Supplemental Information

Activated AMPK boosts the Nrf2/HO-1 signalling axis- a role for the unfolded protein response

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Material

The Hrd1 antibody (#NB100-2526) was from Novus Biologicals.

Methods (detailed protocols)

Preparation of whole cell lysates, SDS- PAGE and Western blot

Cells were seeded in 6-well culture plates. After being treated as indicated cells were washed once with ice cold PBS and then lysed for 10 minutes on ice with 100 µL radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet, 0.5% desoxycholate, 0.1% SDS, complemented with protease and phosphatase inhibitors). The lysates were scraped into microcentrifuge tubes and briefly sonicated. After spinning down the cellular debris at maximal speed in a table top centrifuge for 10 minutes, supernatants were subjected to protein determination according to Bradford. Equal amounts of protein (20-30 µg/ sample) were heat inactivated for 5 minutes at 95°C with 10 mM DTT and then loaded onto polyacrylamide gels of an appropriate percentage (7.5 to 12 % depending on the size of the protein of interest). After separation proteins were transferred to PVDF membranes. After blocking for 1 hour with 5% nonfat

milk in TBS-Tween, membranes were probed at 4°C overnight with the primary antibodies and then for 1 hour at room temperature with the HRP-conjugated secondary antibodies. 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).

Fractionated extraction of nuclear and cytosolic proteins

After treatment and washing with ice-cold PBS, cells were incubated with 100 µL of buffer 1 (10 mM HEPES pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl. Add 1% NP40, 1 mM DTT, 0.5mM PMSF and Complete Protease Inhibitor (Roche)). The obtained lysate was scraped into a microcentrifuge tube and incubated on ice for 15 minutes with vigorous vortexing every 3 minutes. After centrifugation in a table top centrifuge at maximum speed for 5 minutes, the supernatant containing the cytosolic fraction was collected. The pellet containing the nuclear fraction was washed once with 200 µL of buffer 1, followed by 5 minutes centrifugation at maximum speed. The supernatant was completely removed and the pellet was resuspended in 20 µL of Buffer 2 (20 mM HEPES pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 420 mM NaCl, 1 mM DTT, 0.5mM PMSF and Complete Protease Inhibitor (Roche)). After incubating on ice for 15 minutes with vigorous vortexing every 3 minutes followed by centrifugation in a table top cenatrifuge at maximum speed for 5 minutes, the supernatant was transferred to a microcentrifuge tube containing 20 µL of buffer 3 (20 mM HEPES pH 7.5, 1mM EDTA, 0.1 mM EGTA, 100 mM KCl, 20 % glycerol, 1% NP 40 , 1 mM DTT , 0.5mM PMSF and Complete Protease Inhibitor (Roche)) and mixed well, thus obtaining the dissolved nuclear fraction.

Real-time quantitative polymerase chain reaction (RTqPCR)

Total RNA was extracted following the instructions of the peqGOLD Total RNA Kit from Peqlab. RNA concentration and purity was then measured with the Nanodrop. cDNA synthesis was performed according to the protocol of the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor from Applied Biosystems. For each sample 1µg of RNA was used as a template for transcription. The

RT-qPCR was carried out using the LightCycler 480 SYBR Green I Master (Roche Diagnostics) in a reaction volume of 21 µL. Both for the gene of interest, murine HO-1, and the reference gene, murine hypoxanthin-phosphoribosyl-transferase (HPRT), the QuantiTect Primer Assays from Qiagen were ordered and diluted following the instructions of the manufacturer. The following PCR program was run: One denaturation step (10 min at 95°C) followed by up to 40 amplification cycles (annealing step: 30 s at 61°C and elongation step: 15 s at 72°C). The amplification of one specific product was verified by analyzing the melting curves of the amplified DNA and the data were then analyzed using the Light Cycler LC480 Software (Roche Diagnostics) and the $2^{-\Delta\Delta Ct}$ method.

