Supplementary materials for

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

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List of supplementary materials

Fig. S1

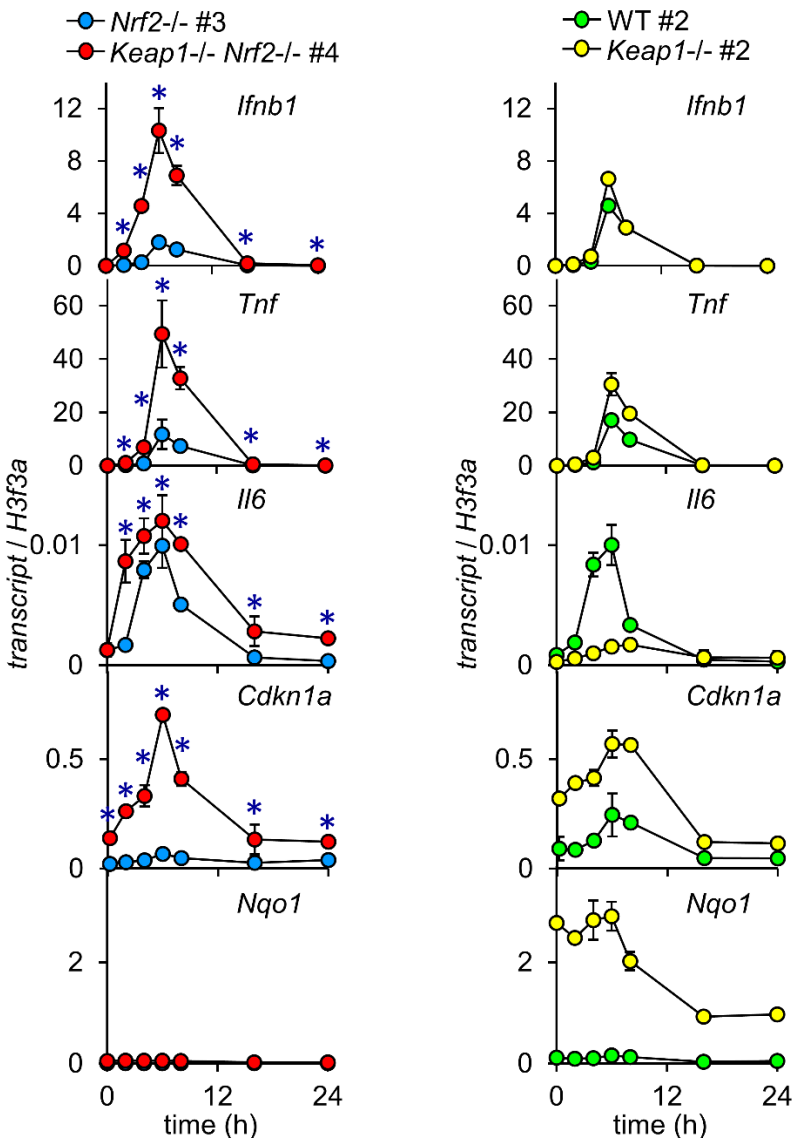

Figure S1. Keap1 moderates the transcription of virus induced genes through mechanisms that did not require Nrf2, and that were distinct from Keap1 regulation of electrophile response gene transcription (related to Fig. 1).

We compared the effects of Keap1 on virus induced (*Ifnb1*, *Tnf* and *Il6*), cell cycle (*Cdkn1a*), and electrophile response gene (*Nqo1*) transcription in MEFs with intact *Nrf2* and in MEFs with *Nrf2-/-* deletions. WT MEFs and MEFs with the *Keap1-/-* and/or *Nrf2-/-* deletions indicated above the graphs were infected with Sendai virus. The levels of the transcripts indicated in the graphs were measured at the times after virus infection indicated at the bottom. The transcripts in *Keap1+/+ Nrf2-/-* #3 and *Keap1-/- Nrf2-/-* #4 MEFs (left graphs, reproduced from Fig. 1A), and in wild type (WT) #2 and *Keap1-/-* #2 MEFs (right graphs) were analyzed in the same experiment. *Keap1-/-* deletions enhanced virus induced gene transcription in MEFs that also had *Nrf2-/-* deletions, whereas *Keap1-/-* deletions caused constitutive *Nqo1* electrophile response gene transcription only in MEFs with intact *Nrf2*. The reproducibility of *Keap1-/-* effects on the RT-qPCR transcript signals were evaluated in 2-7 different sets of MEFs by performing two-factor ANOVA analyses (* $p<0.0005$).

