with inhibitors of ERK (U0126 and PD98059), p38 (SB203580 and SB202190) and JNK (SP600125) to assess their effect on the XN dependent induction of the HO-1 protein. Both in the WT (Fig 17a) and in the AMPK-/- cells (Fig 17b) inhibition of the MAPK signalling did not significantly affect the expression of HO-1, though inhibition of JNK slightly reduced it. Thus, MAPKs do not play a major role in the XN-induced HO-1 induction in our MEF cell system and do not account for the distinct Nrf2/HO-1 response in WT and AMPK-/- cells.
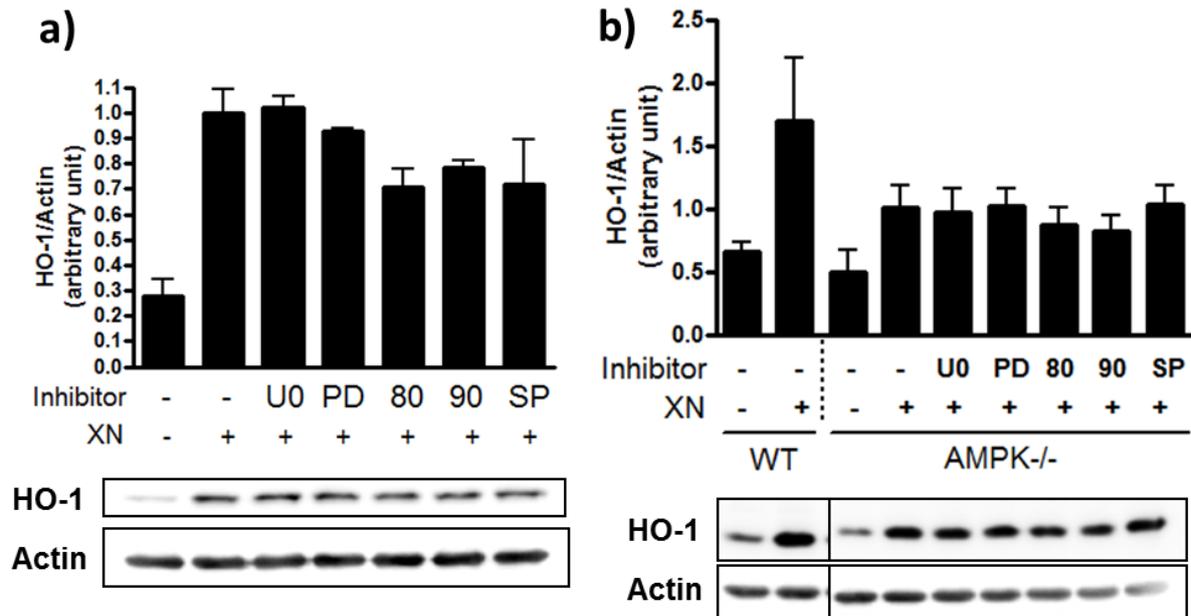
Figure 17: Influence of the MAPK pathway on the activation of HO-1 by XN

a）WT cells were treated with vehicle, XN（5 μM）, U0（U0126 10μM）, PD（PD98059 10μM）, 80（SB203580 10μM）, 90（SB202190 10μM）and SP（SP600125 10μM）for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as a loading control. Representative blots and compiled densitometric analyses of two independent experiments are depicted（mean + SD）. b）AMPK-/- cells were treated with vehicle, XN（5 μM）, U0（U0126 10μM）, PD（PD98059 10μM）, 80（SB203580 10μM）, 90（SB202190 10μM）and SP（SP600125 10μM）for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as a loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD）.

2.2.3 Protein kinase A

PKA activators have been shown to activate Nrf2 in hepatocytes, but in the same cell model it has also been shown that an overexpression of PKA inhibits the ARE signalling [86, 185]. PKA can inactivate AMPK via phosphorylation. [186] We treated the cells with two different inhibitors of PKA, Rp-8-Br-cAMPS and KT 5720. Neither of them showed any effect on the XN-dependent expression of HO-1 (Fig 18) in WT or AMPK-/- cells, ruling out a major role of PKA for the observed AMPK/Nrf2/HO-1 axis.
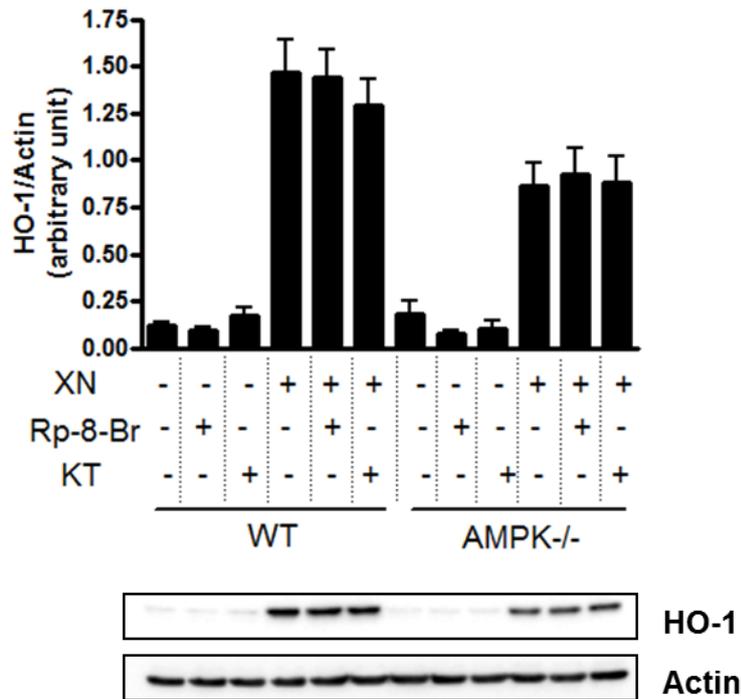
Figure 18: Influence of PKA on the activation of HO-1 by XN

WT and AMPK-/- cells were treated with vehicle, XN (5 μM), KT (KT 5720 10 μM) and Rp-8-Br (Rp-8-Br-cAMPS 50 μM) for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD).

2.2.4 PI3K/Akt and GSK3 beta

a) The PI3K pathway contributes to the upregulation of HO-1 by XN

The PI3K/Akt pathway positively regulates Nrf2: In a number of different cell types it has been shown that inhibition of PI3K interferes with the activation of Nrf2 and that Akt is necessary for the seen effect of PI3K on Nrf2 [70, 71, 112, 187]. To confirm this effect in our cell model we treated the cells with Wortmannin, a well described inhibitor of the PI3K/Akt signalling. Treatment with Wortmannin almost completely abolished the inhibitory phosphorylation of the PI3K downstream target GSK3ß at Ser9 (Fig 19), ensuring the functionality of the used inhibitor. WT and AMPK-/- cells were treated with Wortmannin to test the effect on the HO-1 protein and mRNA upregulation by XN. Both in the WT and in the AMPK-/- cells treatment with Wortmannin diminished the upregulation of HO-1 protein (Fig 20a) and mRNA (Fig 20b) by XN to a comparable extent. As expected, the inhibition of PI3K blunts the upregulation of HO-1, confirming that the PI3K pathway positively regulates Nrf2.
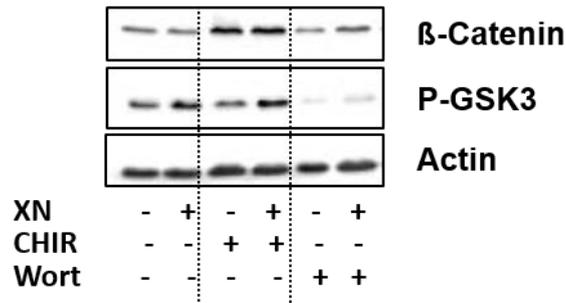
Figure 19: Verification of PI3K and GSK3ß inhibition by Wortmannin and CHIR 99021, respectively

WT cells were treated for 5 hours with vehicle, XN （5 µM）, the GSK3ß inhibitor CHIR 99021 （10 µM） and the PI3K inhibitor Wortmannin （10µM）. Total cell lysates were subjected to immunoblot analyses for P-GSK3ß （Ser9）, ß-Catenin and actin as internal control.
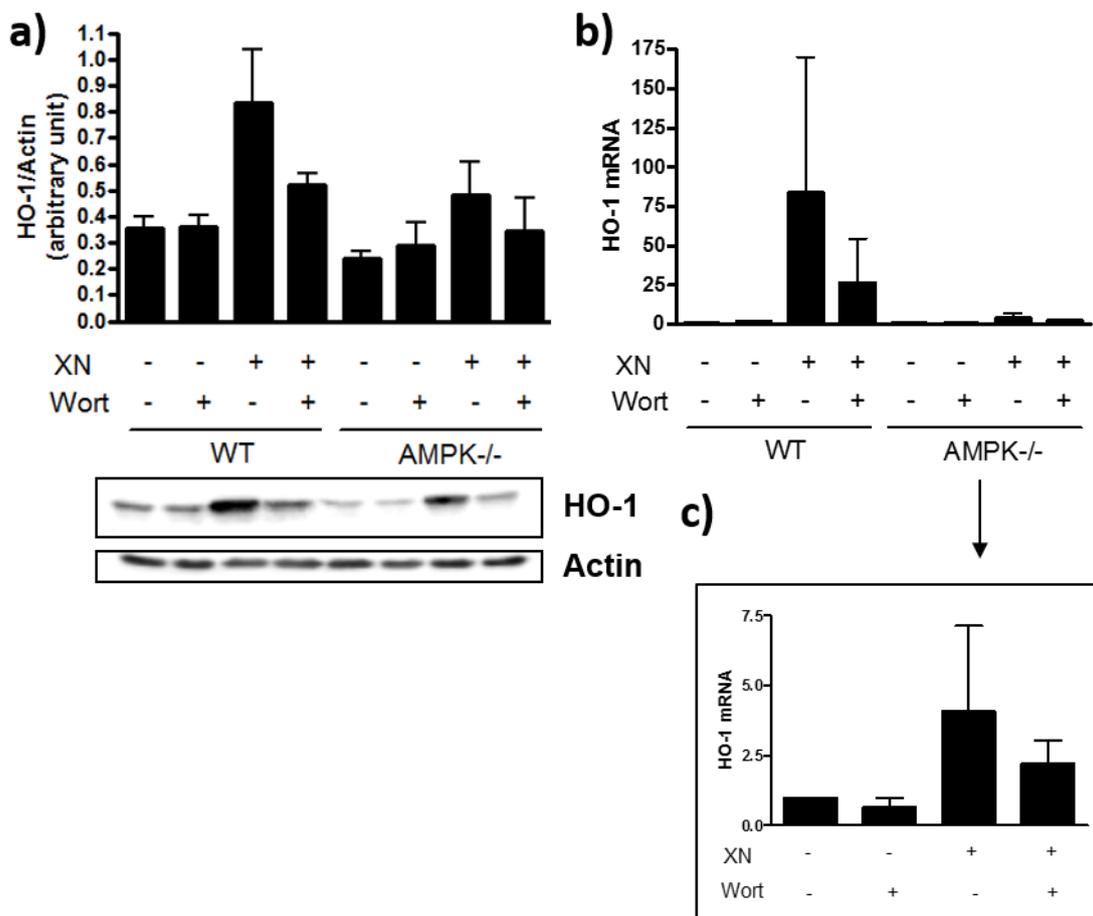
Figure 20: Influence of the PI3K pathway on the activation of HO-1 by XN

a） WT and AMPK-/- cells were treated with vehicle, XN （5 µM） and Wortmannin （10 µM） for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric

analyses of three independent experiments are depicted（mean + SD）. b）WT and AMPK−/− cells were treated with vehicle, XN（5 μM）and Wortmannin（10μM）for 4 hours before the total mRNA was extracted. Levels of HO−1 mRNA were determined by RTqPCR analysis.（n=3; mean + SD）. c）Same as for b）, but only the data for the AMPK−/− cells are shown.

b) The PI3K pathway is blunted in AMPK-/- cells

The reduced Nrf2 response in AMPK-/- cells could be due to an impaired PI3K signalling. To test this we treated the cells with an activator of the PI3K/Akt signalling, YS-49. [188] As a measure for PI3K activation the phosphorylation of Akt at Ser473 was detected. In WT cells treated for 1-5 hours with 50 μM YS-49 already after one hour the phosphorylation of Akt was increased by YS-49 and it reached the highest level after 3 hours, while in solvent treated cells it did not increase (Fig 21a). We compared the effect of YS-49 treatment (1-5 hours) on Akt phosphorylation in WT and AMPK-/- cells. In AMPK-/- cells YS-49 could still induce Akt phosphorylation, but the increase was not significant and the total phosphorylation was blunted compared to the WT cells (Fig 21b). The PI3K dependent signalling appears to be dampened in AMPK-/- cells.
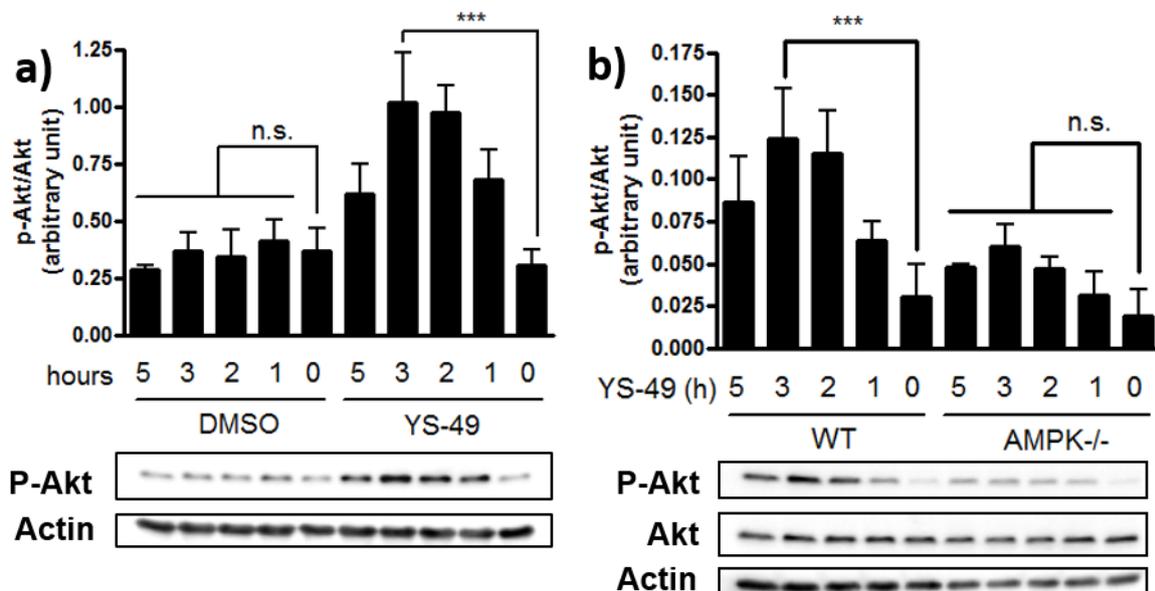
Figure 21: Activation of the PI3K pathway in dependence of AMPK
a）WT cells were treated for 1−5 hours with vehicle（DMSO）or YS−49（50 μM）. Total cell lysates were subjected to immunoblot analyses for Akt, P−Akt（Ser473）

and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD; ∗∗∗ p<0.001; ANOVA, Bonferroni). b) WT and AMPK-/- cells were treated for 1-5 hours with vehicle (DMSO) or YS-49 (50 µM). Total cell lysates were subjected to immunoblot analyses for Akt, P-Akt (Ser473) and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD; ∗∗∗ p<0.001; ANOVA, Bonferroni).