Determination of mitochondrial function by extracellular flux analysis (evaluation and inhibitor concentrations)

Basal respiration was defined as oxygen consumption rate (OCR) without stressor minus OCR after addition of antimycin A and rotenone. *Respiration coupled with ATP production* was defined as Δ(OCR (without stressor)-OCR (+oligomycin)), *maximal respiration* as Δ(OCR (+FCCP)-OCR (+antimycin/rotenone)) and *respiratory spare capacity* as Δ(OCR maximal-OCR basal)). The *proton leak* was calculated as Δ(OCR (+oligomycin) – OCR(+ antimycin /rotenone) and the *residual oxygen consumption* (ROX) was defined as the OCR after addition of antimycin/rotenone. Optimized inhibitor concentrations for MEF were 2 µM oligomycin A, 1.5 µM carbonylcyanid-p-trifluoromethoxyphenylhydrazon (FCCP), 1 µM rotenone A, and 1 µM antimycin A. Correction to biomass was performed by crystal violet staining after the analysis in order to account for potential differences in cell number.

Supplemental Figures

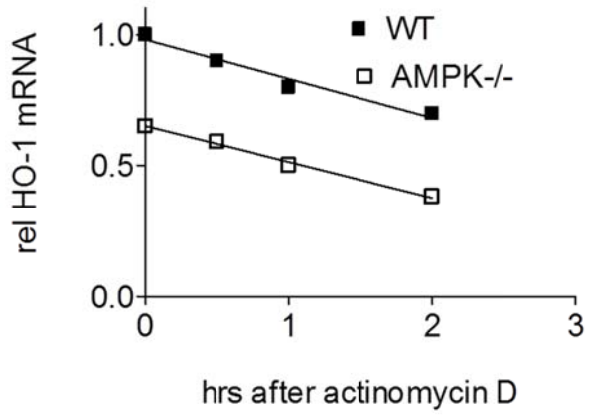

Figure S1: The half –life of HO1 mRNA is comparable in WT and AMPK-/- MEF.

WT and AMPK-/- MEF were treated with XN (5 µM) for 4 hrs. Then the transcriptional inhibitor actinomycin D was added for the indicated periods of time and levels of mRNA levels were determined by qPCR with HPRT as reference gene. Data are expressed as fold of levels at time point of actinomycin addition. A representative result of two independent experiments is shown.

- ➔ **WT and AMPK-/- cells differ in the level of XN-induced HO1 mRNA , but not in the apparent half-life of HO-1 mRNA as evident in the parallel curves between WT and AMPK-/- cells.**