We investigated potential relationships between the effects of Keap1 on virus induced genes and on electrophile response genes by comparing Keap1 effects on Ifnb1, Tnf and Il6 transcription, and on Nqo1 transcription. Keap1 moderated Ifnb1, Tnf and Il6 transcription in MEFs with Nrf2-/- deletions (Fig. 1). In contrast, Keap1 had little effect on Nqo1 transcription in MEFs with Nrf2-/- deletions, but repressed Nqo1 in MEFs with intact Nrf2 (Fig. S1). The smaller effects of Keap1 on Ifnb1, Tnf and Il6 transcription in MEFs with intact Nrf2 are likely to be due to the reduced growth and NFκB induction in MEFs with Keap1-/- deletions and intact Nrf2 (11).

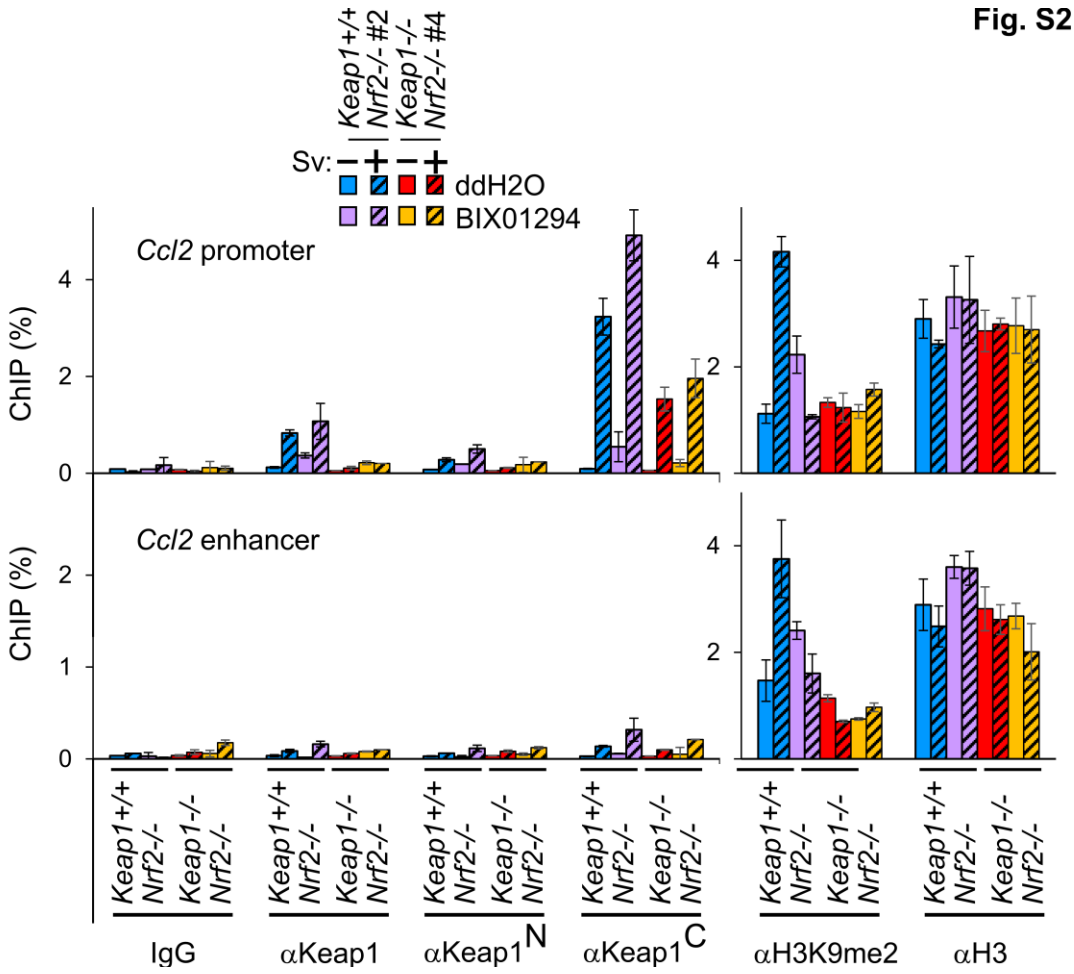

Figure S2. Antibodies that were raised against different regions of Keap1 detect Keap1 binding to chromatin, and the augmentation of Keap1 recruitment by BIX01294 (related to Fig. 1, 2, 3). Several anti-Keap1 antibodies detect Keap1 binding to chromatin and the augmentation of Keap1 recruitment by BIX01294. MEFs with intact Keap1 (cool colors) and MEFs with *Keap1-/-* deletions (warm colors), each with *Nrf2-/-* deletions, were cultured with 20 μM BIX01294 or vehicle starting one hour before infection. Chromatin was isolated 6 h after mock (solid bars) or virus (striped bars) infection. The Keap1, H3K9me2, and total H3 levels were measured at the *Ccl2* promoter (upper graph) and at the *Ccl2* enhancer (lower graph) by ChIP-qPCR using the antibodies indicated at the bottom of the figure. Antibodies that were raised against different regions of Keap1 precipitated the same regions of virus induced genes. The αKeap1 and $\alpha Keap1^{N}$ antibodies produced ChIP signals only in MEFs with intact *Keap1.* The αKeap1 and $\alpha Keap1^{N}$ ChIP signals in *Keap1-/-* MEFs were not significantly different from the signals observed with control IgGs. The $\alpha Keap1^{C}$ antibodies produced ChIP signals also in *Keap1-/-* MEFs. BIX01294 augmented the ChIP signals of all of the anti-Keap1 antibodies, but it had no effect on the αH3 ChIP signals. The αKeap1 ChIP signals were 6- to 22-fold higher on average than the IgG signals at promoters, and 7- to 9-fold higher on average than the αKeap1 ChIP signals in flanking regions. We used the αKeap1 antibodies in subsequent experiments since they were the most selective.

