An inhibitor of interaction between the transcription factor NRF2 and the E3 ubiquitin ligase adapter β-TrCP delivers anti-inflammatory responses in mouse liver

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SUPPLEMENTARY MATERIAL

| Gene product | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|--------------|------------------------|-----------------------|
| Hmox1        | CACAGATGGGCGTCACTTCGTC | GTGAGGACCACTGGAGGAG   |
| Aox1         | CTTTTGACAAAGCATCAGTCTC | CCCCCTCTCCAGTCTATATCGA|
| Nqo1         | GGTACCGGCTCACTGTACTC   | CATCCTCCAGGATCTGCA    |
| Nrf2         | CCCGAAGCAGCTGAAGGCA    | CCAGCGGTGGGTCTCGTA    |
| Gclc         | TTACCGAGGTACGTGTCAGAC  | TATCGATGTCAGGTGATGTC  |
| Gclm         | AATCAGCCGGATTTAGTCAGG  | CCAGCGTCAACTCCAGGAC   |
| Btrc         | AGCGGCTCTCTGCAACACCAT  | AGCACGCGGTCCAAAGCA    |
| Fbxw11       | GTCCGCACTCTGAATGGCACA  | GCACCGGAACATCTTGAG    |
| Ptgs2        | TTCGGGAGCAACAAGAGTG    | TAACCGTCAGGTGTTGAC    |
| Nos2         | CCTCCTTGCCCTCTCACTCTC  | AGTATTAGGCGGTGGCATGT  |
| Il1b         | CTGGTGTTGACGTTCCCATTA  | CCAGACAGCAAGGGCTTT    |
| Tnf          | CATCTTTCTAAAATTCCAGTGACAA | TGGGAGTAGCAAGTGTAACC    |
| Il6          | CCTACCCCAATTTCCAATGCT  | TATTTTCTGACACAGTGAGGATG|
| Actb         | TCCTCCTGGGCAATGGAG     | AGGAGGAGCAAATGATCTCGT |
| Gapdh        | CGACCTCAGCAACTCCCACTTTCC | TGGGAGTCCAGGGTATTCTACTCCT |
| Tbp          | TGCAACAGGAGCCAAGTGAA   | CACATCACAGCTCCCAACCA   |

Supplementary Table 1. Mouse primers used for RT-PCR.
Figure S1. PHAR prevents the decrease in NRF2 levels elicited by the phosphoinositide 3-kinase inhibitor LY294002. Serum-depleted Keap1−/− MEFs were subjected to 20 µM LY294002 or 10 µM PHAR as indicated. A, representative immunoblots of NRF2, phospho-Ser473AKT (pSer473AKT), AKT, phospho-Ser9GSK3β (pGSK-3), GSK3 and VCL as a loading control. B, densitometric quantification of NRF2 protein levels from representative immunoblots from A, expressed as a ratio of NRF2/VCL.
Figure S2. GSK-3 inhibition abolishes further NRF2 accumulation in response to PHAR. Keap1<sup>-/-</sup> MEFs were serum-depleted for 16 h and then subjected to 10 µM PHAR, 10 µM SB216763 (GSK-3 inhibitor) or to both treatments for the indicated times. A, representative immunoblots of NRF2, β-Catenin and VCL as loading control. B, densitometric quantification of NRF2 protein levels from representative immunoblots from A, expressed as a ratio of NRF2/VCL. Data are mean ± S.D. (n=3). A Student t test did not detect statistically significant differences.
Figure S3. PHAR increases NRF2 protein levels in a β-TrCP dependent-manner. Keap1−/− MEFs were transduced with lentivirus encoding shCTRL or sh against mouse β-TrCP1/2. After 5 days, cells were serum-depleted for 16 h and then subjected to 10 µM PHAR for 30 min. 0.1% DMSO was used as vehicle. Then, cells were treated with 20 µM LY294002 for the indicated times. A and C, representative immunoblots of NRF2, pSer473-AKT, AKT, pSer9-GSK3β, GSK3, and VCL as loading control from shCTRL and sh β-TrCP1/2. B-D, densitometric quantification of NRF2 protein levels from representative immunoblots from A and C, expressed as a ratio of NRF2/GAPDH. Data are mean ± S.D. (n=4). *p<0.01 vs LY294002 according to a two-way ANOVA test.
**Figure S4.** Pevonedistat (MLN-4924) promotes the accumulation of NRF2 through NEDD8-activating enzyme (NAE) inhibition. A, Mechanism of action of pevonedistat. Pevonedistat (MLN-4924) is a NAE inhibitor that selectively prevents the activation of cullin-based ring-ubiquitin ligases (CRLs), including CUL1 (connected with β-TrCP) and CUL3 (connected with KEAP1), and alters the ubiquitination and proteasomal degradation of cellular proteins, NRF2 in our case, causing its accumulation in the non-ubiquitinated form. B, HEK293T were serum-depleted for 16h and then subjected to 1 µM MLN-4924 or 20 µM MG132 for the indicated time. Representative immunoblots of NRF2, β-CATENIN, and VCL as a loading control.
Figure S5. Proximity ligation assay (PLA) for KEAP1 and NRF2 in HEK293T cells. HEK293T cells were transfected with expression vectors for NRF2-V5, NRF2\DeltaETGE-V5, HA-KEAP1 and β-TrCP-FLAG for 24h. Then, cells were incubated in the presence vehicle (VEH) or PHAR (10µM) for 6 h. Cells were subjected to the PLA assay using rabbit anti-FLAG/anti-HA and mouse anti-V5. Fluorescent images were captured, and red puncta represent co-localization of KEAP1 and NRF2. To confirm the validity of the assay, we used as negative control ectopic expression of NRF2\DeltaETGE-V5 which lacks the high affinity binding site for KEAP1. Calibration bar = 20 µm. When no antibody was present or when only one plasmid was present (KEAP1-HA only, β-TrCP-FLAG only or NRF2-V5 only), no red fluorescent puncta were detected.
Figure S6. PHAR decreases the inflammatory response in peritoneal macrophages derived from wild type but not from Nrf2-knockout mice. Serum-depleted peritoneal macrophages were pre-treated with 10 µM PHAR for 8h. Then, cells were treated with 100 ng/ml LPS for the indicated times. A, representative immunobLOTS of NRF2, COX2, NOS2, and GAPDH as a loading control. B-C, densitometric analysis of NRF2, COX2, and NOS2 protein levels from representative immunobLOTS from (A), expressed as a ratio of protein levels/GAPDH. Data are mean ± S.D. (n=3). *p<0.05; **p<0.01; ***p<0.001 vs. vehicle or LPS treatment, as indicated, according to a one-way ANOVA test. C, transcript levels of Il1b, Ptgs2, Nos2, Il6, and Tnf were determined by qRT-PCR and normalized by the average of Gapdh, Tbp, and Actb. Data are mean ± S.D. (n=3). *p<0.05; **p<0.01; ***p<0.001 vs. LPS according to a one-way ANOVA test.