

Supplementary Materials

Materials and Methods

Stable knockdown of HDACs in murine embryonic fibroblasts

For establishing a transient knockdown of HDAC1, HDAC2 and HDAC3, immortalized *Hdac3*^{fl/-} Mef cells were transfected with mouse pLKO.1-puro, or pLKO.1 encoding shRNAs derived from the TRC1 library (<http://www.broadinstitute.org/rnai/trc/lib>): pLKO.1-puro-sh*Hdac1* (cloneIDs TRCN0000039399, TRCN0000039400, TRCN0000039402, TRCN0000039403), pLKO.1-puro-sh*Hdac2* (cloneIDs TRCN0000039395, TRCN0000039396, TRCN0000039397, TRCN0000039398), pLKO.1-puro-sh*Hdac3* (cloneIDs TRCN0000039389, TRCN0000039390, TRCN0000039391, TRCN0000039392). For transient transfection 3.5x10⁴ Mef cells were seeded in 48 well plates. 270ng plasmid DNA per well was transfected using Lipofectamine[®] LTX and Plus[™] reagent from Invitrogen[™], following the manufacturer's instructions. 24h post transfection, transfected cells were selected for 48h by adding 1μg/ml puromycin.

Each shRNA-encoding vector was transfected in duplicate wells. One well was kept untreated, while the second well was stimulated with IL-1α (10ng/ml) for 3h. After harvesting the cells on ice in 1x PBS, the samples were prepared for RT-qPCR analysis using the TaqMan[®] PreAmp Cells-to-CT[™] Kit from Ambion[®], following the manufacturer's instructions. The expression of *mCxcl2* (Mm00436450_m1) and *mUbe2l3* (Mm00784559_s1) was determined by qPCR using the TaqMan[®] Fast universal PCR master mix and 7500 Fast real time PCR system from Applied Biosystems. Relative changes of *mCxcl2* mRNA expression compared to the unstimulated pLKO.1 control were normalized to the expression of *mUbe2l3* and quantified using the 2^{-ΔΔCt} method.

Chromatin immunoprecipitation (ChIP)

One 175-cm² flask of confluent KB cells (corresponding to 2.5 - 3.0 x10⁷ cells), treated as described in the figure legends, was used for each condition. Proteins bound to DNA

were cross-linked *in vivo* with 1% formaldehyde added directly to the medium. After 10 minutes incubation at room temperature, 0.1 M glycine was added for 5 minutes to stop the cross-linking. Then, cells were collected by scraping and centrifugation at 1,610 x g (5 minutes, 4°C), washed in cold PBS containing 1mM PMSF and centrifuged again at 1,610 x g (5 minutes, 4°C). Cells were lysed for 10 minutes on ice in 3ml ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.1, 1mM PMSF, Roche protease inhibitor mix). The DNA was sheared by sonication (7 x 30s on / 30s off, 4 times; Bioruptor, Diagenode) and lysates cleared by centrifugation at 16,100 x g at 4°C for 15 minutes. Supernatants were collected and stored in aliquots at -80°C for subsequent ChIP. For determination of DNA concentration 20µl of sheared lysate was diluted with 100µl TE buffer including 10µg/ml RNase A. After 30min at 37°C, 3.8µl proteinase K (20mg/ml) and 1% SDS was added and incubated for at least 2h at 37°C followed by overnight incubation at 65°C. Samples were resuspended in two volumes of buffer NTB (Macherey & Nagel) and DNA was purified using Nucleo Spin columns (Macherey & Nagel) according to the manufacturer's instructions. DNA was eluted with 50µl 5mM Tris pH 8.5 and concentration was determined by Nano Drop. For CHIP, the following antibodies were used: anti-histone H3 (2µg, Abcam; ab1791), anti-acetyl-histone H3 (K9) (2µg, Millipore; 07-352), anti-NF-κB p65 (3µg, Santa Cruz; sc-372), anti-HDAC3 (4µg, Millipore; 17-10238), anti-phospho-Pol II (S5) (1.35µg, Abcam; ab5131), IgG (2µg, Cell Signaling; 2729). Antibodies were added to precleared lysate volumes equivalent to 25µg of chromatin. Then, 900µl of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris/HCl pH 8.1) were added and the samples were rotated at 4°C overnight. Thereafter, 30µl of a protein A/G sepharose mixture, pre-equilibrated in ChIP dilution buffer was added to the lysates and incubation continued for 2h at 4°C. Beads were collected by centrifugation, washed once in 900µl ChIP low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.1, 150mM NaCl), once in 900µl ChIP high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.1, 500mM NaCl), once in 900µl ChIP LiCl buffer (0.25M LiCl, 1% NP40, 1% desoxycholate, 1mM EDTA, 10mM Tris pH 8.1) and twice in 900µl ChIP TE buffer (10mM Tris pH 8.1, 1mM EDTA) for 5 minutes at 4°C. Beads were finally resuspended in 100µl TE buffer including RNase A (10mg/ml). In parallel, 1/10 volume