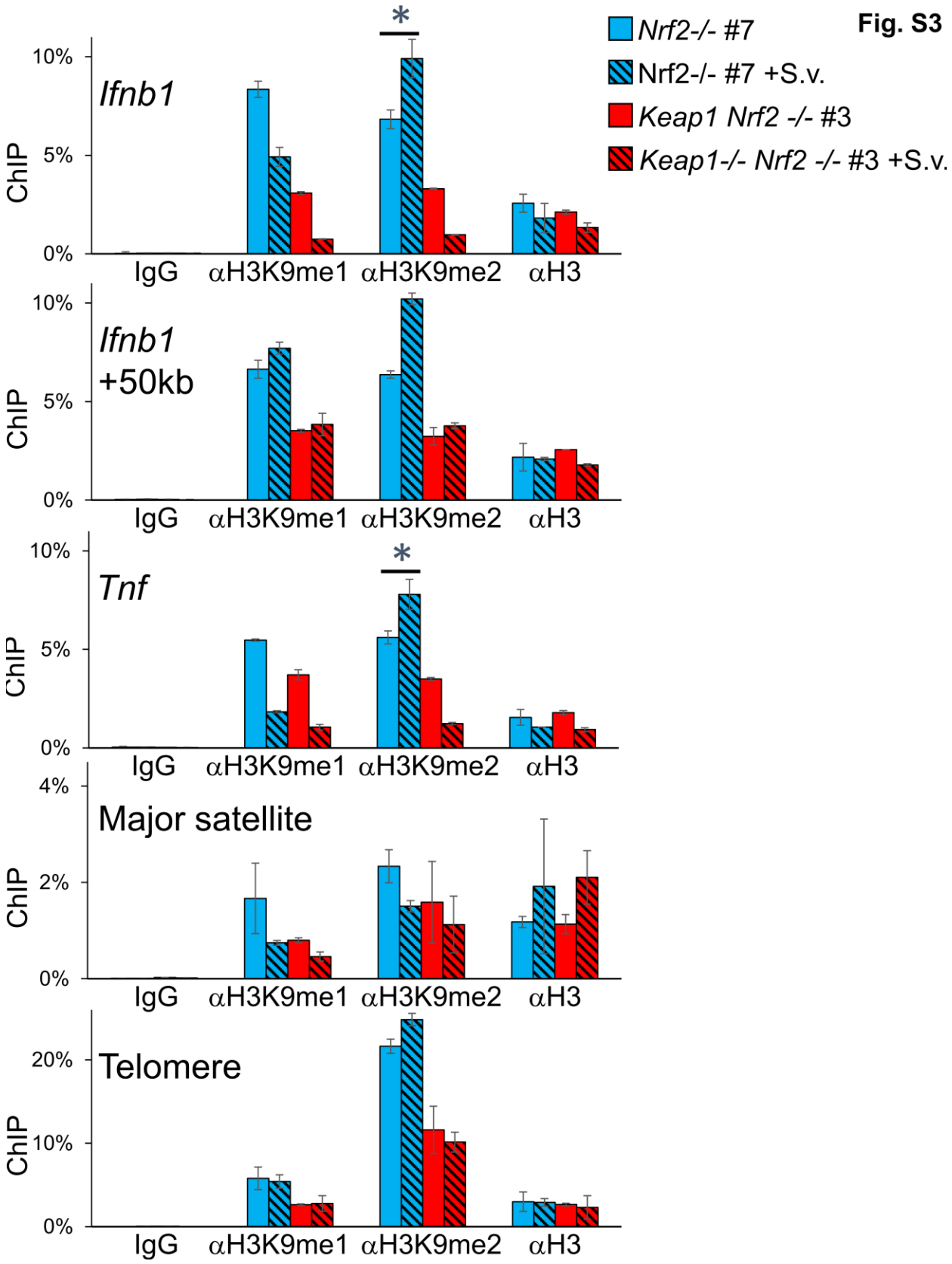

Figure S3. Keap1 is required for virus induced H3K9me2 deposition in regions encompassing virus induced genes (related to Fig. 1, 2, 3).

MEFs with intact Keap1 (blue bars) and MEFs with *Keap1-/-* deletions (red bars), each with *Nrf2-/-* deletions, were infected with mock (solid bars) or Sendai virus (S.v., striped bars). The levels of H3K9me1, H3K9me2 and total H3 were measured 6 hours after infection at the genes and the regions indicated in the graphs using the antibodies indicated. The bar graphs show the results (mean±2*SD) of a representative experiment in which *Keap1+/+ Nrf2-/-* #7 and *Keap1-/- Nrf2-/-* #3 MEFs were compared. The reproducibility of the effects of virus infection on H3K9me2 levels were evaluated in 2-5 different sets of MEFs by two-factor ANOVA analyses (* $p<0.001$, blue – increase, red – decrease).