c) GSK3ß negatively influences the upregulation of HO-1 by XN

The kinase GSK3 is part of the PI3K/Akt pathway: Akt inhibits it via phosphorylation. [72] The GSK3ß isoform can promote a Keap1 independent degradation of Nrf2. It phosphorylates Nrf2 on two serine residues and thus promotes the binding of the ubiquitin ligase β-TrCP that targets Nrf2 for proteasomal degradation [39, 40, 189]. In addition GSK3ß also promotes the nuclear exclusion of Nrf2 via the Fyn kinase. [75] A possible explanation for the reduced Nrf2/HO-1 signalling in AMPK-/- cells could be that the blunted PI3K/Akt signalling in the cells (see above, 2.2.4 b) results in an elevated GSK3ß-mediated inhibition of Nrf2.
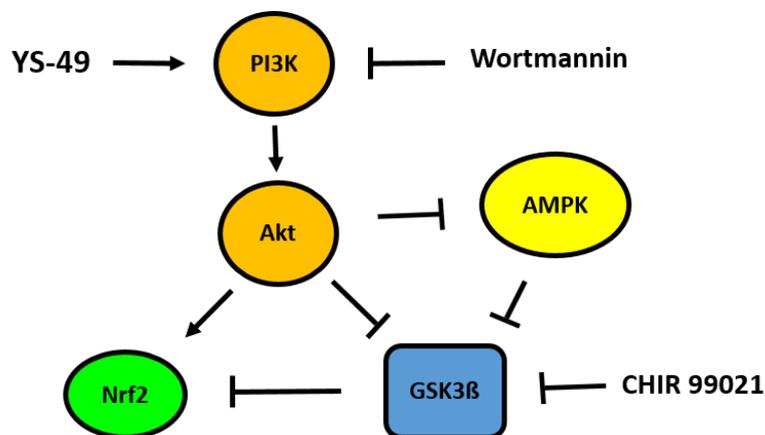
Figure 22: An overview of the PI3K/Akt, GSK3ß and Nrf2 crosstalk
The PI3K activates Nrf2 via Akt. Wortmannin inhibits PI3K while YS-49 activates it. GSK3ß promotes the degradation of Nrf2. Akt inhibits GSK3ß by phosphorylating it at Ser9. CHIR 99021 is an inhibitor of GSK3ß.

To test this hypothesis we employed CHIR 99021, which is a known GSK3 inhibitor. GSK3 promotes the degradation of ß-Catenin and we saw that treatment with CHIR 99021 leads to

the accumulation of ß-Catenin, confirming that GSK3 is inhibited by CHIR 99021 in our cell model. Moreover, we saw that XN increases the inhibitory phosphorylation of GSK3ß at Ser9 (Fig 19). WT and AMPK-/- cells were treated with CHIR 99021 to test the effect on the HO-1 protein and mRNA upregulation by XN. If GSK3 inhibition plays a role for the positive effect of AMPK on Nrf2 signalling, then CHIR 99021 should restore a normal HO-1 expression in AMPK-/- cells. Co-treatment with XN and CHIR 99021 slightly boosted the XN dependent HO-1 protein upregulation both in the WT and the AMPK-/- cells, but the effect was not significant (Fig 23a). In the WT cells CHIR 99021 boosted the XN dependent HO-1 mRNA upregulation, while in the AMPK-/- cells the effect was negligible (Fig 23b). Inhibition of the GSK3 positively influences the HO-1 protein expression both in the WT and in AMPK-/- cells, but the HO-1 level is still lower in the absence of AMPK. Furthermore, inhibition of the GSK3 cannot rescue the mRNA expression of HO-1 in AMPK-/- cells. The GSK3 definitely influences the Nrf2 signalling, but it cannot be the main mediator of the effect of AMPK on the Nrf2/HO-1 signalling.
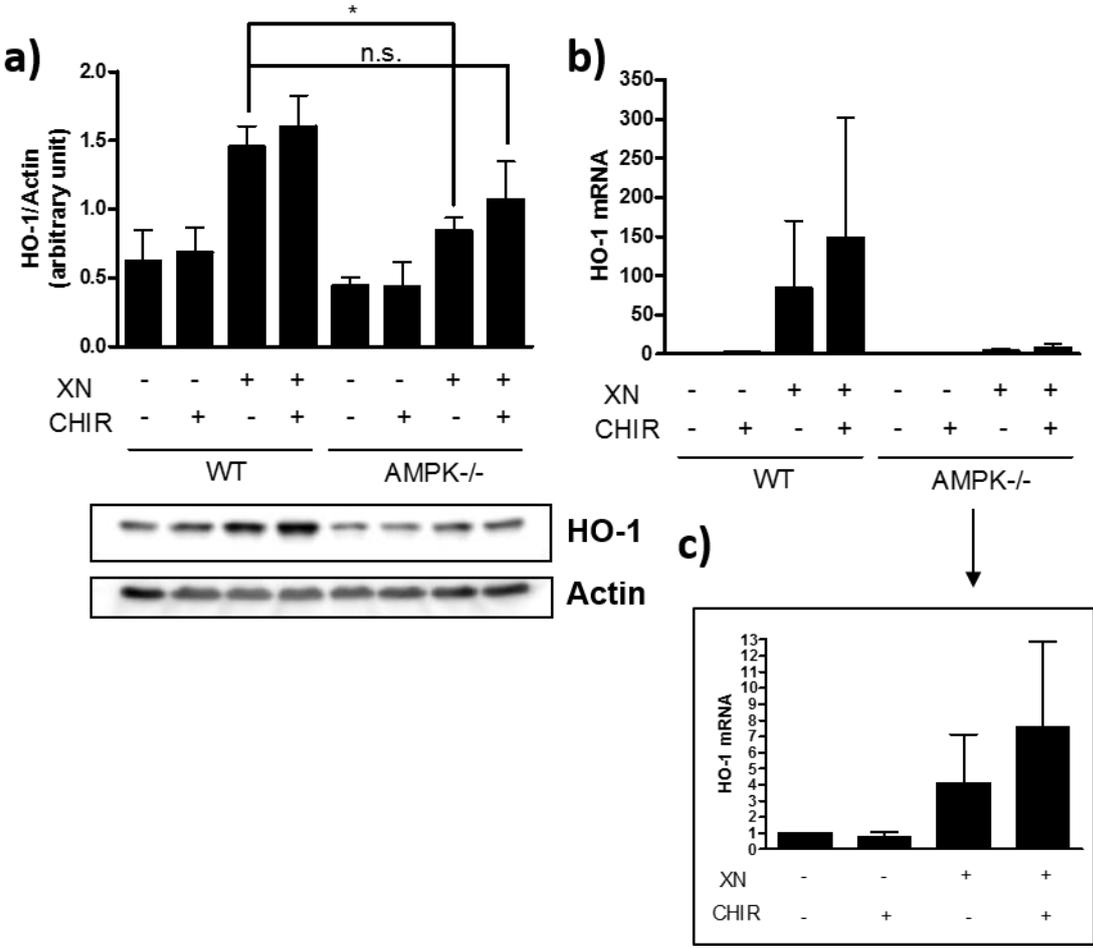
Figure 23: Influence of GSK3ß on the activation of HO-1 by XN

a）WT and AMPK-/- cells were treated with vehicle, XN（5 µM）and CHIR 99021（10 µM）for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD; ANOVA, Bonferroni, ∗ p< 0.05）. b）WT and AMPK-/-cells were treated with vehicle, XN（5 µM）and CHIR 99021（10µM）for 4 hours before the total mRNA was extracted. Levels of HO-1 mRNA were determined by RTqPCR analysis.（n=3; mean + SD）. c）Same as for b), but only the data for the AMPK-/- cells are shown.

d) Nrf2 is required for the activation of the PI3K/Akt pathway

PI3K regulates Nrf2, but Nrf2 has also been shown to in turn regulate the PI3K/Akt pathway [190], implying the existence of a feedback mechanism. To verify this we treated WT and Nrf2-/- cells with the PI3K activator YS-49. We found that in the Nrf2-/- cells the activating phosphorylation of Akt at Ser473 that YS-49 induces in WT cells is completely abolished (Fig 24a), confirming a positive effect of Nrf2 on the PI3K/Akt signalling. It is interesting to note that the PI3K-dependent Akt phosphorylation by YS-49 follows the same pattern as the Nrf2-dependent upregulation of HO-1 by XN: Both are completely abolished in the absence of Nrf2 (Fig. 8a and 24a), while they are blunted in the absence of AMPK (Fig. 12a and 21b). Furthermore, treatment with the Akt activator YS-49 upregulates HO-1 in WT but not in Nrf2-/- cells (Fig 24b), just as treatment with XN (Fig 8a).
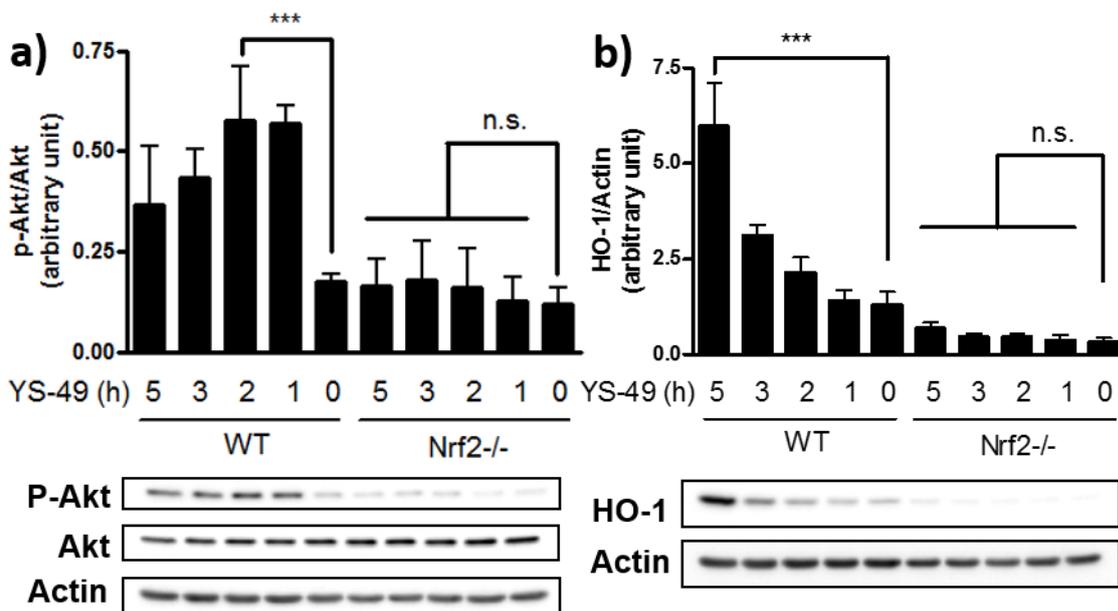
Figure 24: Influence of Nrf2 on the activation of Akt by YS-49

a）WT and Nrf2-/- cells were treated for 1-5 hours with vehicle（DMSO）or YS-49（50 µM）. Total cell lysates were subjected to immunoblot analyses for Akt, P-Akt（Ser473）and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD; ✱✱✱ p<0.001; ANOVA, Bonferroni）. b）WT and Nrf2-/- cells were treated for 1-5 hours with vehicle（DMSO）or YS-49（50 µM）. Total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD; ✱✱✱ p<0.001; ANOVA, Bonferroni）.

2.3 Acetylation

Acetylation has been shown to influence the activity of Nrf2. The histone acetyltransferase (HAT) p300/CBP binds to the transactivation domains of Nrf2 and it increases the binding of Nrf2 to the target DNA, while the deacetylase sirtuin 1 (SIRT1) decreases the acetylation of Nrf2 and reduces the transcription of its target genes [42, 82, 99].

2.3.1 The general acetylation level is increased in AMPK-/- cells

To test whether the deletion of AMPK influences the general acetylation pattern, we detected the acetylated lysine residues in nuclear cell extracts from WT and AMPK-/- cells treated with 5 µM XN for 1-24 hours. We found that the acetylation is generally increased in AMPK-/- cells (Fig 25a) and treatment with XN has a minor positive influence on acetylation in both cell types between 1 and 4 hours incubation. When comparing the acetylation pattern of total cell extracts from WT and AMPK-/- cells treated only with solvent, we saw that in the AMPK-/- cells a band just above the 50 kDa marker is consistently stronger than in the WT (Fig 25b).
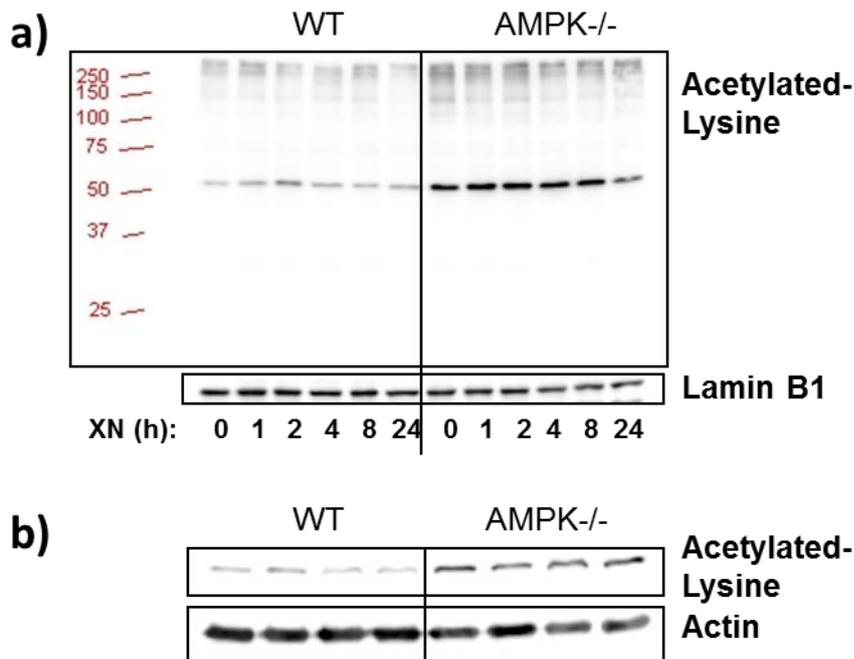
Figure 25: Influence of AMPK on the acetylation status

a) WT and AMPK−/− cells were treated with XN (5 µM) for 0, 1, 2, 4, 8 and 24 hours before nuclear cell lysates were subjected to immunoblot analyses for acetylated-lysine and Lamin B1 as loading control. b) WT and AMPK−/− cells were treated with solvent for 5 hours before total cell lysates were subjected to immunoblot analyses for acetylated-lysine and actin as loading control. Cellular extracts from four independent experiments were used. The detected band was just above the 50 kDa-marker.