(2.5µg) of the initial lysate (input samples) were diluted with 100µl TE buffer including 10µg/ml RNase A. After 30min at 37°C, 3.8µl proteinase K (20mg/ml) and 1% SDS were added and both, input and immunoprecipitates were incubated for at least 2h at 37°C followed by overnight incubation at 65°C. Samples were resuspended in two volumes of buffer NTB (Macherey & Nagel) and DNA purified using Nucleo Spin columns (Macherey & Nagel) according to the manufacturer's instructions. DNA was eluted with 50µl 5mM Tris pH 8.5 and stored at -20°C until further use.

Quantification of ChIP DNA by real-time PCR

PCR products derived from ChIP were quantitated by real time PCR using the Fast ABI 7500 instrument (Applied Biosystems). The following primers were used as described in (37). Hhuman *IL-8* promoter, sense 5'-aagaaaacttcgtcactactccg-3', antisense 5'-tggtttttatatacatcaccctac-3'; human *IL-8* upstream (negative control; gene free region 940bp upstream of *IL-8* transcriptional start site): sense 5'-atcatgggtcctcagaggtcagac-3', and antisense, 5'-ggtgggaggagggtgttatctaatg -3'. The reaction mixture contained 2µl of ChIP or input DNA (diluted 1:10 to represent 1% of input DNA), 0.25µM of primers and 10µl of Fast Sybr Green Mastermix (2x) (Applied Biosystems) in a total volume of 20µl. PCR cycles were as follows: 95°C (20s), 40x (95°C (3sec), 60°C (30sec)). Melting curve analysis revealed a single PCR product.. Calculation of enrichment by immunoprecipitation relative to the signals obtained for 1% input DNA was performed according to the following equation: percent of (input)= $2^{-(Ct_{\text{sample}}-Ct_{\text{input}})}$.

Supplementary Figures

Fig. S1. Apicidin inhibits IL-1-induced *IL-8* expression in tumor cells.

KB cells (upper graph) or A549 cells (lower graph) were pretreated for 24 h with increasing concentrations of the HDAC3 inhibitor apicidin, solvent control (DMSO, 1%), or were left untreated. Thereafter cells were stimulated for 3 h with IL-1 as shown. RT-qPCR was used to determine *IL-8* mRNA expression, bars show the mean *IL-8* expression \pm SEM relative to the unstimulated control (KB cells n=2, A549 cells n=3).

Fig. S2. Tamoxifen has no impact on IL-1-inducible *Cxcl2* expression.

Two immortalized Mef lines were treated for 72h with 10 μ M tamoxifen followed by 30 min of IL-1 treatment as shown or were left untreated as indicated. Total RNA was isolated and *Cxcl2* mRNA expression was quantified by RT-qPCR.

Fig. S3. shRNA-mediated suppression of HDAC3 in murine fibroblasts impairs IL-1-induced *Cxcl2* expression.

Hdac3^{fl/fl} Mefs were transfected with empty pLKO.1 or with pLKO.1 containing the indicated shRNA-expression cassettes directed against HDAC3. Cells were selected for two days in puromycin (1 μ g/ml), stimulated for 3 h with IL-1 or were left untreated. Thereafter mRNA expression of *Cxcl2* was analyzed by RT-qPCR and normalized to the expression of *Ube2l3* as described in the Methods section. Shown are mean fold changes \pm SEM relative to the vector control from duplicate determinations.

Fig. S4. TSA triggers histone H3 acetylation.

The indicated HEK293IL-1R control and HDAC3 knockdown cells were treated with TSA (300ng/ml, 24 h) or IL-1 as shown. Nuclear extracts were analyzed by Western blotting for TSA-mediated hyperacetylation of histones by use of antibodies against acetylated histone H3 (Ac-(K9/14)-H3). Antibodies against beta actin were used as a loading control, a representative experiment is shown.

Fig. S5. HDAC3-knockdown in human cells suppresses expression of several IL-1-response genes.

The indicated HEK293IL-1R control and HDAC3 knockdown cells were stimulated for various periods with IL-1 as indicated. RT-qPCR was used to quantify mRNA expression of the indicated human genes. Changes of mRNA expression are displayed relative to the untreated control. Error bars represent SEM from two independent experiments.

Fig. S6. Microarray-based identification of HDAC3-dependent IL-1-response genes in murine cells.