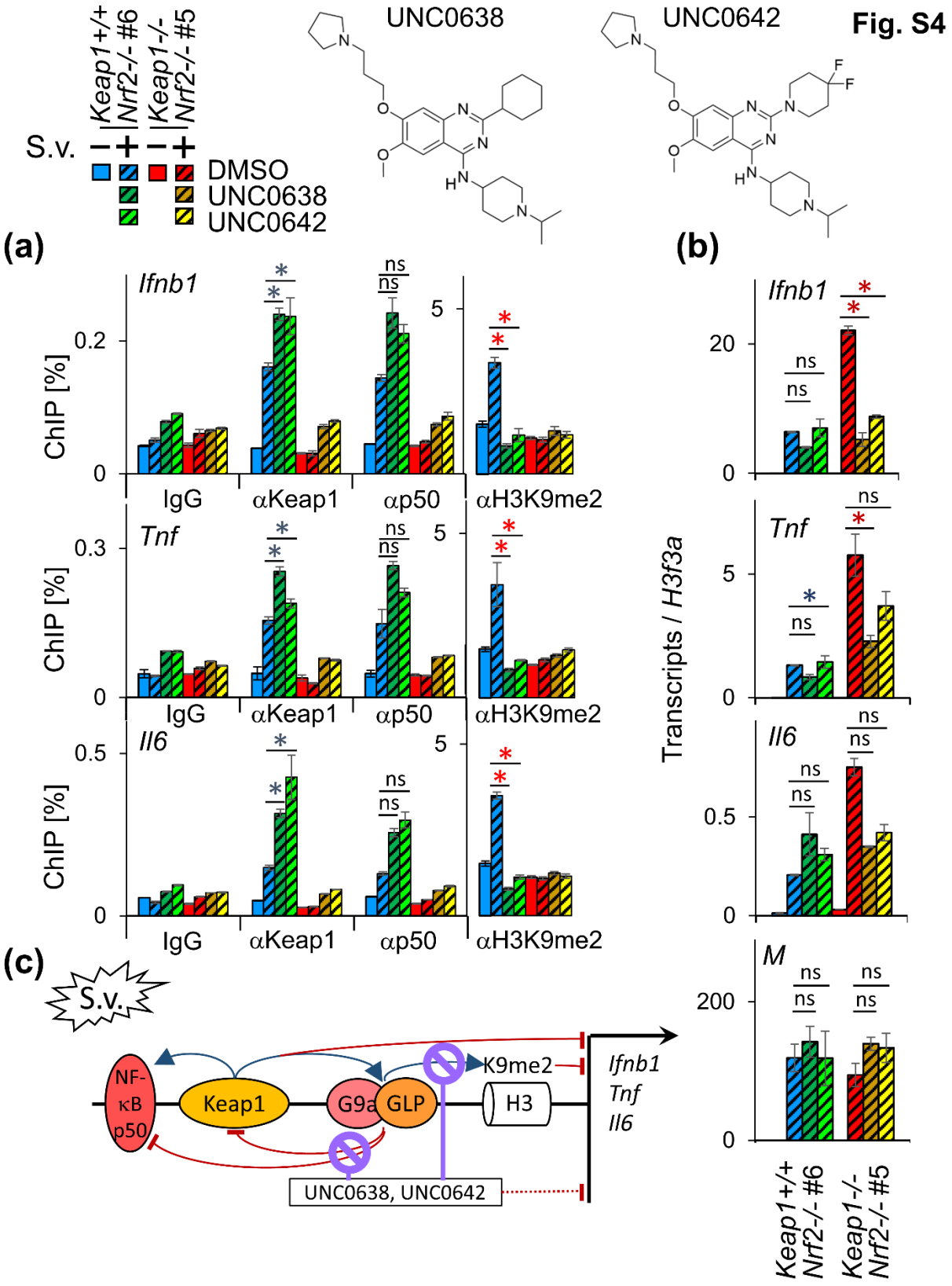

Figure S4. UNC0638 and UNC0642 effects on Keap1 and NFκB p50 recruitment to virus induced genes correlate with their inhibition of H3K9me2 deposition, but not with their effects on transcription (related to Fig. 3).

(a) UNC0638 and UNC0642 effects on Keap1 and NFκB p50 recruitment correlate with their effects on H3K9me2 deposition at virus induced genes. MEFs with intact Keap1 (cool colors) and MEFs with *Keap1-/-* deletions (warm colors), each with *Nrf2-/-* deletions, were cultured with 10 μM UNC0638, 10 μM UNC0642, or vehicle starting 48 hours before infection. The Keap1, NFκB p50, H3K9me2 and H3K27me3 levels were measured 6 hours after mock (solid bar) or virus (striped bars) infection at the genes indicated in the graphs using the antibodies indicated at the bottom. The graphs show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #6 and *Keap1-/- Nrf2-/-* #5 MEFs were compared. The MEFs that were analyzed in panels A and B were cultured in parallel, and the legend at the top of the figure applies to both panels. The reproducibility of UNC0638 and UNC0642 effects on Keap1, NFκB p50, and H3K9me2 ChIP signals were evaluated in 2-3 different sets of MEFs by performing two-factor ANOVA analyses (* $p<0.005$, blue – increase, red – decrease). The effects of BIX01294, MS012 and BRD4700 on Keap1, NFκB p50, and H3K9me2 levels at virus induced genes are shown in Fig. 3A. UNC0642 and UNC0638 differ from