Supplementary materials for

Keap1 moderates the transcription of virus induced genes through G9a-GLP and NFκB p50 recruitment

Veronica Elizabeth Burns and Tom Klaus Kerppola*.

Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-5606

* Corresponding author: Tom Kerppola
E-mail: kerppola@umich.edu

Supplementary Materials and Methods

Supplementary References

List of supplementary figures (separate file)

Figure S1. Keap1 moderates the transcription of virus induced genes through mechanisms that are distinct from Keap1 regulation of electrophile response gene transcription (related to Fig. 1).

Figure S2. Antibodies that were raised against different regions of Keap1 detect Keap1 binding to chromatin, and the augmentation of the Keap1 binding by BIX01294 (related to Fig. 1, 2, 3).

Figure S3. Keap1 is required for virus induced H3K9me2 deposition in regions encompassing virus induced genes (related to Fig. 1, 2, 3).

Figure S4. UNC0638 and UNC0642 effects on Keap1 and NFκB p50 binding to virus induced genes correlate with their inhibition of H3K9me2 deposition, but not with their effects on transcription (related to Fig. 3).

Figure S5. Comparison of the effects of different G9a-GLP inhibitors on Keap1 and NFκB p50 binding to virus induced genes, and on H3K9me2 deposition (related to Fig. 4).

Figure S6. BIX01294 stabilizes Keap1 retention in MEFs during subsequent incubation with buffers containing different detergents (related to Fig. 5).

Supplementary Materials and Methods

Experimental Design. MEFs were derived from embryos with *Keap1-/-* and *Nrf2-/-* deletions, or with *Nrf2-/-* deletions alone. These MEFs were infected with Sendai virus or mock infected. The levels of virus induced gene transcripts and of cell cycle associated gene transcripts were compared at different times after virus infection in multiple independent MEFs of each genotype. The *Ifnb1*, *Tnf* and *Il6* transcripts were selected for analysis since these genes were classified in different categories based on previous studies of their activation (1-3). The *Cdkn1a* and *Ccng1* transcripts were chosen for analysis based on evidence for a role of Keap1 in the cell cycle (4).

Chromatin binding by Keap1 and by other regulators of virus induced gene transcription, and H3K9me2 deposition, were measured by ChIP analyses at virus induced genes and cell cycle associated genes in multiple independent MEFs of each genotype. G9a, GLP, NFκB p50, NFκB p65, cJun, IRF3, H3K9me2 and H3K27me3 were selected for analysis based on previous studies of proteins and histone modifications that correlated with the activation or inhibition of cytokine transcription (2, 5, 6).

The roles of G9a-GLP lysine methyltransferase activities in transcription and in chromatin binding were examined based on the discovery that Keap1 was required for G9a and GLP binding to virus induced genes. Multiple independently derived MEFs of each genotype were cultured with G9a-GLP inhibitors prior to virus infection. The G9a-GLP inhibitors that were tested, and the concentrations and times of culture were selected, based on previous development of compounds that inhibited G9a and/or GLP activities (7-10).

The effects of G9a-GLP inhibitors on the retention of Keap1, NFκB subunits, and other proteins in permeabilized MEFs were tested because of previous findings of Keap1 retention in permeabilized cells (4, 11). The effects of tBHQ alone and in combination with *Keap1-/-* deletions on virus induced gene transcription and regulatory protein binding were examined because of previous studies of Keap1 roles in electrophile response gene regulation.

Derivation of MEFs. Mice with *Keap1* and *Nrf2* deletions (12, 13) were crossed to generate *Keap1-/+ Nrf2-/+* heterozygous mice. Primary MEFs were isolated at about 13 days post coitus from embryos produced by these mice. The MEFs were expanded for up to 5 passages and any compounds or vehicle were added to the cultures. MEFs from different embryos are identified in the figures and figure legends by #number after the genotype. All experiments involving live mice were approved by the Institutional Animal Care & Use Committee at University of Michigan.

MEF culture and Sendai virus infection. The MEFs were cultured in DMEM (GIBCO, cat. no. 11995) supplemented with 10% FBS and 0.1 mg/ml penicillin-streptomycin. MEFs of the indicated genotypes were plated at a density of 200,000/ml. When indicated, compounds or vehicle were added to the MEFs at the times indicated prior to virus infection. The MEFs were infected with Sendai virus by the addition of 200 hemagglutinin units/ml of Sendai virus (Charles River Laboratories, Wilmington, MA) or mock infected. For time course analyses, aliquots of the same Sendai virus stock were thawed and added in an identical manner at different times and the cells were harvested and transcripts were isolated and analyzed in parallel.