Hdac3^{fl/+} or *Hdac3*^{fl/-} Mefs were treated with tamoxifen- or IL-1 as described in the legend for Fig. 2C. RNA was isolated and labeled cRNAs were hybridized to whole murine genome Agilent microarrays. Fluorescence intensity values were used to calculate ratios of gene expression. **(A)** Depicted are color-coded ratio values for 95 genes which were regulated by IL-1 by at least 1.5-fold in the same direction in both independent experiments. Green vertical bars indicate all genes with at least twofold regulation by IL-1. Yellow bars indicate genes whose expression was reduced in the HDAC3 knockout by a least twofold in two independent experiments. **(B)** Depicted are color-coded ratios values for 490 genes which were regulated by at least twofold in response to tamoxifen. In this group, only 7 genes (1.4%) were also responsive to IL-1 as indicated by red bars.

Fig. S7. Identification of p65 K4Q or p65 K5Q-suppressed or -induced genes.

The entire data set which was retrieved by sequential filtering of the expression values of the microarray experiments as described in the legend of Fig. 9C and D is shown. Depicted are color-coded ratio values for IL-1-regulated (green vertical bars) and p65 NF-κB-dependent genes **(A)** and for genes regulated by p65 K4Q or by K5Q mutants **(B)**. Numbers 1-24 indicate experimental conditions as described in the legend of Fig. 9C, D. For further details see the Material & Methods section.

Fig. S8. DNA-binding of p65 is not required for HDAC3-mediated deacetylation.

HEK293T cells were transfected with expression plasmids encoding His-tagged p65 wild type or a DNA-binding mutant (p65 E39I-His) along with YFP-CBP and increasing

concentrations of an expression vector for HDAC3-Flag (0.3 μ g and 1 μ g) as shown. One fraction of cells was purified under denaturing conditions by Ni-NTA columns and analyzed for p65 acetylation as displayed, another fraction was analyzed for adequate protein expression.

Fig. S9. p65 NF- κ B is deacetylated by HDAC1 or HDAC2 but not by HDAC5 and HDAC8.

HEK293IL-1R cells were transiently transfected to express wild type His-tagged p65 along with untagged p300, HA-tagged HDAC3, FLAG-tagged HDAC2, 5, 8 or MYC-tagged HDAC1. Cells were lysed under denaturing conditions, proteins were precipitated by TCA, redissolved and equal amounts were analyzed for general acetylation of p65 with a pan-acetyl-lysine specific antibody or with antibodies specific for acetylated p65 K310, K314 and K315 as shown. Expression of p65, HDACs and p300 was validated as indicated and equal loading of lanes was confirmed by anti- β -actin antibodies. The position of a molecular weight marker is indicated at the left.

Fig. S10. shRNA-mediated suppression of HDAC2, or HDAC3 in murine fibroblasts impairs IL-1-induced *Cxcl2* expression.

Hdac3^{fl/-} Mefs were transfected with empty pLKO.1 or with pLKO.1 containing the indicated shRNA-expression cassettes directed against HDAC1 (A), or HDAC2 (B). Cells were selected for two days in puromycin (1 μ g/ml), stimulated for 3 h with IL-1 (10ng/ml) or were left untreated. Thereafter mRNA expression of *Cxcl2* was analyzed by RT-qPCR and normalized to the expression of *Ube2l3* as described in the Methods section. Shown are mean fold changes +/- SEM relative to the vector control from duplicate determinations.

Fig. S11: HDAC3 deletion does not affect TNF-induced genes.

(A) Wild type (*Hdac3*^{fl/+}) or knockout (*Hdac3*^{fl/-}) Mefs were treated and analyzed exactly as described in the legend of Fig.2B except that cells were stimulated for 30 min with TNF (20ng/ml) instead of IL-1. RT-qPCR was used to quantify *Cxcl2* mRNA expression. Shown are the mean -fold changes compared to the untreated *Hdac3*^{fl/+}

control line, error bars represent SEM from three different experiments performed in duplicates. **(B)** *Hdac3*^{fl/+} or *Hdac3*^{fl/-} Mefs were stimulated as described in (A) followed by RNA isolation and analysis of gene expression on whole murine genome Agilent microarrays. Fluorescence intensity values were used to calculate ratios of gene expression. Depicted are color-coded ratio values for 76 genes which were regulated by TNF by at least 1.5-fold in the same direction in both independent experiments. Purple vertical bars indicate all genes with at least twofold regulation by TNF. Blue bars indicate genes whose expression was reduced in the HDAC3 knockout by a least twofold in two independent experiments. Green bars indicate the overlap with IL-1-induced genes as shown in suppl. Fig.6B.

Supplementary Tables

Table S1. Overlap between HDAC3-dependent IL-1 response genes and p65-dependent genes.

