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	CACAGATGGCGTCACTTCGTC	GTGAGGACCCACTGGAGGAG
Aox1	CTTTTGACCAAAGCATCAGTCTC	CCCTTTCTCCCAGTCTATATTCGA
Nqo1	GGTAGCGGCTCCATGTACTC	CATCCTTCCAGGATCTGCAT
Nrf2	CCCGAAGCACGCTGAAGGCA	CCAGGCGGTGGGTCTCCGTA
Gclc	TTACCGAGGCTACGTGTCAGAC	TATCGATGGTCAGGTCGATGTC
Gclm	AATCAGCCCCGATTTAGTCAGG	CCAGCGTGCAACTCCAAGGAC
Btrc	AGCGGCTCCTCTGACAACACCAT	AGCACGGGGTCCAAAGCA
Fbxw11	GTCCGCACTCTGAATGGGCACA	GCACCGGACCAATTCTTCG
Ptgs2	TTCGGGAGCACAACAGAGTG	TAACCGCTCAGGTGTTGCAC
Nos2	CCTCCTTTGCCTCTCACTCTTC	AGTATTAGAGCGGTGGCATGGT
Il1b	CTGGTGTGTGACGTTCCCATTA	CCGACAGCACGAGGCTTT
Tnf	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Il6	CCTACCCCAATTTCCAATGCT	TATTTTCTGACCACAGTGAGGAATG
Actb	TCCTTCCTGGGCATGGAG	AGGAGGAGCAATGATCTTGATCTT
Gapdh	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT
Tbp	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA

Supplementary Table 1. Mouse primers used for RT-PCR.

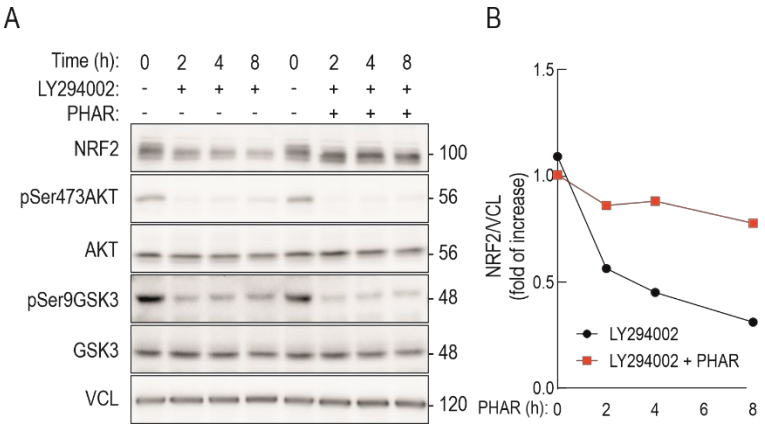

Figure S1. PHAR prevents the decrease in NRF2 levels elicited by the phosphoinositide 3-kinase inhibitor LY294002. Serum-depleted *Keap1*$^{-/-}$ MEFs were subjected to 20 μM LY294002 or 10 μM PHAR as indicated. A, representative immunoblots of NRF2, phospho-Ser473AKT (pSer473AKT), AKT, phospho-Ser9GSK3β (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.