2.3.2 Higher acetylation levels blunt the upregulation of HO-1 by XN

In the absence of AMPK the basal acetylation levels are higher. To test if acetylation influences the Nrf2 dependent signalling in AMPK-/- cells we treated the cells with two different deacetylase inhibitors and an inhibitor of the acetyltransferase HAT p300/CBP. The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) alone promoted the acetylation of two bands below the 25 kDa marker, at the height where histones are expected. EX-527, an inhibitor of the SIRT1 deacetylase, did not influence these bands but instead increased the acetylation of a band just above the 50 kDa marker. This band probably corresponds to the protein p53 that is increasingly acetylated upon inhibition of SIRT1. [191] The Nrf2 activators XN and CDDO-Im did not influence the acetylation of any of these bands when compared to the solvent control DMSO (Fig 26a). WT and AMPK-/- cells were treated with the deacetylase inhibitors to test

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how increased acetylation affects the XN dependent upregulation of HO-1. TSA reduced the upregulation of HO-1 both in WT and AMPK-/- cells in a reproducible manner. EX-527 slightly reduced the HO-1 upregulation only in the AMPK-/- cells (Fig 26b). Deacetylase inhibitors seem to impair the HO-1 upregulation. This is in line with the fact that the XN-induced HO-1 expression is blunted in the AMPK-/- cells that have generally higher protein acetylation levels.
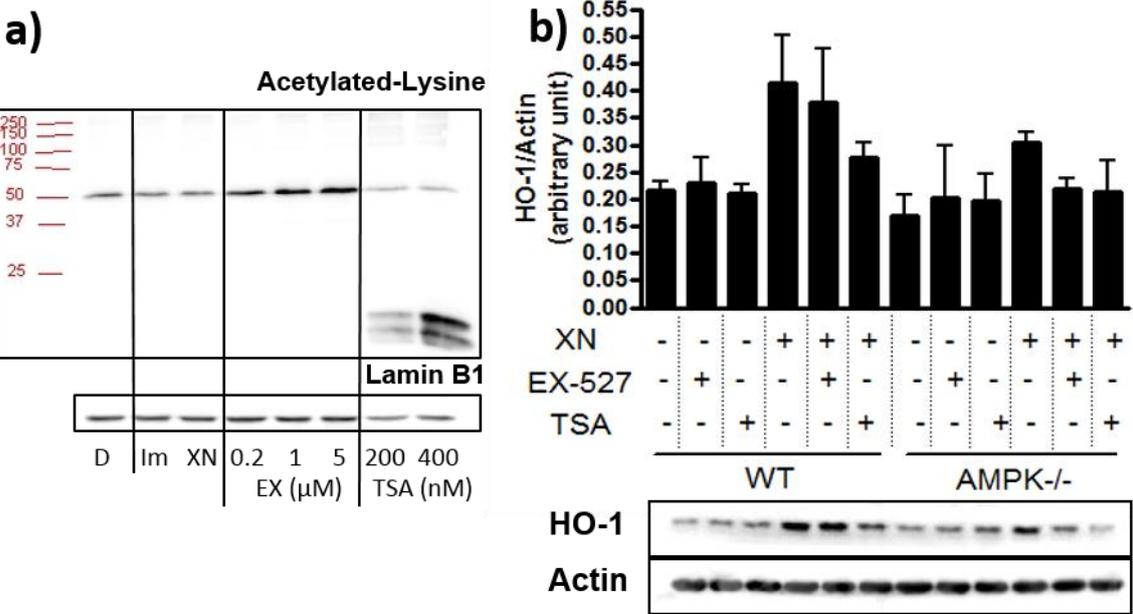
Figure 26: The effect of deacetylase inhibitors on the induction of HO-1 by XN
a) WT cells were treated with D (solvent: DMSO), Im (CDDO-Imidazolide 100 nM), XN (5 μM), EX (EX-527: 0.2, 1 and 5μM) and TSA (Trichostatin A: 200 and 400 nM) for 5 hours before nuclear cell lysates were subjected to immunoblot analyses for acetylated-lysine and Lamin B1 as loading control. b) WT and AMPK-/- cells were treated for 5 hours with vehicle, XN (5 μM), EX-527 (5 μM) and TSA (100 nM). Total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD).

C646 is an inhibitor of the acetyltransferase HAT p300/CBP. In contrast to the effect of the deacetylase inhibitors treatment with C646 increased the HO-1 expression, both in WT and AMPK-/- cells. C646 alone already strongly induced HO-1 and it also boosted the upregulation by XN. At a concentration of 10 μM C646 elicited a comparable upregulation of HO-1 in the WT and AMPK-/- cells (Fig 27a), while at the lower concentrations of 0.5 and 2.5 μM it

promoted a stronger HO-1 upregulation in the WT than the AMPK-/- cells. (Fig 27b) Overall decreased acetylation by p300/CBP appears to be in favour of HO-1 expression, in line with the observed lower acetylation status and higher HO-1 expression in WT compared to AMPK-/- cells. An analysis of the degree of Nrf2 acetylation in WT and AMPK-/- MEF did unfortunately not deliver conclusive results due to difficulties in immunoprecipitating the endogenous Nrf2 from the cells.
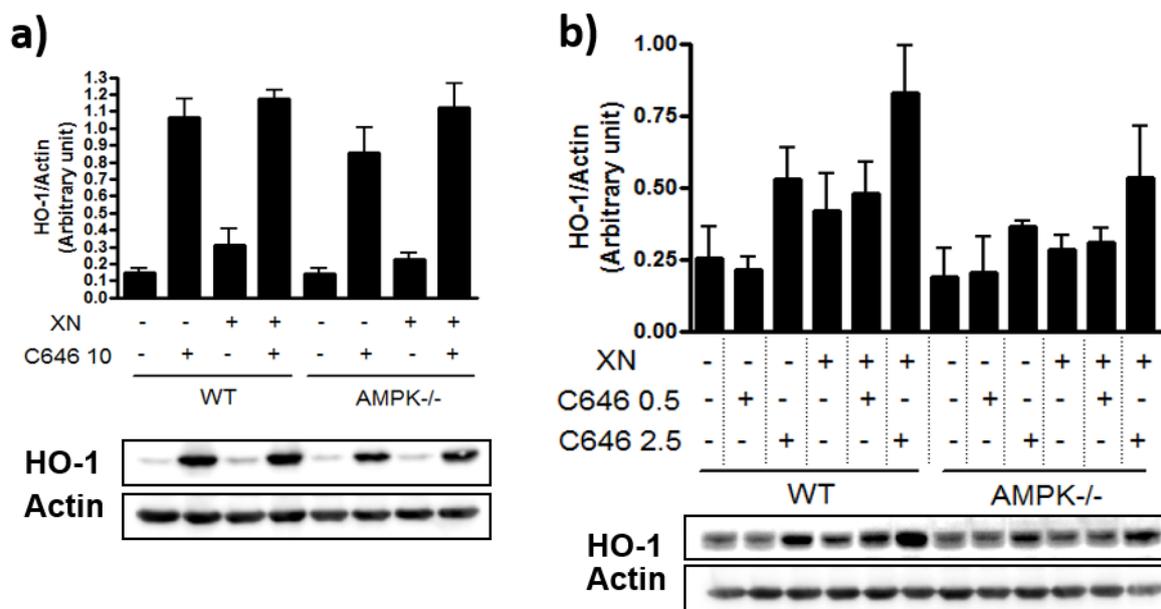
Figure 27: Influence of the HAT p300 on the induction of HO-1 by XN

WT and AMPK-/- cells were treated for 5 hours with vehicle, XN (5 µM) and a) 0.5 and 2.5 µM C646 or b) 10 µM C646. Total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD).

2.4 The p53 levels

P53 has been shown to negatively regulate the expression of Nrf2 target genes by binding to the ARE in the promoter, thus preventing Nrf2 from binding [93]. Upon metabolic stress AMPK can stabilize p53 and phosphorylate it on Ser15 or Ser46, leading to a cell-cycle arrest and eventually promoting apoptosis [127, 178, 192]. Under normal, unstressed conditions AMPK might be preventing the stabilization and activation of p53, thus reducing the amount of p53 available for binding to the ARE. To test how deletion of AMPK affects the p53 levels and

activity, we had a look at the total p53 and at the phosphorylation of p53 at Ser15 in WT and AMPK-/- cells treated with XN for 1-5 hours. Treatment with XN tended to slightly increase the Ser15 phosphorylation in both cell types, indicating that the AMPK is not required for the phosphorylation. However, the total levels of p53 were consistently higher in the AMPK-/- than the WT cells (Fig 28). The higher level of p53 could be involved in lowering the activity of Nrf2 in the AMPK-/- cells by exerting a stronger inhibitory effect on Nrf2-mediated gene expression.
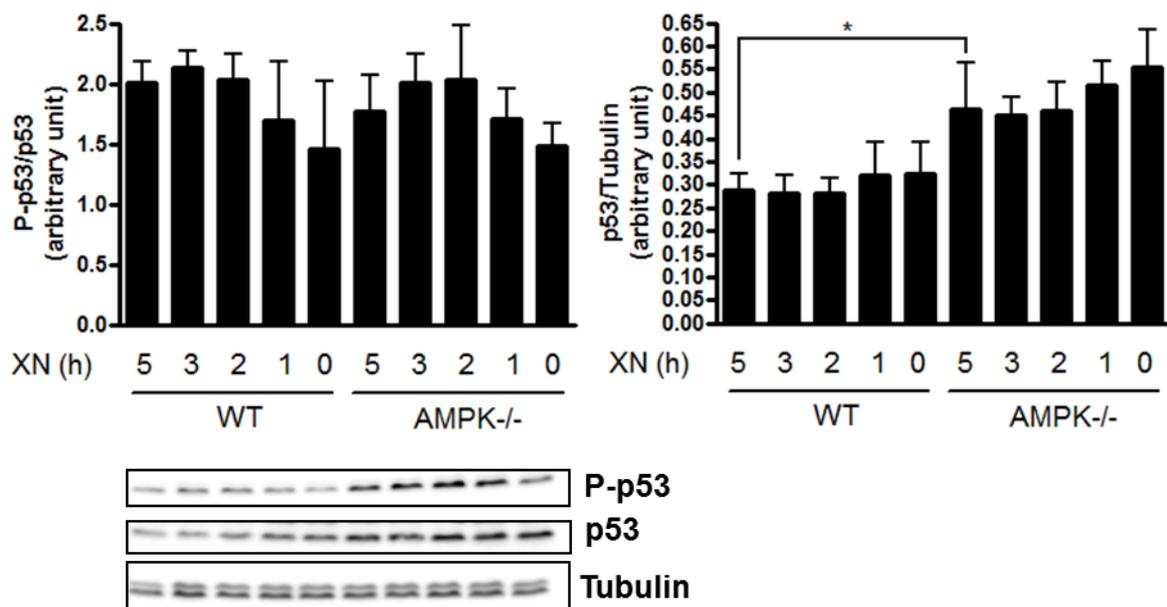
Figure 28: Influence of AMPK on the p53 levels
WT cells were treated for 5 hours with XN（5 μM）. Total cell lysates were subjected to immunoblot analyses for p53，P-p53（Ser15）and tubulin as loading control. The left graph shows the P-p53/p53 ratio，the right graph shows the total p53. Representative blots and compiled densitometric analyses of three independent experiments are depicted（mean + SD；＊ $p < 0.05$；ANOVA，Bonferroni）.

2.5 mTOR and autophagy

AMPK negatively regulates the mTOR complex 1 (mTORC1), another cellular switch between catabolic and anabolic processes. In contrast to and as quasi-opponent to AMPK, the active mTORC1 favours anabolism and impedes catabolic processes. In line with the antagonistic action of AMPK and mTORC1, inhibition of TORC1 has been shown to lead to the activation of protective genes via Nrf2 [180]. Moreover, AMPK and mTORC1 regulate with opposite effects

autophagy, a cellular process that can influence the activity of Nrf2: AMPK promotes autophagy while mTORC1 inhibits it. The autophagy adaptor p62 sequesters Keap1 into protein aggregates that are degraded via autophagy, thus activating Nrf2 by preventing its Keap1 dependent degradation [193]. An mTORC1-dependent phosphorylation of p62 greatly increases the affinity of p62 for Keap1. Therefore, it was investigated whether mTORC1 or autophagy play a role for the AMPK-dependent boost of the Nrf2-mediated HO-1 expression.
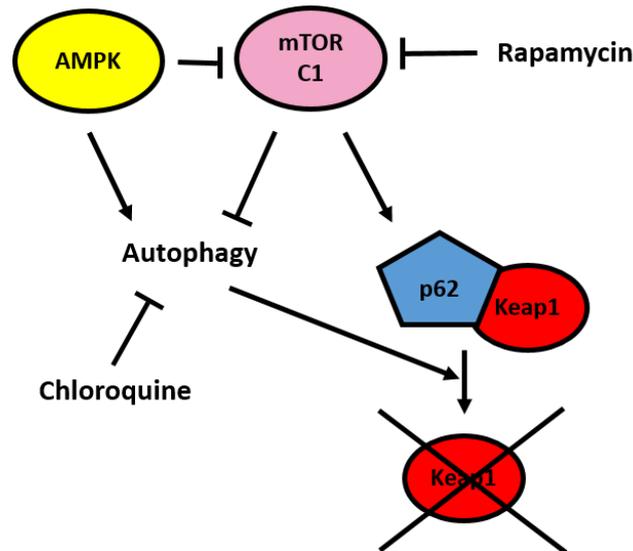
Figure 29: An overview of the connections between mTORC1, autophagy and p62
AMPK activates autophagy while mTORC1 and chloroquine inhibit it. mTORC1 is inhibited by AMPK and rapamycin. p62 sequesters Keap1, the inhibitor of Nrf2, into aggregates that are degraded via autophagy. mTORC1 activates p62 by phosphorylating it and thus increasing its affinity for Keap1.