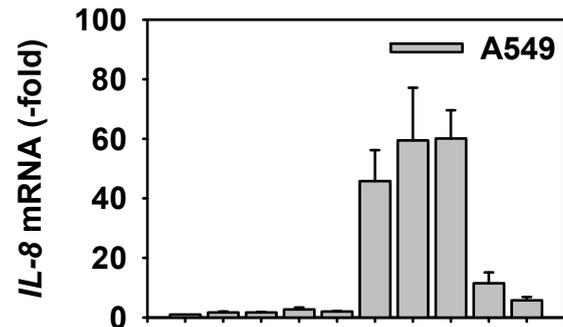
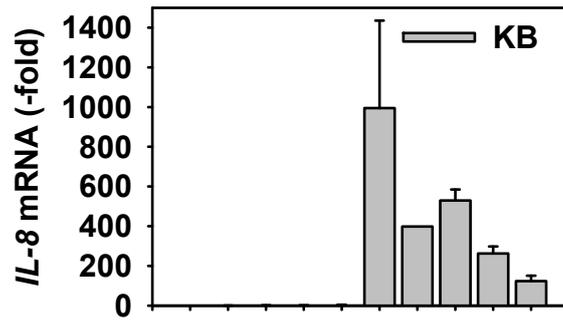
The data set shown in Fig. S6 was used to extract 40 genes which are IL-1-dependent and whose expression is reduced by twofold in at least one of the two experiments. Using data described in Fig.9 and suppl. Fig. 7 the dependency of these genes on p65 NF- κ B or its mutants is depicted.

Table S2. HDAC3 is a global regulator of the transcriptional IL-1 response in human cells.

Shown are gene identifiers, fluorescence intensity values and relative ratios of gene expression corresponding to the results depicted in Fig. 7B. Twofold regulated genes are indicated by yellow or green colors, respectively.

Table S3. Microarray-based identification of HDAC3-dependent IL-1-response genes in murine cells..

Shown are gene identifiers, fluorescence intensity values and relative ratios of gene expression corresponding to the results depicted in Fig. S6A. Twofold regulated genes are indicated by yellow or green colors, respectively.



apicidin (µg/ml, 24h)		.1	1	10		.1	1	10
DMSO	+				+			
IL-1					+	+	+	+

Figure S1

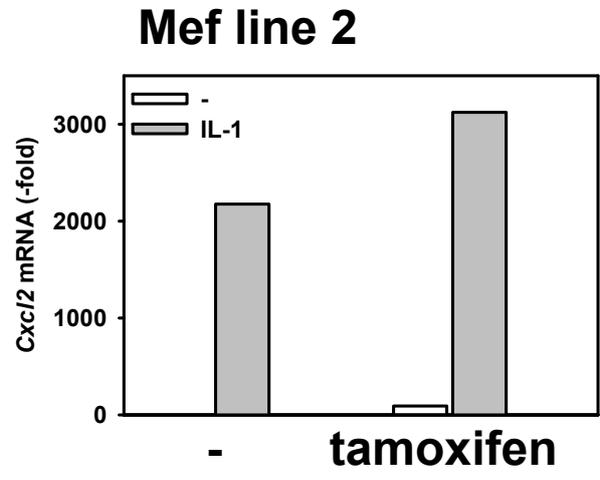
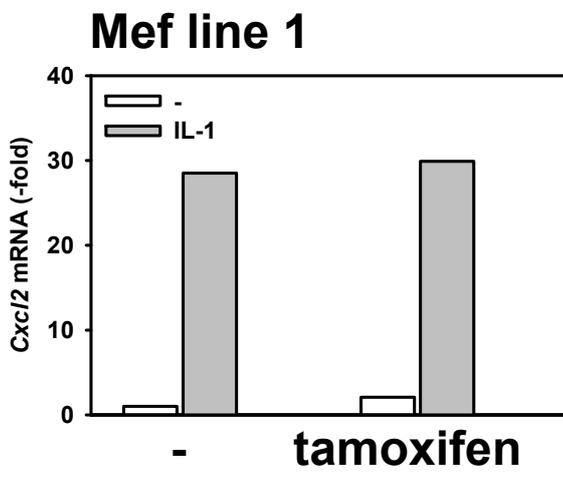