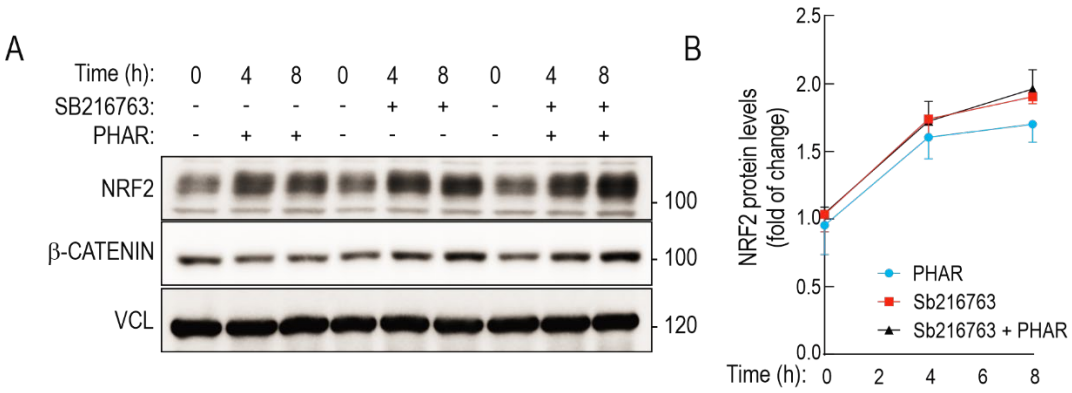

Figure S2. GSK-3 inhibition abolishes further NRF2 accumulation in response to PHAR. *Keap1*$^{-/-}$ MEFs were serum-depleted for 16 h and then subjected to 10 µM PHAR, 10 µM SB216763 (GSK-3 inhibitor) or to both treatments for the indicated times. A, representative immunoblots of NRF2, β-Catenin and 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.