Analysis of transcript levels by RT-qPCR. The MEFs (200,000 cells plated per sample) were washed twice with pre-warmed 1X PBS (Gibco cat. no 10010023), released with 0.05% trypsin-EDTA (Gibco cat no. 25300054), and suspended in culture medium. Total RNA was isolated (RNeasy, QIAgen) and equal amounts of RNA were reverse transcribed (Transcriptor First Strand cDNA synthesis, Roche). The relative amounts of cDNAs corresponding to the indicated transcripts were measured using qPCR. The relative transcript levels were calculated by assuming that they were inversely

proportional to 2^{Ct}, where Ct equals the number of cycles required to reach threshold fluorescence. The amounts of transcripts in each sample were normalized by the amount of *H3f3a* transcripts in the same sample. The average normalized transcript level for duplicate reactions was plotted with upper and lower error bars corresponding to 2*SD. The oligonucleotide primers for RT-qPCR were obtained from IDT, were validated by melt-curve analyses, and had the following sequences:

RT-qPCR	Forward primer sequence	Reverse primer sequence
Ifnb1	cacagccctctccatcaacta	catttccgaatgttcgtcct
Tnf	ttgtcttaataacgctgatttggt	gggagcagaggttcagtgat
Il6	tgccttcatttatcccttgaa	ttactacattcagccaaaaagcac
C*cng1*	cacaccaatcagcgacgta	gggaacaagctggagacct
C*dkn1a*	tccacagcgatatccagaca	ggacatcaccaggattggac
M	tggtgctccactcctaccat	gtgcgaccttgtttgcatta
Nqo1	agcgttcggtattacgatcc	agtacaatcagggctcttctcg
Gapdh	ggccggtgctgagtatgtcgtg	tcggcagaaggggcggagat
H3f3a	gccatctttcaattgtgttcg	agccatggtaaggacacctc

Analysis of protein binding to specific chromatin regions by ChIP-qPCR. The MEFs (2-8 X 10^7 cells plated for each genotype and condition) were harvested as described above for transcript analysis. The cells were washed with PBS and crosslinked for 30 min in 2 ml ice cold 1% formaldehyde containing 2X Complete EDTA-free Protease Inhibitor Cocktail (Roche). Cross-linking was stopped by the addition of glycine to 333 mM. The MEFs were collected and lysed by the addition of 4 ml ice cold cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, and 2X Complete EDTA-free Protease Inhibitor Cocktail (Roche). The nuclei were collected and lysed by 30 min incubation in 4 ml RIPA buffer containing 1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM N-ethylmaleimide, and 2X Complete EDTA-free Protease Inhibitor Cocktail (Roche). The chromatin was sheared by sonication to produce DNA fragments with a size distribution between 500 bp and 1 kb, followed by 14,000 rpm centrifugation at 4 C for 15 min.

The chromatin was divided into 0.5-1 ml aliquots for immunoprecipitation. Each antibody (2-4 μg per reaction) and IgG control were incubated with Dynabeads (Invitrogen) for at least 4 h at 4C with rotation, washed and resuspended in PBS with BSA. The beads were rotated with the cleared lysates for 24 h at 4 C.

The beads with bound immune complexes were washed 5 times in ice cold 100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate, and once with ice cold 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA. Chromatin complexes were eluted by incubating the beads in 1% SDS, 0.1 M NaHCO3 for 1 h at 65 C. The eluted chromatin complexes as well as aliquots of the input chromatin were incubated for 16-20 h at 65 C to reverse the crosslinks. The DNA was purified (QIAquick PCR cleanup, Qiagen). The relative amounts of the genes that were precipitated by each antibody were measured by qPCR. The average percentage of the input DNA that was precipitated (ChIP Efficiency) was plotted for duplicate qPCR reactions with upper and lower error bars corresponding to 2 times the standard deviation. The oligonucleotide primers for ChIP-qPCR were obtained from IDT, were validated by melt-curve analyses, and had the following sequences:

ChIP-qPCR	Forward primer sequence	Reverse primer sequence
Ifnb1	attcctctgaggcagaaaggacca	gcaagatgaggcaaaggctgtcaa
Tnf	aaccctctgccccgcgatg	tcctcgctgagggagcttctgc
Il6	tggggatgtctgtagctcatt	ggaactgccttcacttacttgc
C*cng1*	gcaaaacaaaaaccctcacag	caggtctggcctctattagcc