Figure S2

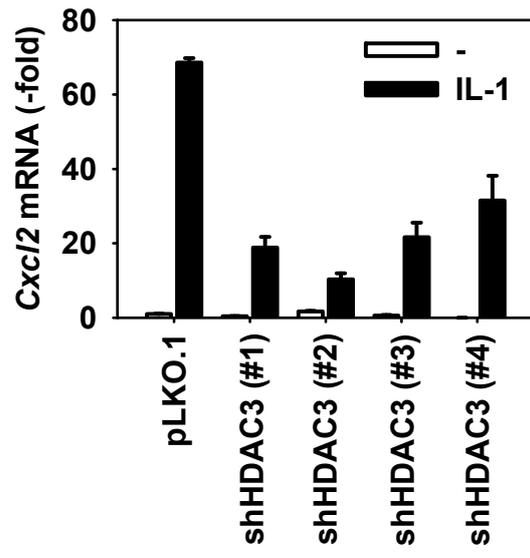


Figure S3

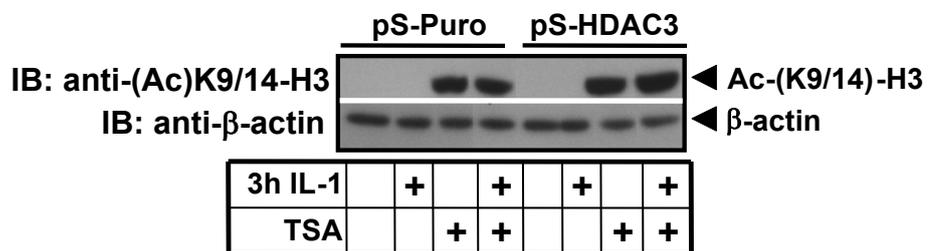