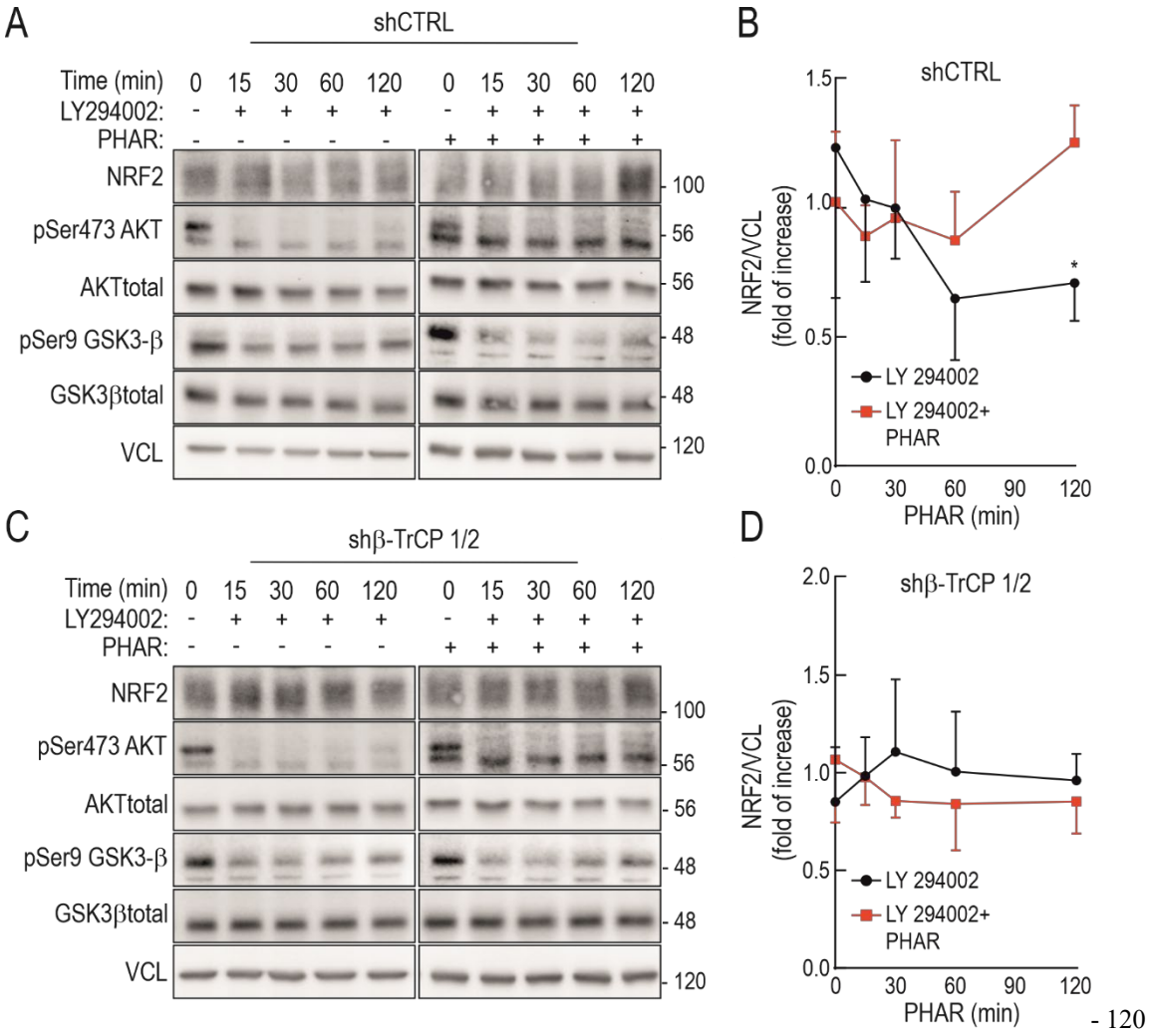

Figure S3. PHAR increases NRF2 protein levels in a β-TrCP dependent-manner. Keap1$^{-/-}$ MEFs were transduced with lentivirus encoding shCTRL or sh against mouse β-TrCP1/2. After 5 days, cells were serum-depleted for 16 h and then subjected to 10 μM PHAR for 30 min. 0.1% DMSO was used as vehicle. Then, 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.

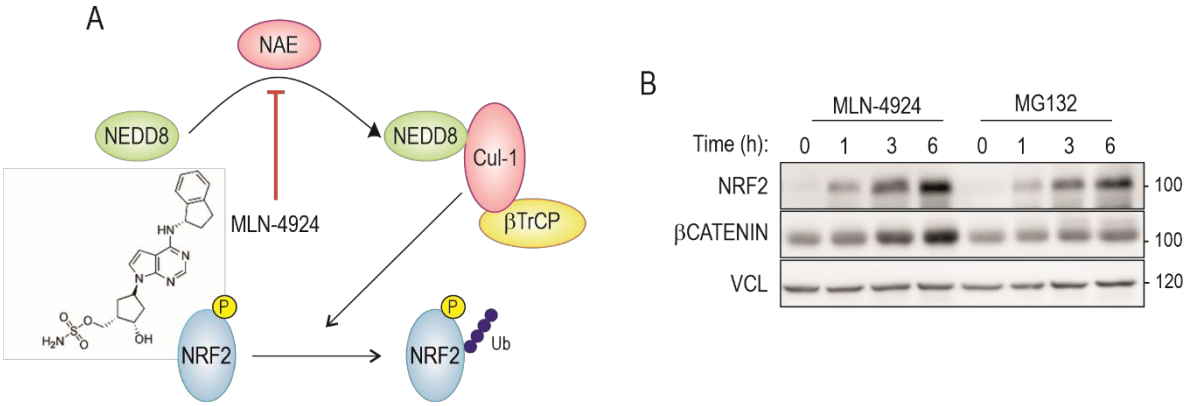

Figure S4. Pevonedistat (MLN-4924) promotes the accumulation of NRF2 through NEDD8-activating enzyme (NAE) inhibition. A, Mechanism of action of pevonedistat. Pevonedistat (MLN-4924) is a NAE inhibitor that selectively prevents the activation of cullin-based ring-ubiquitin ligases (CRLs), including CUL1 (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.