2.5.1 mTORC1 contributes to the XN dependent upregulation of HO-1

AMPK inhibits mTORC1 by phosphorylating Raptor, a binding partner of mTORC1, on Ser792 [194]. Accordingly, we found that in AMPK-/- cells the Ser792 phosphorylation of Raptor is almost gone (Fig 30a). AMPK could boost the Nrf2 signalling by inhibiting mTORC1. If this is the case, then the inhibition of mTORC1 should rescue the XN dependent HO-1 expression in AMPK-/- cells. To test this WT and AMPK-/- cells were co-treated with XN and rapamycin, a well described inhibitor of mTORC1 [195]. Rapamycin alone did not affect the HO-1 protein level, while co-treatment with XN and rapamycin reproducibly lowered the XN dependent upregulation of HO-1 both in the WT and the AMPK-/- cells (Fig 30b). Not only can the

inhibition of mTORC1 not rescue the HO-1 expression in AMPK-/- cells, it even reduces it both in WT and AMPK -/- cells. Possible explanations could be an inhibition of protein synthesis caused by the inhibition of mTORC1, or a decreased binding of p62 to Keap1 in the absence of the activating mTORC1-dependent phosphorylation.
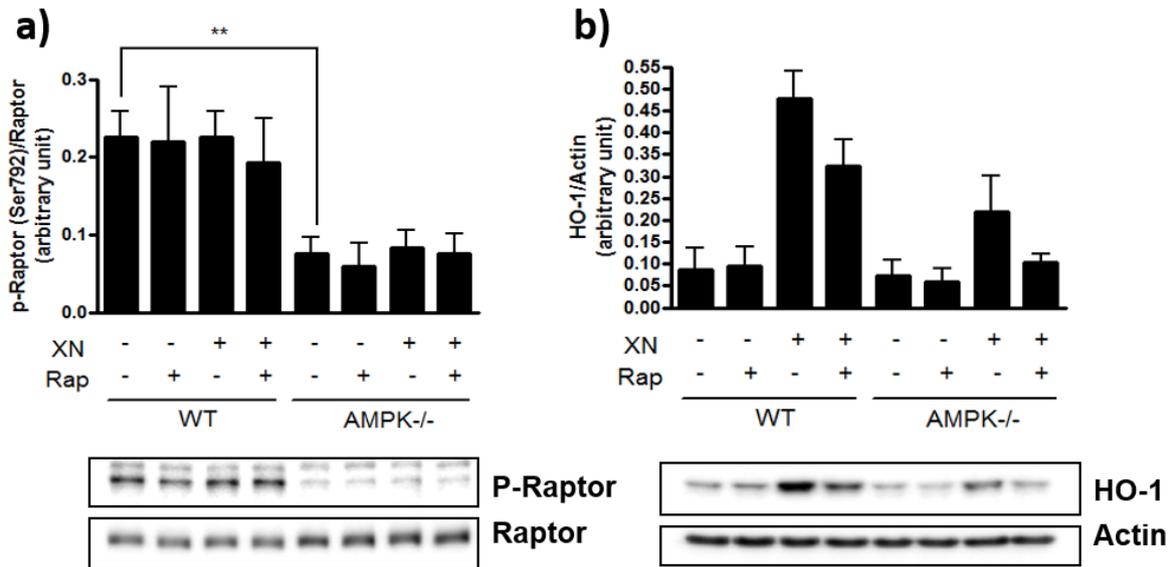
Figure 30: Influence of mTORC1 on the activation of HO-1 by XN

WT and AMPK-/- cells were treated with vehicle, XN (5 μM) and 100 nM Rapamycin for 5 hours before total cell lysates were subjected to immunoblot analyses for a) P-Raptor (Ser792) and Raptor or b) HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of four independent experiments are depicted (mean + SD; ** $p < 0.01$; ANOVA, Bonferroni).

2.5.2 Autophagy does not influence the XN dependent upregulation of HO-1

If the positive effect of AMPK on autophagy plays a role for Nrf2 signalling, then inhibition of autophagy should blunt the induction of HO-1 by XN in WT cells. WT and AMPK-/- cells were co-treated with XN and chloroquine, an inhibitor of autophagy [196]. Treatment with chloroquine alone slightly increased the HO-1 level both in the WT and the AMPK-/- cells. Co-treatment with chloroquine did not influence the XN dependent up regulation of HO-1 (Fig 31) in either cell type.
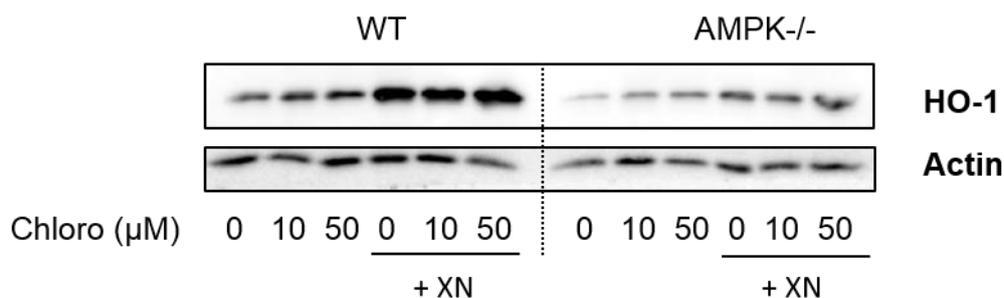
Figure 31: Influence of autophagy on the activation of HO-1 by XN

WT and AMPK-/- cells were treated with vehicle, XN （5 μM） and Chloroquine （10 and 50 μM） for 5 hours before total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots of two independent experiments are depicted. Data were generated and provided by Elke Heiß.

2.6 Lipid metabolism and NADPH

Nrf2 and AMPK both influence the cellular fat metabolism and NADPH level. Nrf2 downregulates genes involved with fatty acid and cholesterol synthesis, anabolic processes that consume NADPH. At the same time it upregulates genes of the PPP pathway, the main cellular source of NADPH. Moreover, Nrf2 has a positive effect on fatty acid oxidation. [35] AMPK directly prevents the synthesis of fatty acids by inhibiting ACC, and instead promotes the beta oxidation of fatty acids. It plays a key role for maintaining the intracellular NADPH homeostasis. [122, 197] Thus, both AMPK and Nrf2 inhibit fatty acid synthesis, boost fatty acid oxidation and regulate the NADPH levels. The latter have been shown by us to play a role for Nrf2-mediated gene transcription under certain circumstances [33]. We investigated whether an altered rate of fatty acid oxidation/synthesis or decreased NADPH levels could account for the distinct Nrf2/HO1 response in WT and AMPK-/- cells.
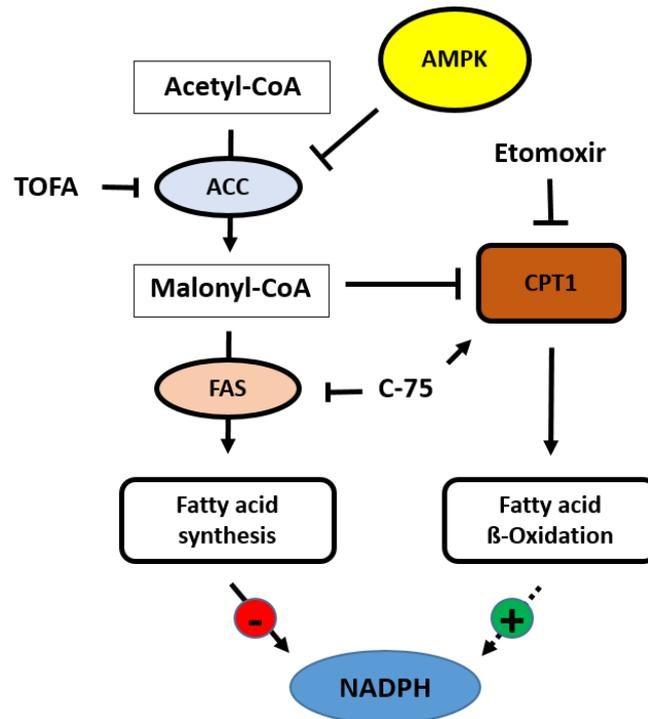
Figure 32: Regulation of fatty acid metabolism by AMPK

ACC carboxylates acetyl–CoA to malonyl–CoA that serves as substrate for the synthesis of fatty acids by FAS and inhibits the ß–oxidation of fatty acids by inhibiting CPT1. AMPK inhibits ACC by phosphorylating it at Ser79, thus preventing the accumulation of malonyl–CoA and activating fatty acid oxidation via CPT1. TOFA also inhibits ACC, while etomoxir inhibits CPT1. C–75 is both an inhibitor of FAS and an activator of CPT1. Fatty acid synthesis consumes NADPH while the ß–oxidation can indirectly contribute to increased NADPH levels.

2.6.1 NADPH and fatty acid oxidation

AMPK plays an important role for maintaining the cellular NADPH levels and it promotes the oxidation of fatty acids that can also indirectly contribute to NADPH generation. [197, 198] To test the influence of fatty acid oxidation and NADPH levels on the Nrf2 dependent signalling, two different approaches were used: We inhibited the fatty acid ß-oxidation by inhibiting CPT1 and we directly supplied the cells with NADPH. CPT1 (Carnitine palmitoyltransferase I) is a rate limiting enzyme for the oxidation of fatty acids that regulates the mitochondrial fatty acid uptake. The compound etomoxir is an inhibitor of CPT1 and has been reported to impair the NADPH generation [199]. WT and AMPK-/- MEF cells were co-treated with etomoxir and XN. If the ß-oxidation of fatty acids plays a positive role for the Nrf2 dependent signalling, then

treatment with etomoxir should impair the XN dependent HO-1 upregulation. Etomoxir alone did not affect the HO-1 expression. Co-treatment with etomoxir and XN raised the XN dependent HO-1 expression both in the WT and the AMPK-/- cells. (Fig 33a) As a reproducible trend this effect was stronger in the AMPK-/- cells. Inhibiting the fatty acid ß-oxidation via CPT1 inhibition does not impair, but on the contrary it boosts the expression of HO-1. To examine the role of the NADPH levels for the AMPK/Nrf2/HO1 signalling the WT and AMPK-/- MEF cells were treated with an NADPH regenerating system. AMPK maintains the intracellular NADPH levels. If lower NADPH levels are impairing the Nrf2/HO-1 signalling in AMPK-/- cells, then supplying them with NADPH should restore a normal response. Co-treatment with NADPH and XN reproducibly decreased the XN dependent HO-1 expression in the WT, but did not affect it in the AMPK-/- cells (Fig 33b). Overall, inhibition of fatty acid oxidation (and presumably NADPH production) seemed to boost XN-induced HO-1 expression in AMPK-/- cells to a greater extent than in WT cells. A comparable reverse trend was seen upon treatment with an exogenous source of NADPH.
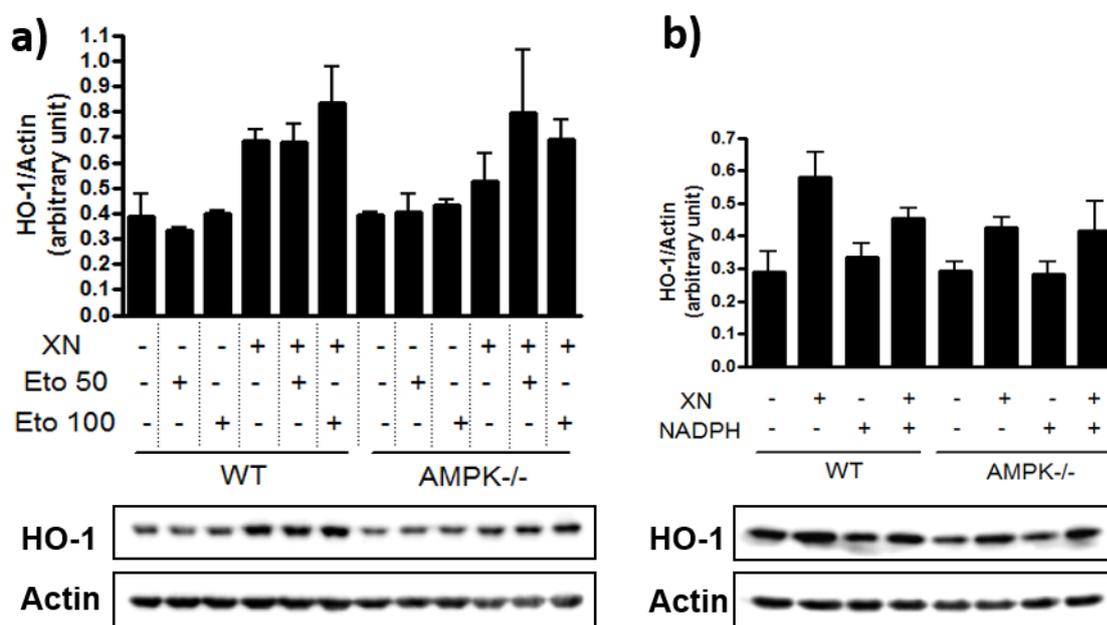
Figure 33: Influence of fatty acid oxidation and NADPH on the induction of HO-1 by XN a) WT and AMPK-/- cells were treated for 5 hours with vehicle, XN (5 µM) and etomoxir (50 and 100 µM). Total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD). b) WT and AMPK-/- cells were treated for 5 hours with vehicle, XN (5 µM) and an NADPH

regenerating system. Total cell lysates were subjected to immunoblot analyses for HO−1 and actin as loading control. Representative blots and compiled densitometric analyses of three independent experiments are depicted (mean + SD).

2.6.2 Fatty acid synthesis

AMPK negatively regulates the synthesis of fatty acids by inhibiting ACC. We tested whether the rate of fatty acid synthesis can influence the XN dependent HO-1 expression by inhibiting it with two different compounds, TOFA and C-75. TOFA is an inhibitor of ACC. If the inhibition of ACC by AMPK plays a positive role for the activity of Nrf2, then treatment with TOFA should increase the HO-1 expression in AMPK-/- cells. Both in the WT and AMPK-/- cells treatment with TOFA could not increase the XN dependent HO-1 upregulation. In the AMPK-/- cells it even slightly reduced it (Fig 34). C-75 inhibits the fatty acid synthesis at a different level than TOFA by inhibiting FAS, an enzyme that catalyses the fatty acid synthesis downstream of ACC. Treatment with C-75 alone already strongly upregulated the expression of HO-1, both in the WT and AMPK-/- cells, and C-75 also boosted the XN-dependent expression of HO-1 (Fig 34). C-75 promotes the accumulation of the cytotoxic FAS-substrate malonyl-CoA which is known to inhibit fatty acid oxidation by inhibiting CPT1. At the same time, C-75 paradoxically activates fatty acid oxidation via CPT1. [200] The observed effect of C-75 on the Nrf2/HO-1 signalling could be due to an activation of Nrf2 by the cellular stress that malonyl-CoA causes. Overall inhibition of fatty acid synthesis by two different inhibitors gave inconsistent results, suggesting that inhibition of fatty acid synthesis itself has no influence on the AMPK-dependent boost of Nrf2 signalling.
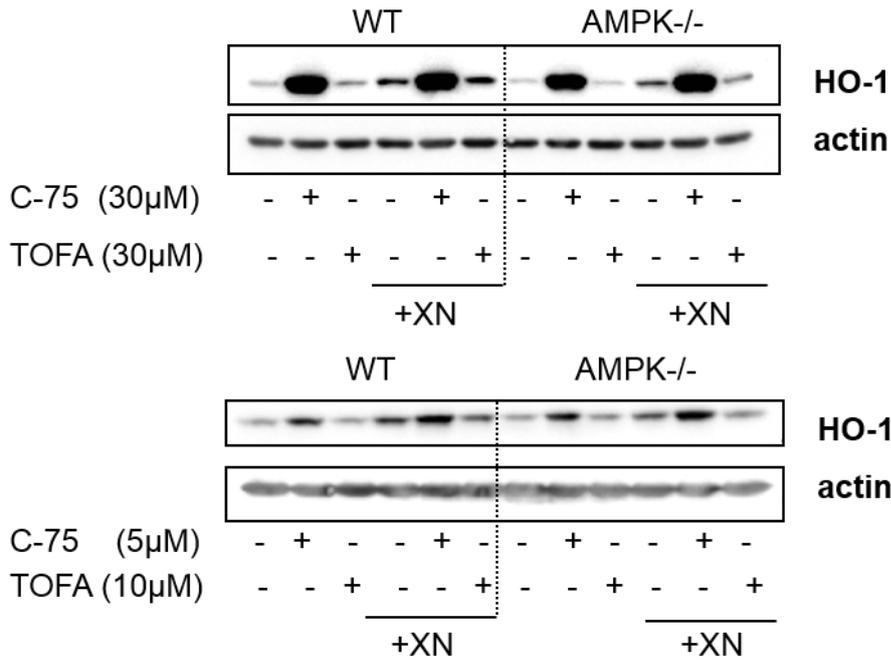
Figure 34: Influence of fatty acid synthesis on the induction of HO-1 by XN

WT and AMPK-/- cells were treated for 5 hours with vehicle, XN (5 μM), C-75 (5 and 30 μM) and TOFA (10 and 30 μM). Total cell lysates were subjected to immunoblot analyses for HO-1 and actin as loading control. Representative blots of two independent experiments are depicted.