BIX01294 and MS012 by a (3-pyrrolidin-1-yl-)propoxy substituent, which binds to the lysine recognition pocket in G9a (24). UNC0638 and UNC0642 augmented Keap1 and NFκB p50 recruitment with different efficiencies in different experiments. These effects correlated with differences in their inhibition of virus induced H3K9me2 deposition (compare Fig. 4 and S4A).

(b) UNC0638 and UNC0642 influence virus induced gene transcription through mechanisms that are independent of Keap1. MEFs with intact Keap1 (cool colors) and MEFs with *Keap1-/-* deletions (warm colors), each with *Nrf2-/-* deletions, were cultured with 10 μM UNC0638, 10 μM UNC0642, or vehicle starting 48 hours before infection. The levels of the transcripts indicated in the graphs were measured by RT-qPCR 6 hours after mock (solid bar) or virus (striped bars) infection. The graphs show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #6 and *Keap1-/- Nrf2-/-* #5 MEFs were compared. The reproducibility of UNC0638 and UNC0642 effects on RT-qPCR transcript signals were evaluated in 2 different sets of MEFs by performing two-factor ANOVA analyses (* $p<0.001$, blue – increase, red – decrease). The effects of BIX01294, MS012 and BRD4700 on virus induced gene transcription are shown in Fig. 3B. UNC0638 and UNC0642 reduced the difference in transcription of these genes between MEFs with intact *Keap1* and *Keap1-/-* MEFs through mechanisms that were, at least in part, independent of Keap1.