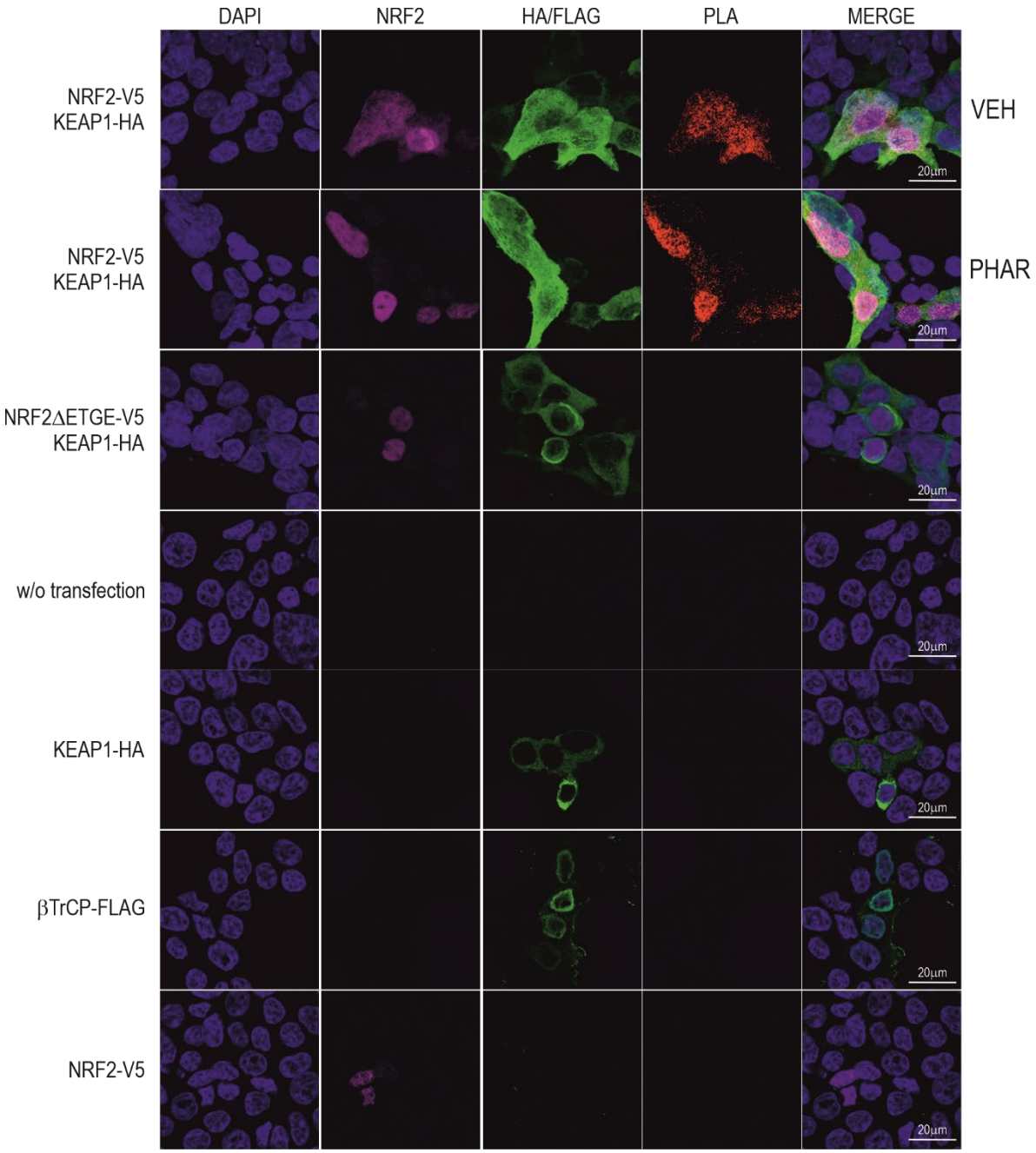

Figure S5. Proximity ligation assay (PLA) for KEAP1 and NRF2 in HEK293T cells. HEK293T cells were transfected with expression vectors for NRF2-V5, NRF2ΔETGE-V5, HA-KEAP1 and β-TrCP-FLAG for 24h. Then, cells were incubated in the presence vehicle (VEH) or PHAR (10μM) for 6 h. Cells were subjected to the 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 NRF2ΔETGE-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.