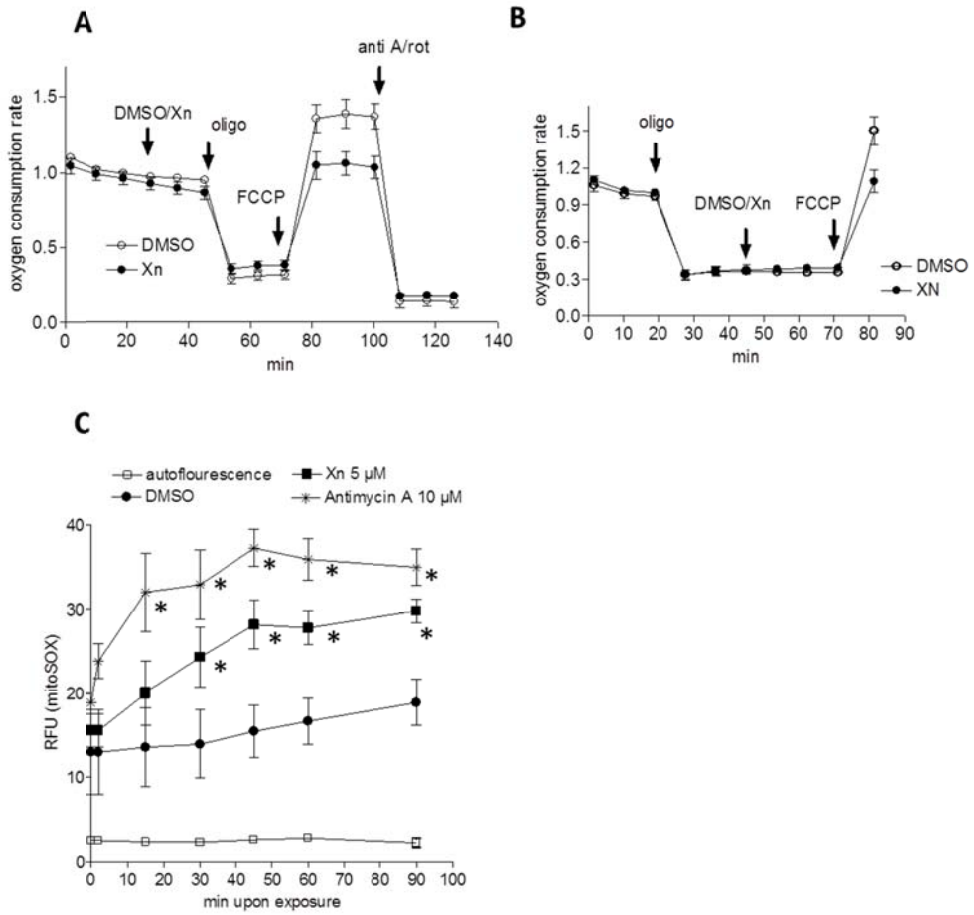

Figure S2: XN interferes with mitochondrial function but does not act as a FCCP-like uncoupler.

(**A**) WT MEF were subjected to a mitochondrial stress test as described in the Methods section. Cells were successively exposed to vehicle or XN (5 µM), to oligo(mycin) (2 µM), FCCP (1.5 µM) and antimycin A + rotenone (A+R; 1+1 µM). The mean oxygen consumption rate (OCR) (corrected for biomass) of three independent experiments is depicted (mean +SD). (**B**) In order to assess the uncoupling potential of XN the OCR (corrected for biomass) of WT MEF was assessed upon successive addition of oligo(mycin) (2µM), vehicle or Xn (5 µM) and FCCP (1.5 µM) as positive control. Compiled data of three independent experiments are shown (mean +SD). (**C**) Mitochondrial ROS production in MEF was recorded over time after exposure to vehicle, XN (5 µM) or antimycin A (10 µM, positive control) by using MitoSox Red and flow cytometric analysis . Compiled data of three independent experiments are depicted (mean + SD ,* $p < 0.05$, (vs vehicle control); ANOVA).

→ Unlike FCCP, XN (5 µM) does not uncouple mitochondrial respiration as evident in the lack of increased OCR upon XN exposure to oligomycin-treated MEF (B). XN, however,

reduces mitochondrial function ((A) and figure 3A in main manuscript) and leads to mitochondrial ROS production (C), features that could be explained by inhibition of electron transport in the OXPHOS: inhibition of the protein-mediated electron flow increases the leakiness of the electron transport chain and direct formation of superoxide. Moreover, an impaired electron flow leads to a reduced proton-motive force and less ATP production as well as a generally reduced maximal electron transport capacity in the mitochondria.

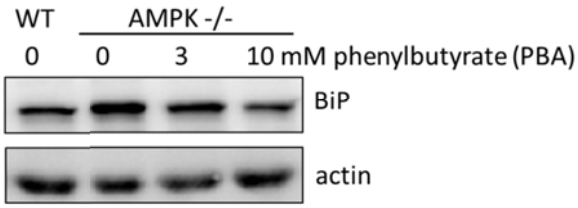

Figure S3: Phenylbutyrate successfully alleviates ER stress in AMPK-/- MEF.

WT cells (control) and AMPK-/- MEF treated with phenylbuyrate (3 and 10 mM) were subjected to western blot analysis for BiP/GRP78 and actin. Representative blots of two independent experiments are depicted.

- ➔ **10 mM phenylbutyrate occurred as optimal concentration restoring WT ER-stress levels in AMPK-/- cells.**