2.7 ER stress

We found that treatment with XN causes mitochondrial stress and lowers the production of ATP (as shown under 1.2.2). In the absence of AMPK cells lack the ability to efficiently react to low energy conditions and this can result in different forms of cellular stress. ER stress is a condition that is caused by the accumulation of unfolded proteins within the ER. [201] Activation of AMPK has been shown to attenuate ER stress [126], while ER stress can both positively and negatively influence the activity of Nrf2: It can activate Nrf2 via a PERK-dependent phosphorylation that promotes the dissociation from Keap1 and the nuclear import, but it can also promote a Hrd1-dependent degradation of Nrf2. [46, 91]

2.7.1 The basal levels of ER stress are higher in AMPK-/- than WT cells

As activation of AMPK has been shown to prevent and reduce ER stress, the deletion of AMPK could potentially lead to higher ER stress levels. To investigate whether this is the case we compared the basal levels of ER stress in WT and AMPK-/- cells by detecting the protein levels

of the ER stress marker BiP. [202] We found that the BiP levels are higher in the AMPK-/- than in the WT cells (Fig 35), confirming that the deletion of AMPK causes an increase in the levels of ER stress.
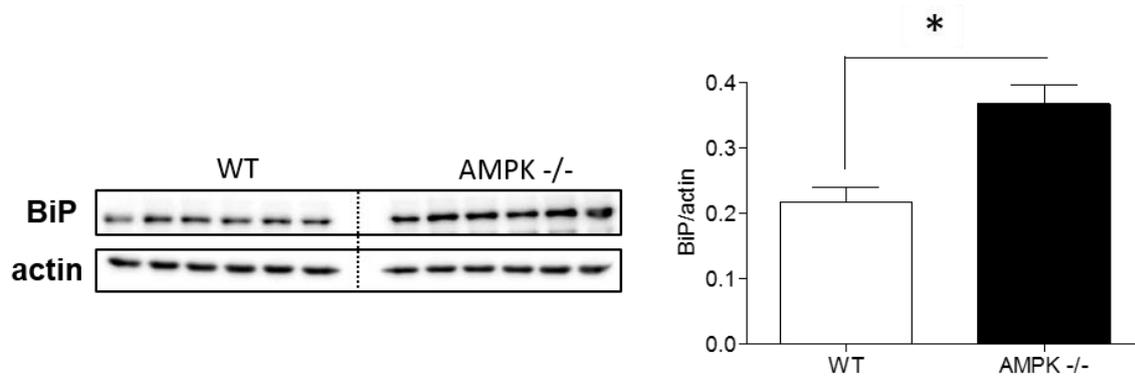
Figure 35: BiP levels in WT and AMPK-/- cells

Total cell lysates of WT and AMPK-/- cells were subjected to immunoblot analysis for BiP and actin as loading control. The graph depicts compiled data of six independently prepared protein extracts. (* $p < 0.05$; Student´s t-test (WT vs AMPK-/-)). Data were generated and provided by Elke Heiß.

2.7.2 ER stress interferes with the upregulation of HO-1 by XN

The higher ER stress levels in the AMPK-/- cells could be responsible for the blunted Nrf2/HO-1 signalling. To test whether ER stress generally affects the Nrf2-dependent upregulation of HO-1 we treated the AMPK-/- and WT cells with two different ER stress inducers, tunicamycin and thapsigargin. Both significantly reduced the XN dependent HO-1 upregulation in the WT, but not in the AMPK-/- cells (Fig 36). ER stress interferes with the Nrf2-dependent upregulation of HO-1 by XN.
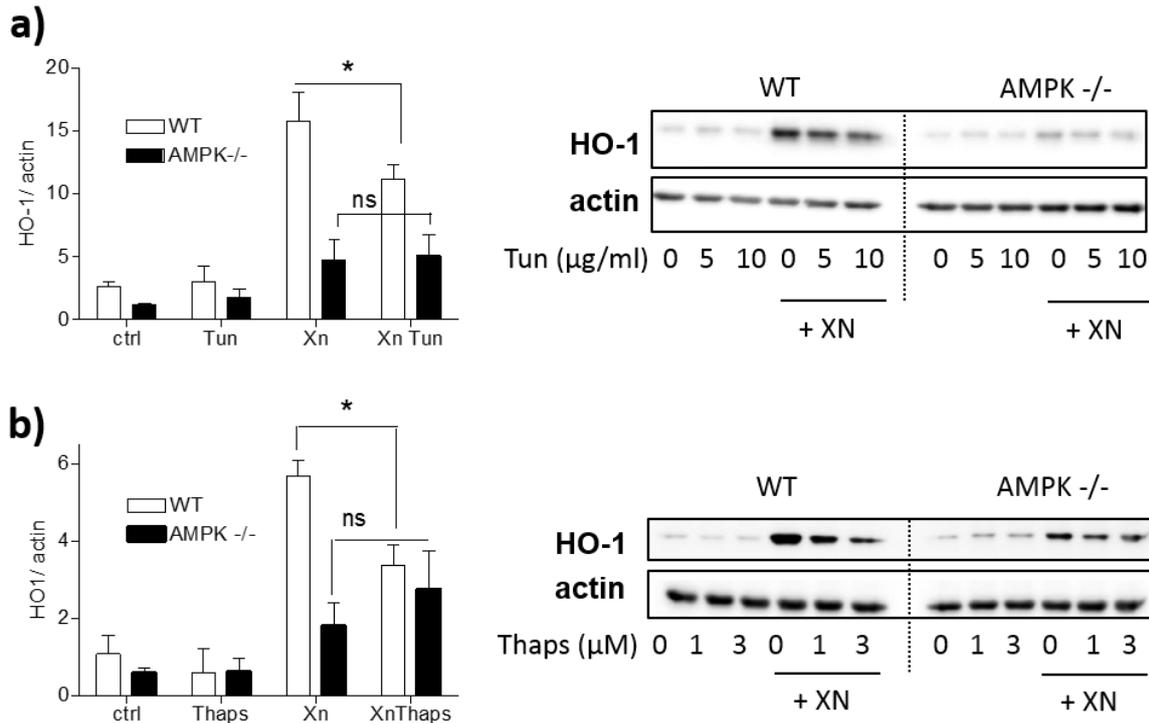
Figure 36: The effect of induced ER stress on the induction of HO-1 by XN

WT and AMPK-/- MEF were pretreated with the indicated concentrations of the ER stress inducers a）Thaps（thapsigargin 1 and 3 μM）and b）Tun（tunicamycin 5 and 10 μg/ml） for 30 minutes and then exposed to vehicle or XN（5 μM）for 6 hrs. Total cell lysates were subjected to immunoblot analysis for HO-1 and actin as loading control. Representative blots are depicted and the graphs shows compiled densitometric data of three independent experiments（using Thaps at 3 μM and Tun at 5 μg/mL）.（mean + SD; ＊ p<0.05; ANOVA）. Data were generated and provided by Elke Heiß.

2.7.3 Alleviation of ER stress rescues the upregulation of HO-1 by XN in AMPK-/- cells

ER stress impairs the upregulation of HO-1 by XN in a comparable way to the inhibition of AMPK. To test if the higher ER stress levels in the AMPK-/- cells affect the upregulation of HO-1 by XN, we treated the WT and AMPK-/- cells with phenylbutyrate, an alleviator of ER stress. Treatment with phenylbutyrate completely restored the HO-1 expression in the AMPK-/- cells, while not influencing it in the WT cells (Fig 37). The higher ER stress levels in the AMPK-/- cells interfere with the protein expression of HO-1 upon XN exposure.
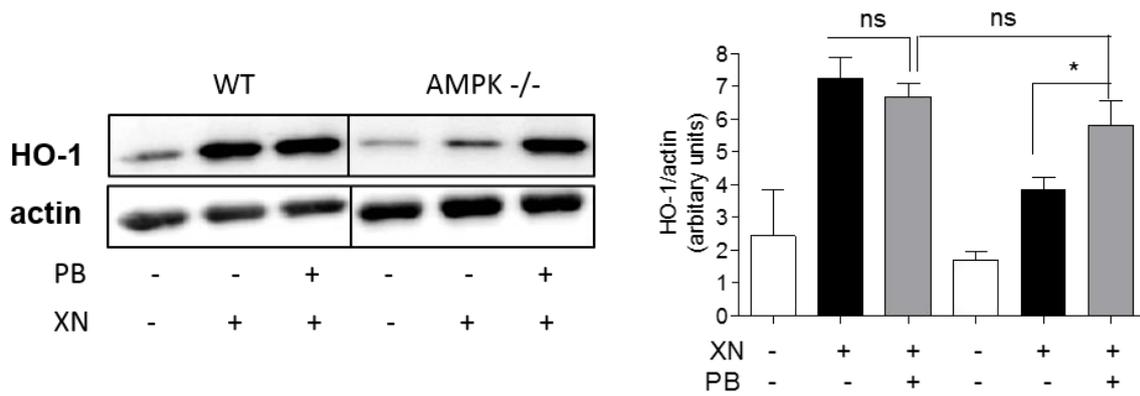
Figure 37: The effect of alleviating ER stress on the induction of HO-1 by XN

WT and AMPK-/- MEF were treated with phenylbuyrate (10 mM), an ER stress inhibitor, and then exposed to vehicle or XN (5 µM) as indicated before total cell lysates were subjected to immunoblot analysis for HO1 and actin as loading control. Representative blots are depicted and the graph shows compiled densitometric data of three independent experiments (mean + SD; * $p<0.05$; ANOVA). Data were generated and provided by Elke Heiß.

D. Discussion

1. AMPK boosts the activation of the Nrf2/HO-1 axis by XN

1.1 Xanthohumol activates both Nrf2- and AMPK signalling

a) XN activates Nrf2 via Keap1

It has been shown in different cell lines that Xanthohumol activates the Nrf2 dependent gene transcription [158, 160, 162]. To verify this we transfected CHO cells with an ARE-promoter-driven reporter gene. Treatment with XN increased the expression of the reporter gene confirming that it leads to Nrf2 dependent gene transcription. As a molecular endogenous readout for the activation of Nrf2 by XN we detected the upregulation of the HO-1 protein, since it is a well described and highly inducible target of Nrf2 [171]. We found that in MEF cells XN induces the protein in a strictly Nrf2 dependent manner: The upregulation was completely gone in Nrf2-/- cells, not only on the protein but also on the mRNA level. The canonical activation of Nrf2 is achieved via modifications of the inhibitor Keap1 that prevent it from binding Nrf2 and targeting it for degradation. But Nrf2 can also be degraded in a Keap1 independent manner via ßTrCP and different factors influence its activity apart from degradation [203]. We tested how Keap1 affects the upregulation of HO-1 by XN. Deletion of Keap1 has been shown to increase both the total levels of Nrf2 and the expression of the Nrf2 target gene NQO1 in MEF cells [35]. However we saw that the basal HO-1 level was not increased in Keap1-/- MEF cells and the same has been reported for the liver of Keap1-/- mice [204]. Higher amounts of Nrf2 do not lead to a higher level of HO-1 in the absence of Keap1. Furthermore we saw that the XN dependent HO-1 upregulation is abolished in the absence of Keap1: In the Keap1-/- cells XN could not upregulate the HO-1 protein and it induced only a minor increase of HO-1 mRNA compared to WT cells. The induction of HO-1 appears to be dependent on a dynamic regulation of the Nrf2 levels by Keap1. There must be a feedback mechanism that prevents the upregulation of HO-1 upon constant activation of Nrf2. Bach1, a transcriptional repressor of HO-1, could be involved: It interferes with the transcription of HO-1 by binding to the promoter [205]. In keratinocytes overexpression of Bach1 has been shown to abolish the induction HO-1 by H_2O_2 [206]. Bach1 could be upregulated in the Keap1-/- cells to prevent the activation of HO-1 by the constantly high levels of Nrf2.

b) XN activates AMPK via LKB1

Supplementation with Xanthohumol has been shown to activate AMPK in ApoE-/- mice: It increases both the activating phosphorylation of AMPK at Thr172 and the AMPK-dependent inhibitory phosphorylation of ACC in the liver. [157] Also in our MEF cells treatment with XN increased the phosphorylation of both AMPK and ACC, confirming the activation of AMPK by XN. LKB1 and CaMKKß are the two main kinases that phosphorylate and activate AMPK. They respond to different stimuli: LKB1 is necessary for the AMP-dependent activation of AMPK while CaMKKβ activates AMPK upon increases in intracellular Ca2+ [207]. Loss of LKB1 in lung adenocarcinomas has been shown to lead to a higher activity of Nrf2, suggesting a potential crosstalk [208]. We had a look at the AMPK and ACC phosphorylation upon treatment with XN in LKB1-/- cells and found that both were gone. LKB1 operates upstream of AMPK in the response to XN by promoting the activating phosphorylation of AMPK at Thr172.

c) XN impairs the respiration and generates ROS in the mitochondria

As discussed above treatment with Xanthohumol activates both Nrf2 and AMPK signalling in MEF cells. Interference of XN with mitochondrial function could be a potential explanation. The mitochondria are a major cellular source of both energy and ROS [209]. XN could be activating AMPK by interfering with the mitochondrial respiration and ATP production while activating Nrf2 by increasing the mitochondrial ROS production. In myocytes and preadipocytes it has been shown that low concentrations of XN (5 and 8 µM) promote mitochondrial uncoupling while high concentrations (25 and 70 µM) decrease the respiration via ROS; in hepatocytes already the low concentrations inhibited the respiration. [165]. Based on these findings it seems that low concentrations of XN uncouple oxygen consumption from ATP production while high concentrations inhibit the mitochondrial electron flow. The sensitivity to XN varies between cell lines. We found that treatment with 5 µM XN impairs mitochondrial function in MEF cells: It did not promote mitochondrial uncoupling but it lowered the basal, total, spare and ATP-synthesis-related oxygen consumption. XN inhibited the respiration in MEF cells even though the used concentration of XN is rather low and not cytotoxic upon 24 hrs treatment. It appears that MEF cells are rather sensitive to treatment with XN. The respiration could be affected by an XN-dependent increase in mitochondrial ROS production: In the study mentioned above antioxidant pre-treatment prevented the inhibition of respiration by XN and in cancer cell lines XN has been reported to transiently increase the formation of superoxide anion radical in the mitochondria [166]. We found that treatment

100

with XN increases the mitochondrial ROS in MEF cells and it does so to a comparable extent in WT and AMPK -/- cells (data not shown). It is likely that through the ROS generation XN affects the general function of the mitochondria, thus lowering the ATP production and activating AMPK, while at the same time the higher ROS level activates Nrf2 (in addition to the XN-mediated Keap1 modification).