C*dkn1a*	ttgcctctcggagaccag	ccacccaggactgaacaga
Ifnb1 -10 kb	tcctgcagcattcgtacaag	cattcctctctccccttgc
Ifnb1 +50 kb	tttctgttttctgtcggatcac	accaatagcgttgagagacca
Ccl2 promoter	tgtcgtggattgtgatagcatag	ggctctttgtccttccttctt
Ccl2 enhancer	cagagtgaagtgagagggaaac	aagcttcagttagcacaggag
Major satellite	catccacttgacgacttgaaaaatgacgaaatc	gccatattccaggtccttcagtgtgc
Telomere	cggtttgtttgggtttgggtttgggtttgggtttgggtt	ggcttgccttacccttacccttacccttacccttaccct

Analyses of protein retention in MEFs. The MEFs (200,000 cells plated per sample) were cultured as described above and G9a-GLP inhibitor or vehicle was added at the times indicated. Two different protocols were used to evaluate protein retention in MEFs:
MEF incubation in buffers with successively stronger detergents. The MEFs were permeabilized by resuspension in 0.2 ml of ice cold CSK buffer containing 0.1% triton X-100, 300 mM sucrose, 100 mM NaCl, 10 mM imidazole pH 7, and 2X Complete EDTA-free Protease Inhibitor Cocktail (Roche) for 30 min on ice. The MEFs were collected by 5 min centrifugation at 4 C. The MEFs were resuspended in in 50 μl ice cold CSK buffer with 0.5% triton X-100, for 30 min on ice. The MEFs were collected by 5 min centrifugation at 4 C. The MEFs were suspended in 50 μl CSK buffer with 1% SDS, for 30 min at RT. The MEFs were collected by 5 min centrifugation at RT. Each of the supernatants and the final pellet were adjusted to 1X SDS loading buffer (58.33 mM Tris·Cl, 5% glycerol, 2.5% SDS, 0.1 M DTT, 0.002% bromophenol blue, pH 6.8) and boiled for 10 min. The proteins were analyzed by polyacrylamide gel electrophoresis and immunoblotting.
MEF incubation in RIPA buffer. The MEFs were permeabilized by resuspending them in 50 μl ice cold RIPA buffer containing 1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM N-ethylmaleimide, and 2X Complete EDTA-free Protease Inhibitor Cocktail (Roche) for 30 min on ice. The MEFs were collected by 5 min centrifugation at 4 C. The supernatant and pellet were adjusted to 1X SDS loading buffer, boiled for 10 min, and analyzed by polyacrylamide gel electrophoresis and immunoblotting.
Antibodies: The αKeap1 (Aviva Systems Biology OACD04962, lot A20190909988) antibody was raised using recombinant Keap1 Met1-Gln286 for immunization. The $\alpha Keap1^{N}$ (Santa Cruz sc-15246, lot H2603) antibody has an epitope mapping near the N-terminus of human Keap1. The $\alpha Keap1^{C}$ (Proteintech 10503-2-AP, lot 00051883) antibody was raised using a recombinant human Keap1 325-624 fragment for immunization. The specificities of the three αKeap1 antibodies were evaluated by immunoblotting and by performing parallel RT-qPCR as well as ChiP-qPCR experiments using MEFs with *Keap1-/-* and intact *Keap1* alleles. The sources of the other antibodies were as follows: αp50/p105 (Cell Signaling 13586, D4P4d lot 2), αp65 (Cell Signaling 8242, lot 9), αphospho-p65-Ser536 (Cell Signaling 3031, lot 11), αG9a (Cell Signaling 3306, lot 2), αGLP (Abcam ab41969, lot GR191907-41), αH3K9me2 (Abcam ab1220, lots GR3228498-2, GR325223-3), αH3 (Abcam ab1791, lot GR3198255-1), αIRF3 (Santa Cruz sc-9082, lot G0910), αJun (Santa Cruz sc-1694, lot E0207), αvinculin (Cell Signaling 4650T, lot 4), αlaminB1 (Cell Signaling 13435, lot 2), normal rabbit IgG (Santa Cruz sc-2027, lot L2414), normal mouse IgG (Santa Cruz sc-2025, lot I2208).
Cell culture supplies and chemicals: DMEM (Gibco 11995) was supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals S11550 lot H1030) and 0.1 mg/ml penicillin-streptomycin (Gibco 15140). PBS (10010) and 0.05% trypsin-EDTA solution (25300) were from Gibco. The following compounds were obtained from Cayman Chemical: BIX01294 (13124, ≥98%), BRD4770 (11787 ≥98%), UNC0638 (10734, ≥98%), UNC0642 (14604, ≥95%). The following chemicals were obtained