Figure S4

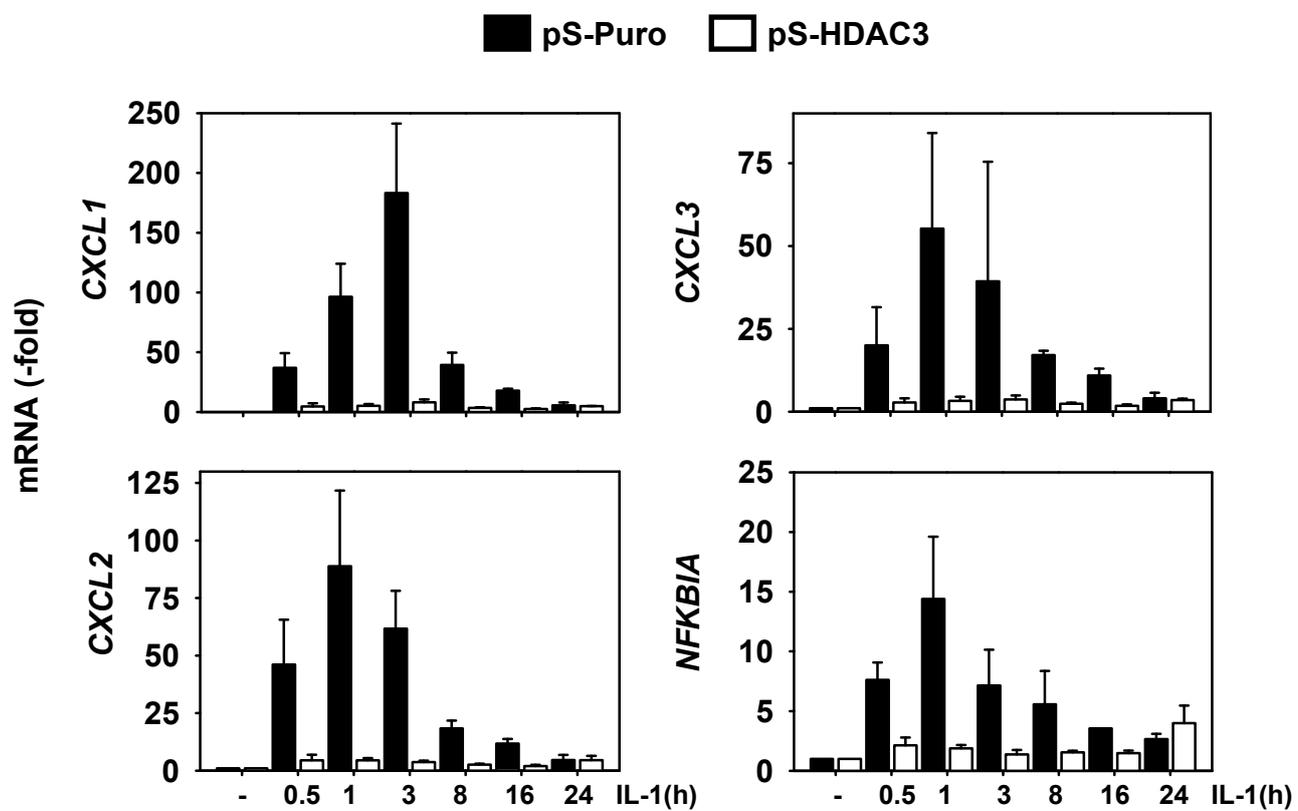
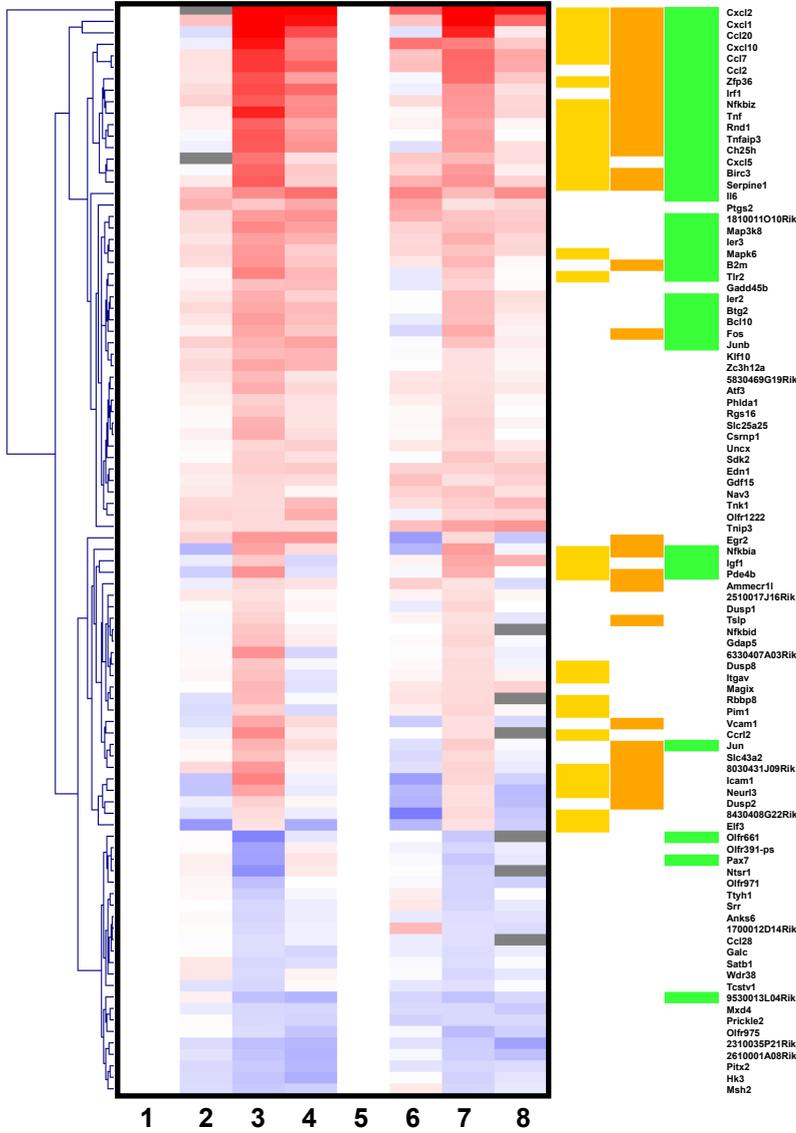