(c) The diagram shows effects of UNC0638 and UNC0642 on Keap1, NFκB p50 and H3K9me2 levels, and on transcription at virus induced genes. The blue arrows indicate effects that are required for recruitment or for deposition. The red arcs and bars indicate effects that inhibit or moderate recruitment, deposition, or transcription. The dotted line reflects the observation that UNC0638 and UNC0642 inhibited virus induced gene transcription by mechanisms that were independent of Keap1 and H3K9me2 deposition.

Fig. S5

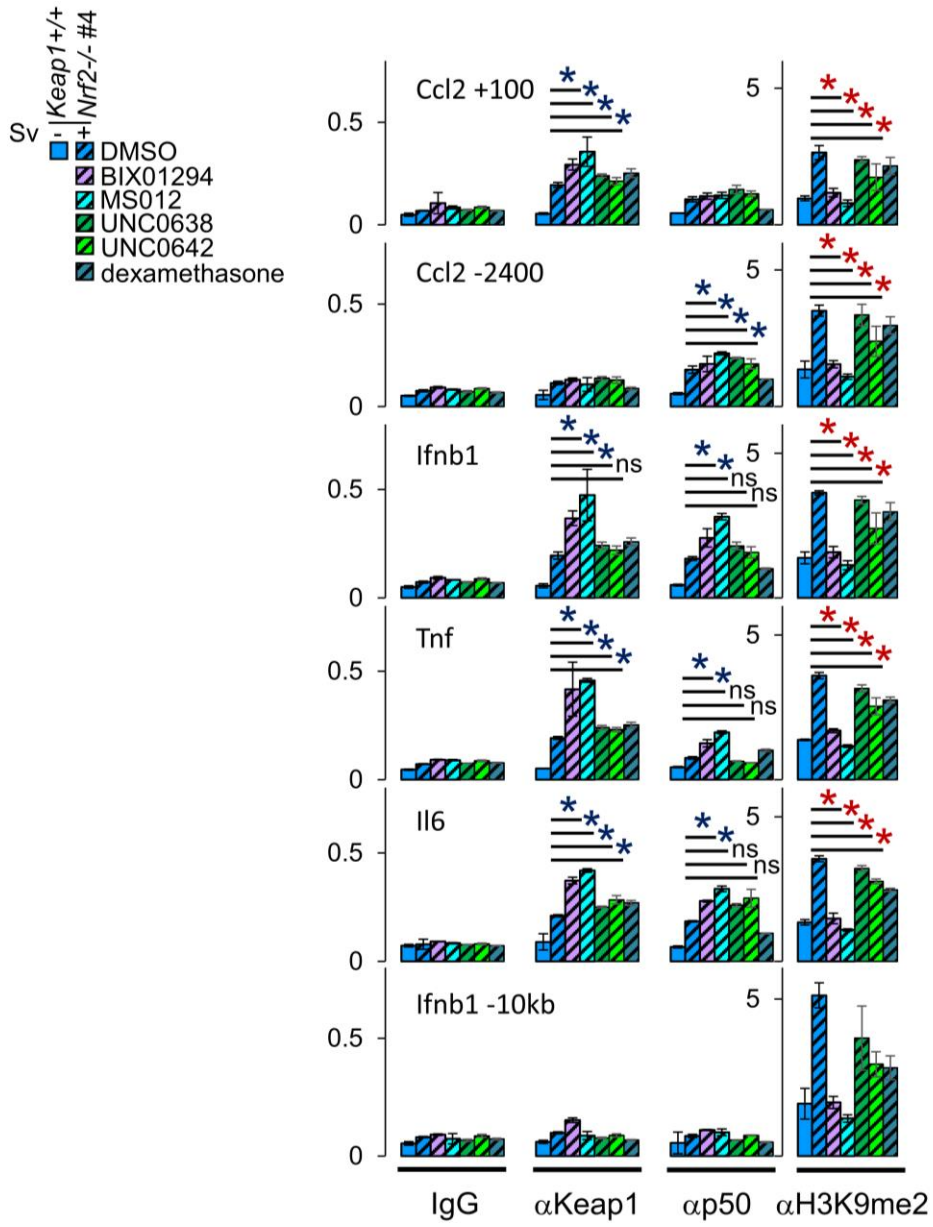

Figure S5. Comparison of the effects of different G9a-GLP inhibitors on Keap1 and NFκB p50 binding to virus induced genes, and on H3K9me2 deposition (related to Fig. 4).
Different G9a-GLP inhibitors have different effects on Keap1 and NFκB p50 recruitment that correlate with differences in their inhibition of H3K9me2 deposition at virus induced genes. The MEFs were cultured for one hour with 20 μM BIX01294, or for 48 hours with 1 μM MS012, 10 μM UNC0638, 10 μM UNC0642, or vehicle before infection. Chromatin was isolated 6 h after mock (solid bars) or virus (striped bars) infection. The Keap1, NFκB p50, and H3K9me2 levels were measured at the genes and the regions indicated in the graphs by ChIP-qPCR using the antibodies indicated at the bottom of the figure. The graphs show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #2 MEFs were compared. The reproducibility of the effects of different G9a-GLP inhibitors on Keap1, NFκB p50 and H3K9me2 ChIP signals were evaluated in 1-3 different sets of MEFs by performing two-factor ANOVA analyses (* $p<0.005$, blue – increase, red – decrease).