from Millipore Sigma: tBHQ (112941, ≥98%) and MS012 (SML2174 ≥98%). These compounds were dissolved in ddH2O (BIX01294) or DMSO (all other compounds) at concentrations ranging from 20 to 50 mM. The final concentration of DMSO in experiments using this vehicle was 0.1%. Other chemicals and detergents were purchased from Millipore Sigma and Thermo Fisher Scientific.

Statistical analyses of the likelihood of chance occurrence of observed results. The data that are shown in each figure are representative of data that were obtained from multiple independent experiments that were performed using different sets of MEFs (numbers of different MEFs are specified in the Figure legends). Each graph shows the means and two times the standard deviation for replicate measurements from one experiment. The results of ANOVA analyses to test hypotheses using data from multiple independent experiments with different sets of MEFs are also indicated in each graph. The p-values for each set of experiments were corrected for multiple testing error by the method of Šidák. These p-values were compared to the value indicated in each figure legend to determine if the test supported the acceptance (ns) or rejection (*) of the null hypotheses. The ANOVA tests were performed using the Real Statistics Microsoft Excel plug-in v. 6.8 (Copyright (2013 – 2020) Charles Zaiontz. www.real-statistics.com). ANOVA analyses were performed using data that were obtained from independent experiments, and where the mean value for one of set of measurements was at least 3-fold higher than the value for the negative control (e.g. IgG in ChIP experiments).

Supplementary References

1. Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, et al. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell. 2009;138(1):114-28.
2. Burns V, Kerppola TK. Opposite orientations of a transcription factor heterodimer bind DNA cooperatively with interaction partners but have different effects on interferon-beta gene transcription. The Journal of biological chemistry. 2012;287(38):31833-44.
3. Tong AJ, Liu X, Thomas BJ, Lissner MM, Baker MR, Senagolage MD, et al. A Stringent Systems Approach Uncovers Gene-Specific Mechanisms Regulating Inflammation. Cell. 2016;165(1):165-79.
4. Mulvaney KM, Matson JP, Siesser PF, Tamir TY, Goldfarb D, Jacobs TM, et al. Identification and Characterization of MCM3 as a Kelch-like ECH-associated Protein 1 (KEAP1) Substrate. Journal of Biological Chemistry. 2016;291(45):23719-33.
5. Ea C-K, Hao S, Yeo KS, Baltimore D. EHMT1 Protein Binds to Nuclear Factor-κB p50 and Represses Gene Expression. Journal of Biological Chemistry. 2012;287(37):31207-17.
6. Fang TC, Schaefer U, Mecklenbrauker I, Stienen A, Dewell S, Chen MS, et al. Histone H3 lysine 9 di-methylation as an epigenetic signature of the interferon response. The Journal of Experimental Medicine. 2012;209:661-9.
7. Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro ML, et al. Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell. 2007;25(3):473-81.
8. Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, et al. A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat Chem Biol. 2011;7(8):566-74.
9. Yuan Y, Wang Q, Paulk J, Kubicek S, Kemp MM, Adams DJ, et al. A Small-Molecule Probe of the Histone Methyltransferase G9a Induces Cellular Senescence in Pancreatic Adenocarcinoma. ACS Chemical Biology. 2012;7(7):1152-7.
10. Xiong Y, Li F, Babault N, Dong A, Zeng H, Wu H, et al. Discovery of Potent and Selective Inhibitors for G9a-Like Protein (GLP) Lysine Methyltransferase. J Med Chem. 2017;60(5):1876-91.

11. Mildenberger J, Johansson I, Sergin I, Kjobli E, Damas JK, Razani B, et al. N-3 PUFAs induce inflammatory tolerance by formation of KEAP1-containing SQSTM1/p62-bodies and activation of NFE2L2. Autophagy. 2017;13(10):1664-78.
12. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997;236(2):313-22.
13. Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, et al. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. Nat Genet. 2003;35(3):238-45.