1.2 In the absence of AMPK the Nrf2 dependent upregulation of HO-1 is impaired

a) AMPK boosts the activity of the Nrf2/HO-1 signalling axis

AMPK has been shown to positively influence the activity of Nrf2. Genetic or pharmacologic inhibition of AMPK interferes with the accumulation of Nrf2 and the Nrf2-dependent expression of HO-1 induced by different compounds [135, 136, 138, 140, 210]. We found that AMPK also takes part in the XN-dependent upregulation of HO-1: XN could still induce HO-1 in the absence of AMPK and the time course of the induction was comparable to the one seen in WT cells. But the total HO-1 protein levels were significantly lower. The deletion of AMPK leads to a blunted expression of HO-1. Moreover, it affects the induction of HO-1 already on the mRNA level: As for the protein, XN could still upregulate the HO-1 mRNA in AMPK-/- cells but the total levels were lower than in the WT. Pharmacologic inhibition of AMPK with compound C confirmed the effects of AMPK activity on the upregulation of HO-1 mRNA and protein. These results show that while AMPK is not strictly necessary for the induction of HO-1, it boosts it both on the mRNA and the protein level. The kinase LKB1 that activates AMPK takes part in this effect: We found that also in LKB1-/- cells the upregulation of the HO-1 protein by XN is blunted, just as in the AMPK-/- cells. Treatment with an inhibitor of the CAMKKß that also operates upstream of AMPK did not significantly affect the upregulation of HO-1 by XN in the WT, further supporting the central role of LKB1 in the XN-dependent activation of AMPK. Both AMPK and LKB1 crosstalk with the Nrf2 dependent signalling. The fact that when AMPK is activated via LKB1 it vigorously enhances the Nrf2 dependent signalling might be a mechanism to ensure that under conditions of energy stress the cell is better protected against oxidative insults. AMPK is likely to influence the upregulation of HO-1 on the transcriptional level: Inhibition or deletion of AMPK reduces the XN-dependent increases in HO-1 protein and mRNA, while the stability of the mRNA and the protein are comparable in WT and AMPK-/- cells (data from Elke Heiß). The easiest explanation for the

effect of AMPK on Nrf2 signalling would be that it increases the Nrf2-dependent gene transcription by increasing the total or nuclear Nrf2 levels.

b) AMPK does not influence the total level or nuclear accumulation of Nrf2

AMPK boosts the induction of HO-1 both on the protein and the mRNA level. It could be promoting the Nrf2 dependent gene transcription by increasing the total amount or the nuclear import of the transcription factor. The deletion of AMPK does not influence the total amount of Nrf2 in MEF cells: We saw that the total level of Nrf2 that accumulates upon treatment with XN is comparable in AMPK-/- and WT cells. Furthermore we found that the deletion of AMPK does not affect the nuclear accumulation of Nrf2: Treatment with XN promoted a comparable increase of nuclear Nrf2 in the AMPK-/- and WT cells. The Nrf2 levels even appeared to be slightly higher in the AMPK-/ cells. In the absence of AMPK neither the total level nor the XN-dependent nuclear accumulation of Nrf2 are affected. The AMPK does not stabilize Nrf2 nor does it increase its nuclear localization in response to treatment with XN. These findings contradict the observations made by other groups: Treatment with the AMPK inhibitor compound C has been shown to reduce the nuclear localization of Nrf2 and expression of HO-1 induced by treatment with thymoquinone, berberine and dehydrodiconiferyl alcohol [135, 136, 140]. Compound C itself has been shown to activate Nrf2 and upregulate HO-1 [211]; it could have an AMPK-independent effect on the nuclear import of Nrf2. In our study AMPK boosted the Nrf2 dependent signalling without obviously influencing the nuclear amounts of the transcription factor. As it does not increase the Nrf2-dependent gene transcription by increasing the level of Nrf2, AMPK could instead be regulating factors that synergize or antagonize the transcriptional and/or DNA binding activity of Nrf2.

2. Investigating the contribution of different factors to the effect of AMPK on Nrf2/HO-1

AMPK positively influences the Nrf2 dependent upregulation of HO-1 by XN and as discussed above it does not do so by increasing the total or nuclear level of Nrf2. There is a number of different ways through which AMPK could positively influence the activity of Nrf2 instead. Nrf2 is subject to posttranslational modifications: Phosphorylation of Nrf2 by different kinases

and cellular pathways can regulate its activity and acetylation of Nrf2 influences the Nrf2 dependent gene expression [99, 203]. High levels of the tumour suppressor p53 can prevent the Nrf2 dependent gene transactivation by competing for the promoter binding site [92, 93]. The maintenance of the NADPH levels, which Nrf2 influences by regulating the PPP (pentose phosphate pathway) and fatty acid synthesis, is important for the Nrf2-dependent gene expression [31, 33]. Furthermore, ER stress has been shown to both positively and negatively influence the activity of Nrf2 [46, 89]. The potential contribution of all these factors to the effect of AMPK on Nrf2 signalling will be discussed in the following sections.

2.1 Cellular pathways and kinases that regulate Nrf2

a) PKC, PKA and the MAPK pathway are not involved

The **PKC** is a very well described activator of Nrf2 [66]. The PKC-delta isoform phosphorylates it on Ser40 and thus promotes the release from the inhibitor Keap1 [67]. To test a possible role of PKC in the crosstalk between Nrf2 and AMPK we inhibited the PKC with two different compounds. Treatment with GÖ 6983, a selective inhibitor of several PKC isozymes [212], slightly lowered the expression of HO-1 by XN both in WT and AMPK-/- cells and thus in an AMPK-independent manner. This is in agreement with the fact that PKC positively regulates Nrf2. However, treatment with the PKC-delta inhibitor rottlerin upregulated HO-1 and rottlerin also boosted the XN dependent HO-1 upregulation both in WT and AMPK-/- cells. Rottlerin is a mitochondrial uncoupler [184]. Though traditionally reported as an inhibitor of the PKC-delta, it now appears that this effect is not very strong and rather a side activity [213]. Since PKC positively regulates Nrf2, rottlerin should reduce the HO-1 expression, but it appears that the ROS generated by the uncoupling override this effect by strongly activating Nrf2. Together with the minor effect of GÖ 6983 on the expression of HO-1 this indicates that PKC does not play an important role for the upregulation of HO-1 by XN and is not responsible for the positive effect of AMPK on the Nrf2-dependent signalling.

The **PKA** is activated by cAMP and it promotes lipolysis and gluconeogenesis. It has been shown to activate Nrf2 in the liver of mice under conditions of fasting [86]. Both PKA and AMPK can activate the NAD(+)-dependent deacetylase SIRT1 [214, 215], that the PKA regulates via phosphorylation [88]. SIRT1 can negatively regulate the Nrf2-dependent gene transcription by de-acetylating Nrf2 [99] and the PKA could take part in the effect of AMPK on Nrf2 by modulating the activity of SIRT1. However treatment with two different inhibitors of PKA, KT

5720 and Rp-8-Br-cAMPS, did not influence the upregulation of HO-1 by XN at all. Most likely PKA influences the activity of Nrf2 only under nutrient starvation conditions. It takes no part in the effect of AMPK on HO-1 upregulation in our experimental setup.

The **MAPK** pathway is a signalling cascade comprising several kinase families, the three major ones being JNK, p38 and ERK. AMPK has been shown to activate p38 and JNK [216, 217] and to antagonize ERK [218]. The MAPKs can directly phosphorylate Nrf2, but this has been shown to have only a minor effect on its activity [82]. In our hand treatment with inhibitors of ERK (U0126 and PD98059), p38 (SB203580 and SB202190) and JNK (SP600125) did not significantly affect the upregulation of HO-1 by XN, confirming that they play a minor role for Nrf2 activity.

b) PI3K/Akt positively regulates and is in turn regulated by Nrf2

The PI3K/Akt pathway plays an important role for regulating the cell cycle, cellular growth and proliferation. Activation of the pathway has been shown to take part in the Nrf2 dependent upregulation of HO-1 by different compounds [112, 219, 220]. Also in the case of XN the PI3K/Akt positively influences the upregulation of HO-1: Inhibition of PI3K with Wortmannin affected the upregulation of HO-1 on the protein and the mRNA level both in WT and AMPK-/- cells. PI3K activates Akt that in turn activates mTORC1 and inhibits GSK3 [221], a kinase known to phosphorylate Nrf2 [40]. The roles of mTORC1 and GSK3 will be separately discussed below. Since PI3K/Akt positively regulates Nrf2 we wanted to know if the activation of the pathway is affected in the absence of AMPK. To test this we treated the cells with a small compound, YS-49, that has been reported to activate PI3K and Akt [188]. Treatment with YS-49 promoted the activating phosphorylation of Akt at Ser473 in WT cells. In AMPK-/- cells the phosphorylation of Akt by YS-49 was impaired. Interestingly this recalls the pattern seen for the induction of HO-1 by XN in the AMPK-/- cells. The deletion of AMPK impairs both the activation of the PI3K/Akt pathway and the Nrf2 dependent signalling, suggesting that a reduced PI3K/Akt signalling in the absence of AMPK might be at least partly responsible for the blunted blunted Nrf2-signalling. To test this hypothesis it would be interesting to see how exogenous expression of a constitutively active Akt affects the HO-1 expression in WT and AMPK-/- cells. A further layer of complexity is added by the fact that Nrf2 itself has been reported to regulate PI3K: In human melanocytes overexpression of Nrf2 markedly activates the PI3K/Akt signalling [190]. We treated Nrf2-/- cells with YS-49, and found that the phosphorylation of Akt on Ser473 that it promotes in WT cells was completely abolished: Nrf2

is necessary for the phosphorylation of Akt at Ser473 and thus influences its activity. The PI3K/Akt pathway positively regulates Nrf2 and is in turn subject to a positive regulation by Nrf2.

c) GSK3ß negatively regulates Nrf2

GSK3ß phosphorylates Nrf2 on two serine residues thus targeting it for a Keap1 independent degradation via the ubiquitin ligase adaptor β-TrCP [40]. In addition GSK3ß also phosphorylates the Fyn kinase. This leads to the migration of Fyn into the nucleus where it promotes the nuclear export and degradation of Nrf2 by phosphorylating it at Tyr568 [75]. Although we did not see altered stability or nuclear localization of Nrf2 between WT and AMPK-/- cells, GSK3β may still be involved in our observed AMPK-boost of Nrf2. The Akt pathway negatively regulates GSK3ß via a phosphorylation at Ser9 that prevents the binding of GSK3ß to substrates bearing a priming phosphate [222]. Furthermore, AMPK and GSK3ß appear to crosstalk in an Akt dependent manner: GSK3 inhibits the catabolic action of AMPK via a phosphorylation that requires the PI3K-Akt signalling and is independent of the phosphorylation state of GSK3ß at Ser9 [125]. Activation of AMPK with AICAR increases the inhibitory phosphorylation of GSK3ß at Ser9 and this is prevented by an inhibitor of Akt [223]. The reported informations suggest a close interrelationship between AMPK, Akt, GSK3ß and Nrf2 signalling. It is therefore conceivable that AMPK could be positively influencing the activity of Nrf2 via increased Akt activity and/or decreased GSK3ß activity. We saw that treatment with XN increases the inhibitory Ser9 phosphorylation of GSK3ß. Furthermore, inhibition of GSK3ß with the selective GSK3 inhibitor CHIR 99021 [224] increased the upregulation of HO-1 by XN both in WT and AMPK-/- cells. But the total protein levels of HO-1 were still lower in the AMPK-/- cells. On the RNA level inhibition of GSK3ß boosted the upregulation of HO-1 in WT cells but could not rescue the strongly affected HO-1 upregulation in the AMPK-/- cells. These results confirm that GSK3ß negatively regulates Nrf2. They also show that GSK3ß may be involved in the Nrf2-AMPK crosstalk, but it does not play a central role for the blunted Nrf2/HO-1 signal in AMPK-/- cells.

d) mTORC1 positively regulates Nrf2

The mTOR complex 1 (mTORC1) plays a central role in controlling cellular growth: It promotes anabolic and inhibits catabolic processes, thus acting as a switch between cellular proliferation

and quiescence. AMPK negatively regulates mTORC1 [190]. Nrf2 also crosstalks with mTOR: In C. elegans genetic deletion of TORC1 leads to the protective activation of genes via the Nrf2 orthologue SKN-1, that also upregulates genes of the TORC1 pathway itself [180]. Transfecting cells with a 'gain of function' mutant Nrf2, as it occurs in a wide range of human cancers, promotes proliferation and does so by activating mTOR: Treatment with rapamycin prevents the proliferation [225]. Taken together these data suggest that Nrf2 and TORC1 operate in a feedback system where TORC1 negatively influences the activity Nrf2 that in turn positively regulates the expression of TORC1 itself. AMPK inhibits mTORC1 by phosphorylating its binding partner raptor, and we found that in the AMPK-/- cells this phosphorylation is largely gone. To test if mTORC1 is involved in the effect of AMPK on the Nrf2 dependent HO-1 expression we inhibited mTORC1 with rapamycin. Treatment with rapamycin blunted the XN dependent HO-1 upregulation both in WT and AMPK-/- cells. An over activation of mTORC1 in the absence of AMPK is obviously not the reason for the impaired Nrf2/HO-1 signalling. Protein synthesis is reduced upon inhibition of mTORC1 and this could explain why treatment with rapamycin further lowers the induction of HO-1 protein by XN. In our system, the mTORC1 plays an overall positive role for the Nrf2 dependent signalling and HO-1 protein expression. This finding is in agreement with two studies reporting that inhibition of mTOR with temsirolimus prevents the nuclear translocation of Nrf2 [226] and inhibition with rapamycin decreases the total cellular Nrf2 levels [227]. The positive role of mTOR also fits with the fact that the PI3K pathway, that activates mTOR via Akt, positively affects Nrf2 signalling.