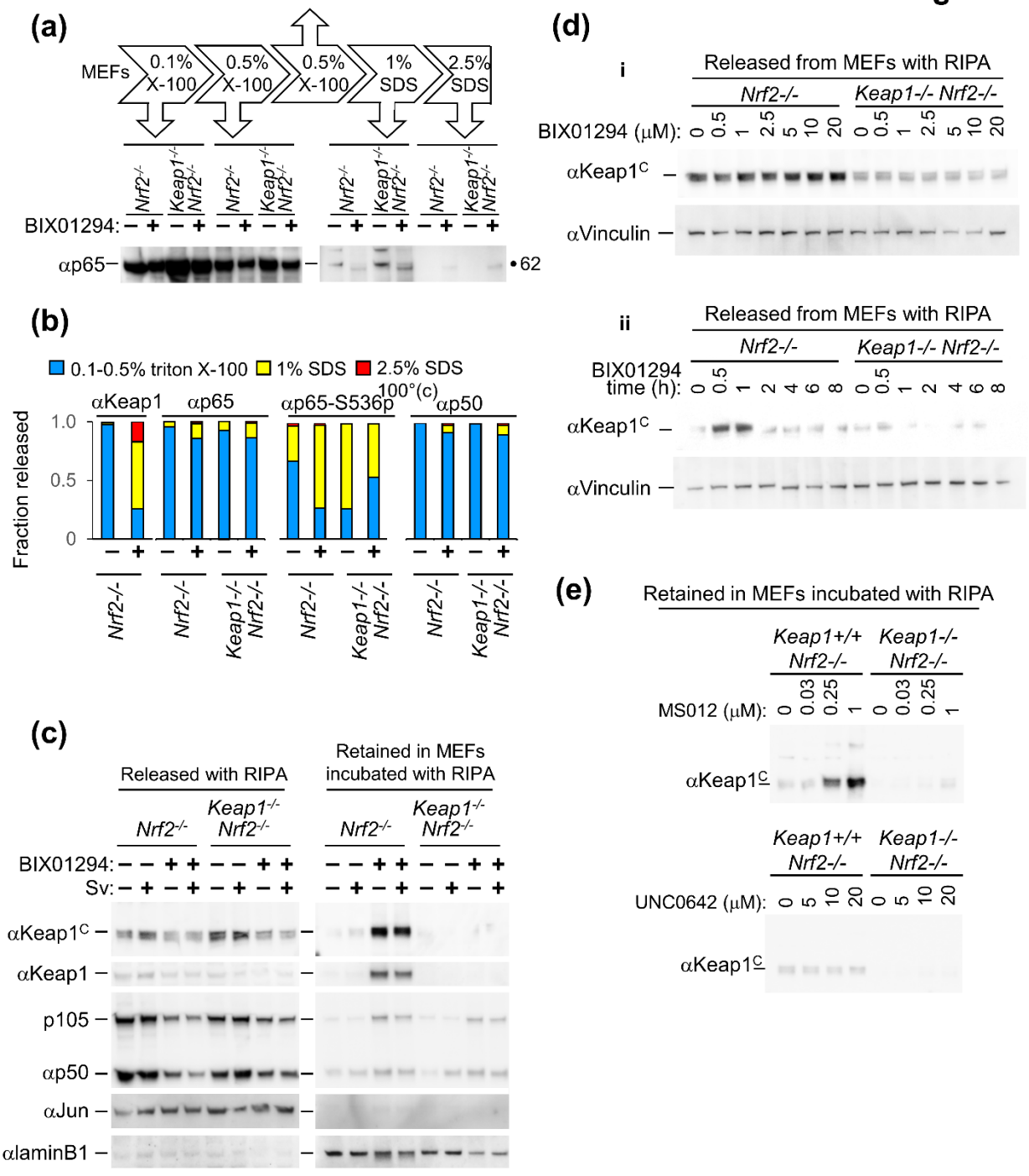

Figure S6. BIX01294 stabilizes Keap1 retention in MEFs during subsequent incubations with detergents (related to Fig. 5).

(a) NFκB p65 that was retained in MEFs that were cultured with and without BIX01294 has different electrophoretic mobilities. The samples that were prepared as described in Fig. 5A were separated on another gel to evaluate the reproducibility of the differences in the electrophoretic mobilities of NFκB p65 that was retained in MEFs that were cultured with *versus* without BIX01294. (b) Quantitation of the proportions of proteins that were released during sequential incubation with buffers containing the detergents that are indicated in the legend. The proportions of the proteins indicated above the bars that were released from MEFs that were cultured with BIX01294 (+) or with vehicle (-) were quantified using ImageJ. The proportions of the proteins that were released in the buffers that contained different detergents are indicated by the colors of the bar segments. The increased retention of Keap1 in MEFs that were cultured with BIX01294 is consistent with augmentation of Keap1 interactions by BIX01294.

(c) BIX01294 stabilizes Keap1 retention in MEFs during incubation in RIPA buffer and is detected by different anti-Keap1 antibodies. The MEFs that are indicated above the images were cultured with vehicle (-) or with 20 μM BIX01294 (+) starting 1 hour before infection. At 6 h after mock (-) or virus (+) infection, the MEFs were incubated in RIPA buffer (see Materials and Methods). The proteins that were released during the incubation (left images), and the proteins that were retained (right images) were analyzed by immunoblotting using the antibodies indicated to the left of the images. The retention of Keap1, NFκB p50, NFκB p105, cJun and lamin B is compared in the same MEFs. The images show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #8 and *Keap1-/- Nrf2-/-* #5 MEFs were compared. BIX01294 stabilized Keap1 retention in MEFs during incubations with different combinations of detergents.