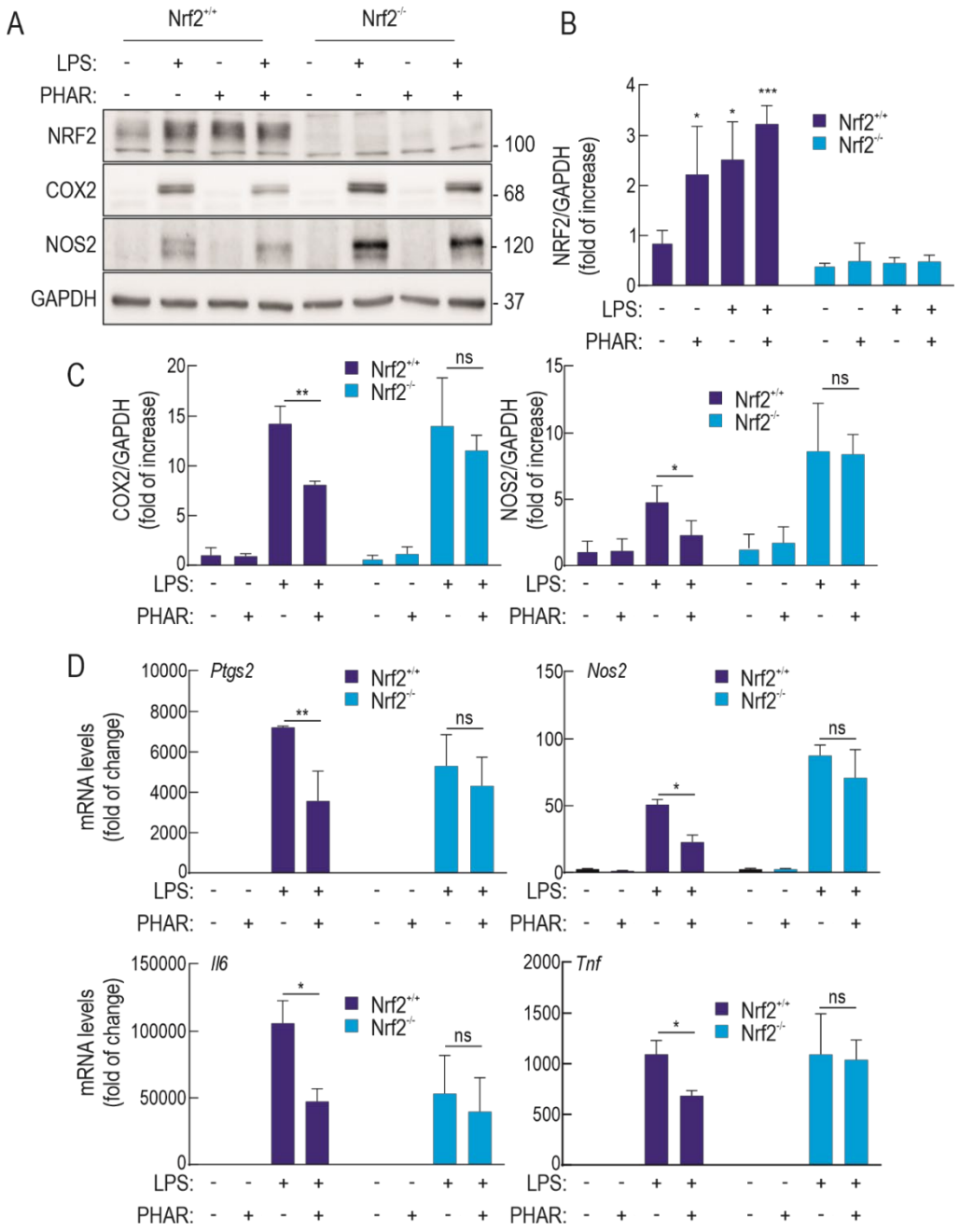

Figure S6. PHAR decreases the inflammatory response in peritoneal macrophages derived from wild type but not from Nrf2-knockout mice. Serum-depleted peritoneal macrophages were pre-treated with 10 μM PHAR for 8h. Then, cells were treated with 100 ng/ml LPS for the indicated times. A, representative immunoblots 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.