e) Autophagy is not involved

Together AMPK and mTOR regulate the recycling of cellular material via autophagy: AMPK directly promotes autophagy by phosphorylating the autophagy inducer Ulk1 and by inhibiting mTORC1 that in turn negatively regulates autophagy [116]. The activity of Nrf2 can be regulated via an autophagy-dependent mechanism: The autophagy adaptor p62 directly binds the Nrf2 inhibitor Keap1 and sequesters it into aggregates that are then subjected to autophagic degradation, thus activating Nrf2 [193]. Moreover, p62 is a target gene of Nrf2 and thus there is a positive feedback regulation between p62, autophagy and Nrf2 accumulation. The role of mTOR signalling in this setting is complex: while mTORC1 is a general inhibitor of autophagic degradation, phosphorylation of p62 by mTORC1 greatly increases the affinity of p62 for Keap1 [55]. The deletion of AMPK could affect the Nrf2-dependent signalling by

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causing a general decrease in autophagy that interferes with the p62-dependent activation of Nrf2. To test how autophagy affects the XN dependent induction of HO-1 we treated the cells with chloroquine, a compound that disrupts autophagy by preventing the acidification of the lysosomes. Treatment with chloroquine had no effect on the induction of the HO-1 protein either in WT or AMPK-/- cells. Under the tested conditions autophagy does not appear influence the activity of Nrf2. This is in line with the unaltered Nrf2 protein levels, which appear as main target of autophagy within the Nrf2 signalling pathway, between WT and AMPK-/- cells. However autophagy is a complex regulatory network. To completely exclude that it plays a role for the effect of AMPK on the Nrf2-dependent signalling it would be good to directly inhibit Ulk1, the kinase that AMPK phosphorylates to activate autophagy, and see how this affects the Nrf2-dependent signalling in WT and AMPK-/- cells.

f) PI3K/Akt, AMPK and Nrf2: The broader picture

The PI3K pathway positively influences the Nrf2 dependent signalling, since inhibition of PI3K blunts the upregulation of HO-1 by XN. Furthermore the PI3K signalling is affected in AMPK-/- cells and this could be a potential explanation for the blunted upregulation of HO-1. We tried to understand how PI3K activates Nrf2. Negative regulation of GSK3ß would be the easiest explanation, as it is an inhibitor of Nrf2 that is inhibited by Akt. [40, 222] But though inhibition of GSK3 could increase the upregulation of HO-1 in the AMPK-/- cells, it was not sufficient to significantly rescue the response. Akt positively regulates mTORC1 [228], an inhibitor of autophagy that is inhibited by AMPK [116, 194]. Inhibition of mTORC1 blunted the upregulation of HO-1, indicating a positive role for Nrf2 dependent signalling. Autophagy is not obviously involved in the signalling, since inhibiting it by chloroquine did not influence the upregulation of HO-1. Overall the data suggest that in the MEF cells the PI3K/Akt pathway may be promoting a dual activation of Nrf2 via inhibition of GSK3ß and positive regulation of mTORC1. How mTORC1 acts on Nrf2 remains yet to be understood: It may be doing so simply by generally promoting protein synthesis. Alternatively the fact that mTORC1 promotes the autophagic degradation of Keap1 via p62 may play a role, though we saw that inhibition of autophagy with chloroquine does not affect the Nrf2-dependent signalling. It would be interesting to test whether upon siRNA mediated knockdown of p62 treatment with rapamycin can still blunt the XN-dependent upregulation of HO-1. Moreover, direct phosphorylation and transcriptional activation of Nrf2 by mTORC1 cannot be excluded.
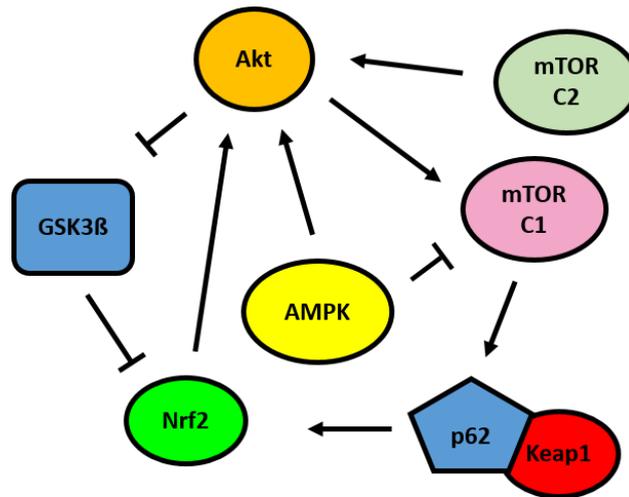
Figure 38: The connections between Akt, AMPK and Nrf2

Akt activates Nrf2 by inhibiting GSK3ß that promotes the degradation of Nrf2. At the same time Akt activates mTORC1, while AMPK inhibits it. mTORC1 phosphorylates p62 and thus increases its binding affinity for the Keap1, the inhibitor of Nrf2. p62 targets Keap1 for autophagic degradation and thus activates Nrf2. Nrf2 is required for the activating phosphorylation of Akt at Ser473 and AMPK also influences this phosphorylation. mTORC2 directly phosphorylates Akt at Ser473.

What remains to be explained is the precise role of AMPK herein. The whole picture gets even more complicated by the fact that Nrf2 itself positively influences the activity of the PI3K/Akt pathway: We saw that in Nrf2-/- cells the activating phosphorylation of Akt at Ser473 is completely abolished. Interestingly the same phosphorylation was blunted in AMPK-/- cells. There could be a feedback mechanism between Nrf2 and Akt in which AMPK takes part. Nrf2 synergizes with Akt and mTOR in favouring cellular proliferation [32, 225], a process that AMPK counteracts [229]. But both AMPK and Nrf2 require glucose, for generation of ATP via glycolysis and NADPH production via the PPP respectively [33, 230], and Akt mediates the insulin dependent glucose uptake [231]. Activation of AMPK has been shown to increase the responsiveness to insulin and to increase the phosphorylation of Akt at Ser473 while at the same time inhibiting mTOR [232]. Perhaps this could explain how AMPK influences the activity of Nrf2: Under energy stress conditions it becomes active and positively regulates PI3K/Akt to promote glucose uptake, thus also indirectly activating Nrf2. The mTOR complex 2 (mTORC2) has been shown to phosphorylate Akt at Ser473 and could thus be involved in this potential mechanism. [233, 234]. If this is the case, then treatment with mTORC2 inhibitors should

impair the Nrf2-response in WT cells while expressing a constitutively active Akt should restore a normal response in the AMPK-/- cells.

2.2 The tumour suppressor p53

The tumour suppressor p53 is activated by cellular stress and it displays a dual regulation of Nrf2: Low levels of p53 activate Nrf2 while high levels suppress the Nrf2 dependent gene expression [92]. p53 negatively regulates the expression of Nrf2 target genes by competing with Nrf2 for the binding to the ARE sequence in the promoter [93]. AMPK positively regulates p53: It can induce a cell-cycle arrest by phosphorylating p53 at Ser15 [127] and under glucose starvation it promotes apoptosis by stabilizing p53 and phosphorylating it at Ser46 [192]. We found that in AMPK-/- cells the total level of p53 is significantly higher. It seems that the inability of the cells to respond to energy stress in the absence of AMPK causes cellular stress that promotes the accumulation of p53. It is likely that p53 contributes to the impaired Nrf2 signalling in the AMPK-/- cells by preventing Nrf2 from binding to the ARE. To test this the amount of p53 associated to the ARE in promoters of Nrf2-regulated genes could be compared in WT and AMPK-/- cells by chromatin immunoprecipitation.

2.3 Acetylation

Inside the nucleus Nrf2 is subject to acetylation events that regulate its activity and influence its binding to target promoters. CBP acetylates several lysine residues within the DNA-binding Neh1 domain of Nrf2. Mutations in these residues affect the DNA-binding activity of Nrf2 [82], while the CBP dependent acetylation has been shown to increase the target gene promoter binding and the transcriptional activity of Nrf2 [99]. Deacetylation of Nrf2 by SIRT1 negatively influences the Nrf2 dependent gene transcription [99]. AMPK crosstalks with SIRT1 [235] and can thus influence protein acetylation: Under conditions of metabolic stress AMPK promotes the acetylation of p53 by inhibiting SIRT1 [235], but it can also enhance the activity of SIRT1 by increasing cellular NAD+ levels [215]. To investigate how the deletion of AMPK affects the protein acetylation we detected the acetylated lysine residues in total cellular protein extracts from untreated WT and AMPK-/- cells. In the AMPK-/- cells a prominent band stood out just over the 50 kDa marker that was much weaker in the WT. Since acetylation mainly takes place in the nucleus, we also detected the acetylated lysine residues in nuclear extracts after treatment with XN. Again acetylation levels were higher in the AMPK-/- cells and the band just over the 50 kDa marker stood out. Treatment with XN did not change this pattern. The band

above the 50 kDa marker could correspond to p53: We found that the cellular p53 levels are higher in AMPK-/- than WT cells and acetylation is necessary for the activation of p53 in response to stress [236]. It is interesting that in AMPK-/- cells, where the activity of Nrf2 is impaired, the total acetylation levels are higher than in the WT cells. This stands in contrast to the outcome of acetylation of Nrf2 itself. As already mentioned above generally acetylation increases while deacetylation reduces the activity of Nrf2: p300/CBP promotes the transcriptional activity of Nrf2 by acetylating it, while NF-κB has been shown to repress the transcription of Nrf2 dependent genes by depriving CBP from the ARE [185]. In RAW 264.7 cells and tumour cell lines increasing acetylation by inhibiting histone deacetylases promotes Nrf2 activation [101, 237]. Deacetylation of Nrf2 by SIRT1 decreases the activity of Nrf2 [99] and decreased histone acetylation goes along with an impaired Nrf2 response in astrocytes that is rescued by treatment with the HDAC inhibitor TSA [100]. Only one publication reports a negative influence of acetylation on Nrf2 activity: The HDAC inhibitor TSA increased the acetylation and thus negatively affected the stability of Nrf2 in bronchial epithelial cells, and this was confirmed in mice [102]. It is possible that the total acetylation levels do not reflect the acetylation status of Nrf2. We tried to immunoprecipitate endogenous Nrf2 to gain further insight, but two different Nrf2 antibodies failed to precipitate the protein. To further test how general protein acetylation affects the Nrf2 dependent signalling in our model we treated the cells with two different deacetylase inhibitors: The general HDAC inhibitor TSA and a specific inhibitor of SIRT1. Increasing the acetylation status negatively affected the expression of HO-1. Treatment with TSA slightly lowered the XN dependent HO-1 expression both in WT and AMPK-/- cells, inhibition of SIRT1 only in the AMPK-/- cells. Decreasing acetylation by inhibiting the acetylase p300/CBP with C646 had the opposite effect: C646 alone already strongly upregulated HO-1 both in WT and AMPK-/- cells. It also boosted the XN-dependent upregulation of HO-1, but at low concentrations of C646 the response was weaker in the AMPK-/- than WT cells. Based on these results we may conclude that p300/CBP-dependent acetylation reduces while deacetylation promotes the upregulation of Nrf2/HO-1 signalling by XN. What has to be considered, though, is that HO-1 could be the wrong target to unambiguously study the influence of acetylation on Nrf2 signalling. One study reports that out of several analysed Nrf2 target genes HO-1 was the exception in being the only one that was not regulated via acetylation [82]. To confirm the effects of acetylation on the Nrf2 signalling pathway it would be good to repeat the study with several different Nrf2 target

genes, to specify which proteins are susceptible to the AMPK-mediated decrease in acetylation. As a strategy to overcome the problems encountered in immunoprecipitating the Nrf2 protein and study the acetylation pattern of Nrf2 itself in WT and AMPK-/- cells could be the overexpression of a tagged version of the Nrf2 protein.

2.4 Fat metabolism and NADPH

The regulation of fat metabolism and NADPH homeostasis are processes that both Nrf2 and AMPK influence. Nrf2 down regulates genes involved in fat and cholesterol synthesis, anabolic processes that consume NADPH [181]. At the same time it upregulates genes of the pentose phosphate pathway (PPP), the main cellular source of NADPH [32]. AMPK directly prevents the synthesis of fatty acids by inhibiting ACC, and instead promotes the beta oxidation of fatty acids that can indirectly contribute to raising the cellular NADPH levels [122]. Thus AMPK raises the cellular NADPH level in a short term, transient fashion, while Nrf2 raises it in a slower and more stable way. These partially overlapping actions could mean that upon the occurrence of redox stress AMPK is necessary for maintaining the cellular NADPH level until Nrf2 works the slower metabolic adjustments. Cellular NADPH levels play an important role for the activity of Nrf2: Lowering them through inhibition of the PPP interferes with the Nrf2 dependent gene expression [33]. We investigated whether fatty acid metabolism or NADPH levels constitute the interface of the AMPK/Nrf2 crosstalk.

a) The NADPH level and fatty acid oxidation

AMPK inhibits the synthesis of fatty acid and favours their breakdown via mitochondrial beta oxidation. Through this dual action it raises the cellular NADPH level [122]. By inhibiting the ACC it prevents the accumulation of malonyl-CoA, a negative regulator of fatty acid oxidation that prevents the uptake of fatty acids into the mitochondria by inhibiting CPT1 (Carnitine palmitoyltransferase I). To test the importance of fatty acid oxidation for the upregulation of HO-1 by XN we inhibited it by treating the cells with etomoxir, an irreversible inhibitor of CPT1. Etomoxir alone had no effect on the upregulation of HO-1, but it slightly boosted the XN-dependent upregulation both in the WT and AMPK-/- cells. Since AMPK promotes fatty acid oxidation the opposite effect was to be expected. The generation of ROS is the most likely explanation for the positive effect of etomoxir on HO-1 expression: In glioblastoma cells etomoxir has been shown to impair the NADPH production and promote ROS generation [199] and in HepG2 cells to generate oxidative stress and thus upregulate HO-1 [238]. To further

test the influence of cellular NADPH levels on the expression of HO-1 by XN we supplied the cells with a NADPH regenerating system. If low NADPH levels impair the response to XN in the AMPK-/- cells this should rescue it. The exogenous NADPH could not increase the expression of HO-1 either in the WT or the AMPK-/-cells. Together with the response to etomoxir this indicates that the AMPK does not exert a positive influence on the Nrf2 dependent HO-1 expression by raising the NADPH levels or boosting fatty acid oxidation.

b) Fatty acid synthesis

AMPK negatively regulates the synthesis of fatty acids by inhibiting ACC. To mimic the inhibitory effect of AMPK we treated the cells with TOFA, an inhibitor of ACC. TOFA showed no effect on the XN dependent HO-1 up regulation either in the WT or the AMPK-/- cells. We also inhibited the fatty acid synthesis downstream of ACC by treating the cells with C-75, an inhibitor of FAS. C-75 alone already induced a strong upregulation of HO-1 both in the WT and AMPK-/- cells and it also boosted the XN-dependent upregulation. A likely explanation could be that C-75 generates metabolic stress: Inhibition of FAS leads to the accumulation of the cytotoxic substrate malonyl-coA [239] that can trigger the Nrf2-dependent cellular stress response. What makes the role of C-75 even more complex is that it inhibits the fatty acid synthesis and at the same time it activates fatty acid oxidation by activating CPT1 [240]. AMPK also has this dual action. Thus it would be interesting to test whether the combination of the two actions can upregulate the Nrf2/HO-1 axis. The inhibition of fat synthesis alone has no effect on the Nrf2 dependent HO-1 expression.