(D) Effects of the concentration of BIX01294 and the time of culture on the release of Keap1 from MEFs. The MEFs that are indicated above the images were cultured with the indicated concentrations (μM) of BIX01294 for 4 h (i, upper panel), and for the indicated times (h) with 20 μM BIX01294 (ii, lower panel). After culture, the MEFs were incubated in RIPA buffer, and the proteins that were released from the MEFs were analyzed by immunoblotting. The images show αKeap1^{C} and αVinculin immunoblots of the supernatants containing the proteins that were released from the MEFs. The images show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #8 and *Keap1-/- Nrf2-/-* #5 MEFs were compared. A cross-reactive band that co-migrated with Keap1 was detected by the αKeap1^{C} antibodies in the fractions that were released from *Keap1-/- Nrf2-/-* MEFs that were incubated RIPA. This cross-reactive band was detected at lower levels in the fractions that were retained in the MEFs that were incubated with RIPA (Fig. 5B).

(e) Comparison of the effects of different G9a-GLP inhibitors on Keap1 retention in MEFs. The MEFs were cultured with the concentrations (μM) of the G9a-GLP inhibitors indicated above the lanes for 48 hours. After culture, the MEFs were incubated in RIPA buffer, and the proteins that were retained in the MEFs were analyzed by immunoblotting with αKeap1^{C} antibodies. The images show the results of a representative experiment in which *Keap1+/+ Nrf2-/-* #8 and *Keap1-/- Nrf2-/-* #5 MEFs were compared. Different G9a-GLP inhibitors enhanced Keap1 retention in MEFs with different potencies, consistent with the differences in their augmentation of Keap1 binding to specific genes.