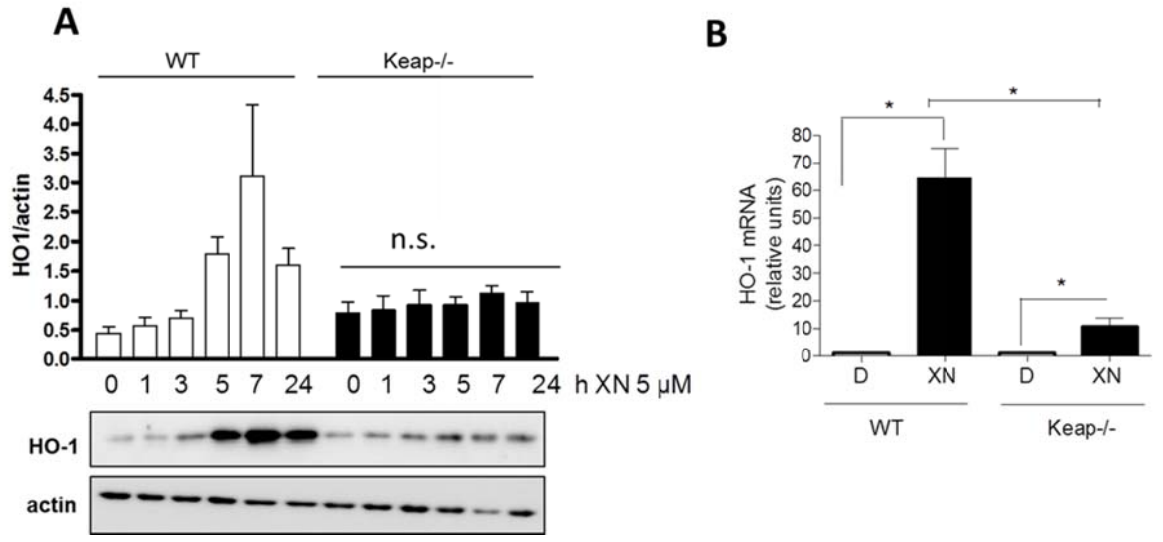

Figure S4: XN induces HO1 predominantly via the KEAP1-dependent canonical activation pathway. (**A**) WT and Keap-/- MEF were treated with XN (5 µM) for the indicated periods of time before total cell lysates were subjected to immunoblot analysis for HO-1 and actin, respectively. Respective blots of three independent experiments are depicted with the bar graph showing compiled densitometric data from all performed experiments (mean + SD; * $p < 0.05$; ANOVA). (**B**) WT and Keap-/- MEF were treated with vehicle (DMSO, D) or XN (5 µM) for 4 hours before their mRNA was isolated and subjected to qPCR analysis for quantification of HO-1 (target) and HPRT (reference) transcripts . The bar graph shows compiled data of three independent experiments expressed as fold HO1 induction (normalized to HPRT levels upon XN exposure. (mean + SD; * $p<0.05$; Student´s t-test).

→ KEAP-/- cells do not respond with a significantly elevated HO-1 protein expression upon XN exposure. On the HO-1 mRNA level, XN causes a slight elevation in KEAP1-/- cells, however, to a much weaker extent than in WT cells. Thus, XN mainly activates Nrf2 via the KEAP1 pathway.

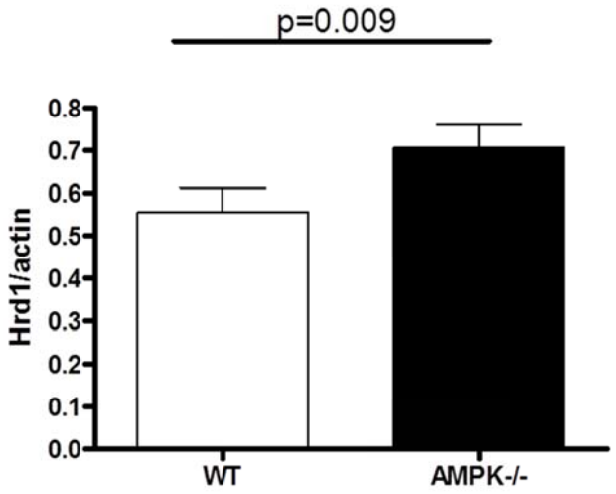

Figure S5: Hrd1 levels are slightly, but significantly higher in AMPK-/- than WT cells.

Total cell lysates of WT and AMPK-/- were subjected to immunoblot analysis for Hrd1 and actin. Graph depicts compiled densitometric data of six independent experiments. (*p<0.05; Student´s t-test).

- ➔ **In line with the elevated ER stress AMPK-/- MEF show a little higher levels of Hrd1, a ubiquitin ligase also involved in negative Nrf2 regulation. However, the higher Hrd1 levels could not be linked to reduced total levels of Nrf2 in our test system (see Fig. 5 main manuscript). This finding suggests that the negative impact of ER stress on Nrf2/HO-1 axis in our model is Hrd1-independent.**