2.5 ER stress

ER stress has been reported to influence the activity of Nrf2 both in a positive and in a negative way: PERK is activated by ER stress and directly phosphorylates Nrf2, leading to the dissociation from Keap1 and thus promoting the Nrf2-dependent gene transcription [91]. However ER stress can also promote the degradation of Nrf2 via the E3 ubiquitin ligase Hrd1 [46]. The effect of AMPK on ER stress is less ambiguous: Activation of AMPK has been shown to protect against ER stress induced by different factors and in different cell lines. Among others, it protects HK-2 cells against the effect of chemical ER stress inducers, it attenuates hypoxia induced ER stress in cardiomyocytes and it reduces palmitate induced ER stress in retinal pericytes [126, 241, 242]. Since activation of AMPK alleviates the higher ER stress levels caused by different forms of cellular stress it is possible that AMPK also influences the basal

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ER stress level. In human and mouse endothelial cells deficient in AMPKα2 the ER stress levels have been shown to be higher [182]. We found that also in MEF cells the deletion of AMPK (α1) lead to higher ER stress levels: The amounts of the ER stress marker BiP were higher in untreated AMPK-/- than WT cells. The higher basal levels of ER stress could be interfering with the upregulation of HO-1 by XN. To test this we induced ER stress with two different chemical inducers of ER stress, i.e. thapsigargin that interferes with ER calcium homeostasis and tunicamycin that interferes with proper protein glycosylation. We found that the induced ER stress blunted the XN-induced upregulation of the HO-1 protein in WT cells but did not further reduce it in the AMPK-/- cells, where already the basal ER stress levels are higher. Furthermore treatment with phenylbutyrate, an alleviator of ER stress, did not influence the upregulation of HO-1 by XN in the WT cells but restored a normal response in the AMPK-/- cells. ER stress blunts the upregulation of HO-1 by XN in WT cells, while alleviation of ER stress is sufficient to rescue it in AMPK-/- cells. These results show that ER stress interferes with the Nrf2 dependent signalling while AMPK boosts it by alleviating ER stress. The next step will be to find out how AMPK alleviates the ER stress, to understand how it influences the Nrf2/HO-1 axis. ER stress is caused by the accumulation of misfolded proteins in the ER and it activates the unfolded protein response (UPR) to restore ER homeostasis. The UPR attenuates protein translation, upregulates proteins required for protein folding and promotes the degradation of misfolded proteins. PERK, IRE1α and ATF6 are the three key players that orchestrate this response [243]. AMPK activation has been shown to be necessary for the activation of IRE1α and PERK by phenformin [244], making them likely targets, especially PERK since it is known to regulate Nrf2. The PI3K/Akt pathway positively influences the Nrf2 signalling and could also be involved in the AMPK-mediated alleviation of ER stress: Metformin protects NIT-1 cells against ER stress-induced apoptosis via activation of AMPK and PI3K [245]. ER stress has been shown to promote a GSK3β-dependent phosphorylation of mTORC2 that interferes with the activating phosphorylation of Akt by mTORC2 [246]. This phosphorylation is blunted in the AMPK-/- cells. Thus it could be that AMPK positively influences the activation of the PI3K/Akt signalling and indirectly of Nrf2 by preventing the ER stress-dependent phosphorylation of mTORC2.

Summary and Conclusions

Main findings

- XN activates both Nrf2 and AMPK signalling, the latter via LKB1. It impairs the respiration and promotes the generation of ROS in the mitochondria.

- AMPK boosts the Nrf2-dependent upregulation of HO-1 by XN both on the protein and mRNA level, but it does not increase the total level or nuclear accumulation of Nrf2.

- The PI3K/Akt pathway boosts the XN induced HO-1 upregulation and in AMPK-/- cells the blunted expression of HO-1 correlates with a blunted PI3K signalling. Nrf2 in turn positively regulates Akt.

- Degradation of Nrf2 via GSK3 is not sufficient to explain the reduced XN-dependent HO-1 expression in AMPK-/- cells.

- The levels of p53, a transcriptional repressor of Nrf2, are higher in AMPK-/- than WT cells.

- The ER stress levels are higher in AMPK-/- than WT cells. Alleviation of ER stress is sufficient to rescue the upregulation of HO-1 by XN in AMPK-/- cells.

Conclusions

The findings of this work clearly show that Nrf2 and AMPK crosstalk: AMPK boosts the Nrf2-dependent upregulation of HO-1 by XN in MEF cells both on the mRNA and protein level. Since AMPK does not affect the total level or nuclear import of Nrf2 (and does not influence the half-life of HO-1 mRNA and protein) it is very likely to favour the upregulation of HO-1 by increasing the promoter binding and/or the transcriptional activity of Nrf2. AMPK could achieve this by promoting posttranslational modifications of Nrf2 itself, or by regulating factors that influence the transcriptional activity of Nrf2. Due to problems with the immunoprecipitation of endogenous Nrf2 protein we could not directly compare the phosphorylation or acetylation status of Nrf2 in WT and AMPK-/- cells. Overexpression studies of a tagged Nrf2 version could overcome the antibody problem and bring further insights. Instead we tested the effect of different kinases that have been reported to regulate Nrf2 on the AMPK-Nrf2 crosstalk. These experiments made use of pharmacological inhibitors/activators which are well established but still bear the risk of unknown off-target action. Thus, knockdown and overexpression approaches could be considered for confirmation. We found that PKC, PKA and the MAPKs do not play a major role for the

crosstalk, while the PI3K/Akt pathway could be potentially involved, as it also boosts the XN-dependent HO-1 upregulation and is blunted in the AMPK-/- cells. Akt influences the activity of Nrf2 both via GSK3 and mTORC1. Nrf2 in turn positively regulates the mTORC2-dependent activating phosphorylation of Akt at Ser473, implying a positive feedback mechanism between Nrf2 and PI3K/Akt. Overexpression studies of constitutively active and dominant negative Akt protein in WT and AMPK-/- cells can clarify whether the PI3K pathway plays a role for the AMPK-Nrf2 crosstalk. Increased acetylation levels appears to negatively influence the AMPK-Nrf2 signalling: The general protein acetylation levels are higher in the AMPK-/- cells and treatment with HDAC inhibitors blunts the upregulation of HO-1. Notably, acetylation of Nrf2 itself has been reported to activate the transcription factor, but the overall cellular acetylation levels do not necessarily reflect the Nrf2-acetylation. The regulation of NADPH levels, fatty acid synthesis and fatty acid oxidation by AMPK are not relevant for the upregulation of HO-1 by XN in our test model. Both the levels of ER stress and of p53, a transcriptional repressor of Nrf2, are increased in AMPK-/- cells. p53 is stabilized in response to stress and could thus be activated as a consequence of the ER stress. AMPK clearly plays a positive role for Nrf2 signalling by alleviating ER stress: Inducing ER stress impairs the HO-1 upregulation, while alleviating it is sufficient to rescue the upregulation of HO-1 in AMPK-/- cells, while not influencing it in WT cells. For the future it will be interesting to find out how alleviation of ER stress influences the Nrf2-dependent signalling. There are several branches of the UPR that AMPK could be regulating. By influencing the ER stress response AMPK could also potentially be modulating the activity of Akt that has been shown to be negatively regulated by ER stress. Thus it would be interesting to test in future studies whether the ER stress affects the PI3K signalling, possibly via mTORC2. Overall, using a chemical biological approach we could corroborate the existence of an AMPK/Nrf2 signalling axis that was revealed to integrate signals from various different pathways, including PI3K/Akt, UPR and p53. A complex picture emerges where cellular metabolism and the Nrf2-dependent stress response take influence on each other through shared signalling networks. A better understanding of how these networks integrate the metabolic and antioxidative responses will ultimately contribute to a better understanding of the role of Nrf2 beyond cellular protection and the action spectrum of Nrf2 activators.

Concluding remarks about the study design

In this work we studied the effect of AMPK on Nrf2 signalling by comparing the response to XN in WT and AMPK-/- MEF and gained important insights and hints to the complex molecular network linking AMPK and Nrf2. For the design of follow-up studies it has to be considered that the complete deletion of AMPK as in our knockout MEF can lead to metabolic adjustments that partially compensate for the loss of the protein, or to a dysregulation of cellular processes. Out of these reasons it will be interesting to study how a transient downregulation of AMPK via the expression of a dominant negative AMPKα or treatment with siRNAs targeting AMPKα affects the Nrf2 signalling. As a readout for the activation of Nrf2 we detected the levels of the antioxidant stress protein HO-1. Different Nrf2 target genes underlie different regulations. To make a general statement about how AMPK influences Nrf2 signalling studies with several different Nrf2 target proteins are necessary, preferably by unbiased microarrays. It will be of particular interest to test whether AMPK also influences the reported negative regulation of metabolic target proteins, such as ACC or FAS, by Nrf2. To study the regulation of these metabolic proteins and confirm the effects of AMPK on Nrf2 signalling cell types specialized in glucose and lipid metabolism should be employed, such as liver or muscle cells. In the long run, corroboration of the AMPK/Nrf2 crosstalk in appropriate in vivo models would be desirable.

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Appendix

Abbreviations

A

ACC	Acetyl-CoA carboxylase
AHR	Aryl hydrocarbon receptor
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
ApoE	Apolipoprotein E
ARE	Antioxidant responsive element
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
AUC	Area under the curve

B

Bach1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
beta-TrCP	beta-transducin repeat-containing protein
BiP	Binding immunoglobulin protein
BRCA1	Breast Cancer gene 1
BSA	Bovine serum albumin

C

CAMKKß	Ca2+/Calmodulin-Dependent Kinase Kinase ß
cAMP	cyclic AMP
CBP	cAMP response element-binding protein (CREB)-binding protein
CC	Compound C
cDNA	Complementary DNA
CDDO-Im	1-(2-Cyano-3,12,28-trioxooleana-1,9(11)-dien-28-yl)-1H-imidazole
C/EBP-β	CCAAT/enhancer-binding protein beta
CHD6	Chromo-ATPase/helicase DNA binding protein

CHO	Chinese hamster ovary
CK2	Casein kinase 2
CNC	Cap 'n' collar
CO	Carbon monoxide
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase I
CRIF1	CR6-interacting factor 1
CV	Crystal violet
Cys	Cysteine

D

D	DMSO
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

E

ECL	Enhanced chemiluminescence
EDTA	Ethylene-diamine-tetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
EH	Epoxide hydrolase
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase

F

FADH2	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FBS	Fetal bovine serum

136

FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

G

G6PD	Glucose-6-phosphate dehydrogenase
GCL	Glutamate-cysteine ligase
GCS	Glutamylcysteine synthetase
GLUT	Glucose transporter
GSH	Glutathione
GSK3	Glycogen synthase kinase 3
GSTA1	Glutathione S-Transferase Alpha 1

H

HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HO-1	Heme oxygenase 1
HPR	Horseradish peroxidase
HPRT	Hypoxanthin-phosphoribosyl-transferase
Hrd1	HMG-CoA reductase degradation protein 1

I

IgG	Immunoglobulin G
IRE1α	Inositol-requiring enzyme 1α
ITC	Isothiocyanate

J

JNK	Jun amino terminal kinase

K

Keap1   Kelch-like ECH-associated protein 1

L

LKB1   Liver kinase B1

M

Maf   v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog

MAPK   Mitogen-activated protein kinase

MEF   Mouse embryonic fibroblast

miR, miRNA   Micro RNA

mRNA   Messenger RNA

MRP   Multidrug resistance-associated protein

mTOR   Mammalian target of rapamycin

mTORC   Mammalian target of rapamycin complex

N

NAD+/NADH   Nicotinamide adenine dinucleotide (oxidized/reduced form)

NADPH   Nicotinamide adenine dinucleotide phosphate

Neh   Nrf2−ECH homology

NFkB   Nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1   NAD(P)H quinone oxidoreductase 1

Nrf2   Nuclear factor (erythroid-derived 2)-like 2

ns   non-significant

O

OCR   Oxygen consumption rate

P

PAA   Polyacrylamide

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDK1	3'-phosphoinositide dependent kinase-1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PGD	Phosphogluconate dehydrogenase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPP	Pentose phosphate pathway
PVDF	Polyvinylidene fluoride

R

RAC3/AIB1/SRC-3	Receptor-associated coactivator 3/AIB-1/steroid receptor coactivator-3
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNF4	RING finger protein 4
ROS	Reactive oxygen species
Rp-8-Br-cAMPS	8-Bromoadenosine 3',5'-cyclic Monophosphothioate, Rp-Isomer
RTqPCR	Real-time quantitative PCR
RXRα	Retinoid X receptor alpha

S

SAPK	Stress activated protein kinase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Siah2	Seven in absentia homolog 2

SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SQSTM1	Sequestosome 1

T

TALDO1	Transaldolase 1
TBC1D1	TBC1 domain family member 1
tBHQ	Tertiary butylhydroquinone
TBS-T	Tris-Buffered Saline-Tween 20
Thr	Threonine
TKT	Transketolase
TOFA	5-(Tetradecyloxy)-2-furoic acid
TSA	Trichostatin A
TSC2	Tuberous Sclerosis Complex 2

U

Ulk1	Unc-51 Like Autophagy Activating Kinase 1
UPR	Unfolded protein response

W

WT	Wild type

X

XN	Xanthohumol

List of Figures

Publications and Presentations

Publications

Kristin Zimmermann, Johannes Baldinger, Barbara Mayerhofer, Atanas G Atanasov, Verena M Dirsch, Elke H Heiss. Activated AMPK boosts the Nrf2/HO-1 signaling axis—A role for the unfolded protein response. *Free Radic Biol Med*. 2015 Apr 3. pii: S0891-5849(15)00154-9.

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In preparation:

Jarolim K, del Favero G, Pahlke G, Dostal V, Zimmermann K, Dirsch V, Heiss E, Ellmer D, Stark T, Hofmann T, Marko D. Impact of the *Alternaria alternata* Mycotoxins Altertoxin I and II on the Nrf2-ARE Pathway.

Poster presentation

Kristin Zimmermann, Verena M. Dirsch, Elke H. Heiss. DOES NRF2 CROSS-TALK WITH METABOLIC REGULATORS? An investigation into whether the central metabolic sensor AMPK plays a role for Nrf2 activation by xanthohumol. Metabolism 2014 - Alterations of metabolic pathways as therapeutic targets, 29–31 January 2014, Esch-sur-Alzette, Luxembourg.

Oral presentation

Kristin Zimmermann, Verena M Dirsch and Elke H Heiss. Using the natural polyphenol xanthohumol as a molecular probe for studies to the interplay between Nrf2 and AMPK Konference Trends in Natural Products Research 2014, Young Scientists Meeting, June 23 - 25, 2014, Olomouc, Czech Republic.

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"And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about."

(Haruki Murakami)