Figure S5

A



tamoxifen		+		+		+		+
IL-1			+	+			+	+
<i>Hdac3</i> ^{fl/-} <i>Cre-ER</i> ⁺	+	+	+	+	+	+	+	+
exp.	1	1	1	1	2	2	2	2

- 29 IL-1-regulated genes suppressed by 2-fold in *Hdac3*^{-/-} cells in exp. 1
- 31 IL-1-regulated genes suppressed by 2-fold in *Hdac3*^{-/-} cells in exp. 2
- 35 IL-1-regulated genes by 2-fold in exp. 1 and exp. 2

Figure S6

B

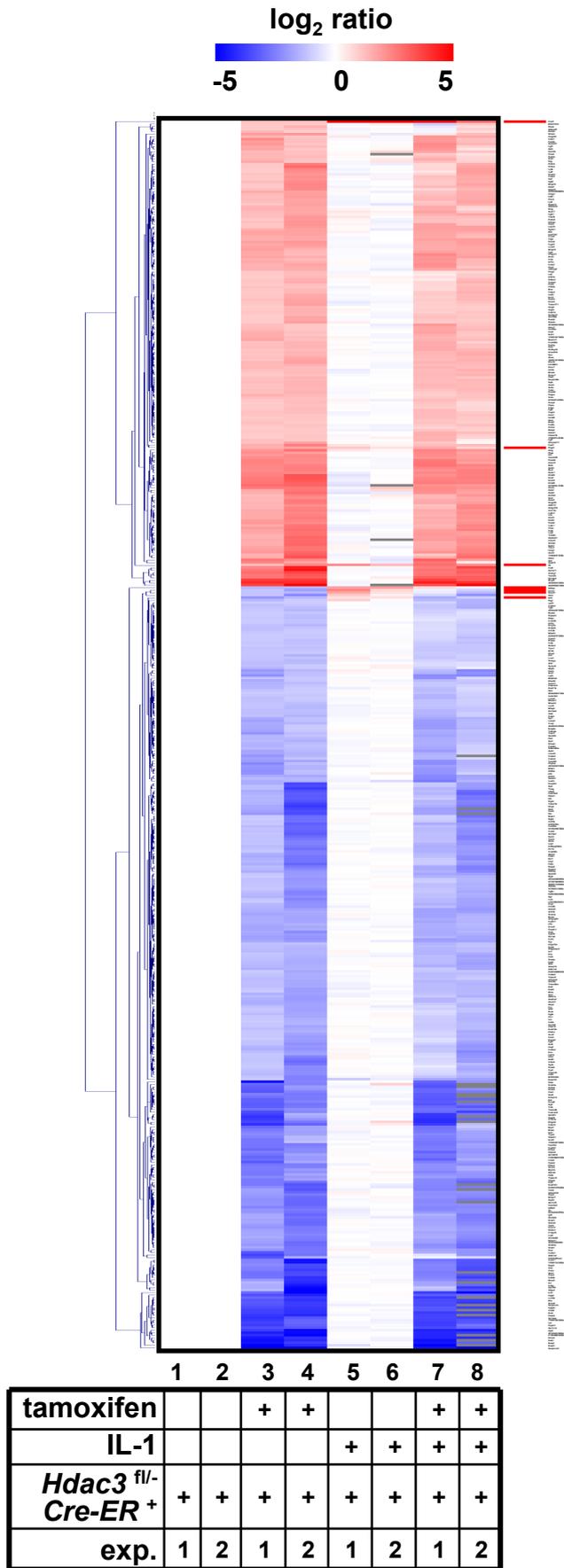


Figure S6

A

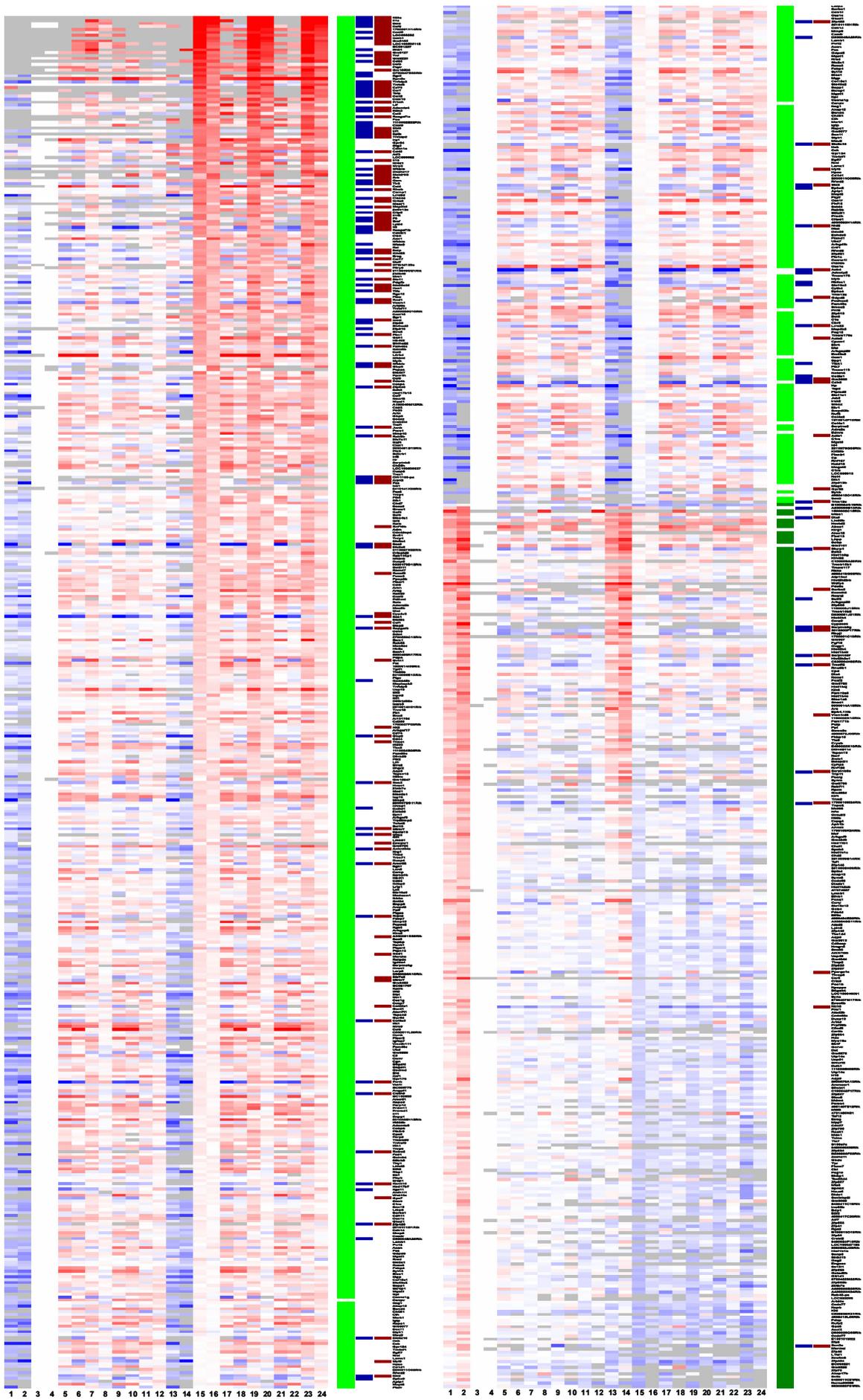


Figure S7

B

- p65 K4Q-induced genes
- p65 K4Q-repressed genes
- p65 K5Q-induced genes
- p65 K5Q-repressed genes
- IL-1-induced genes

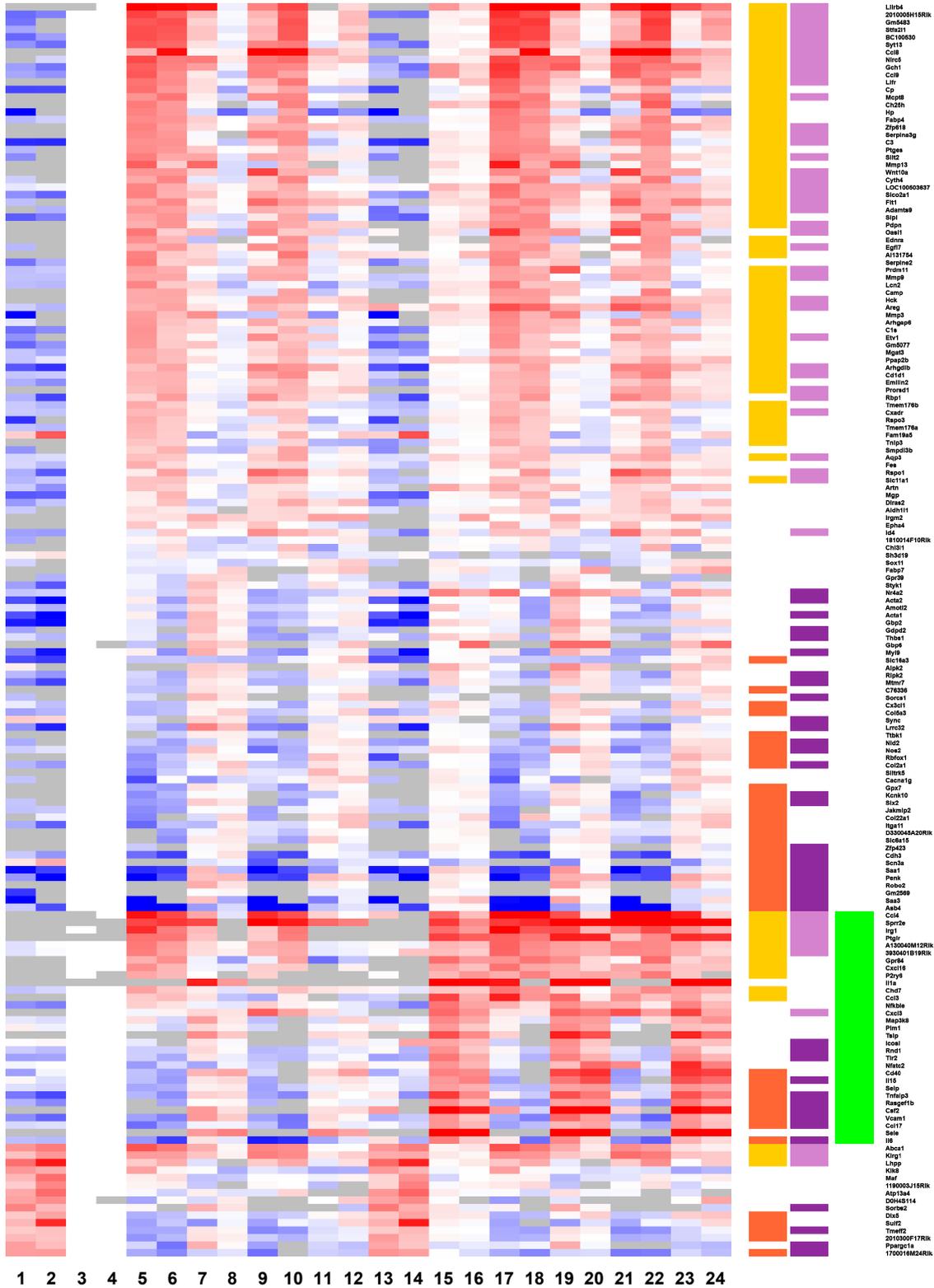


Figure S7

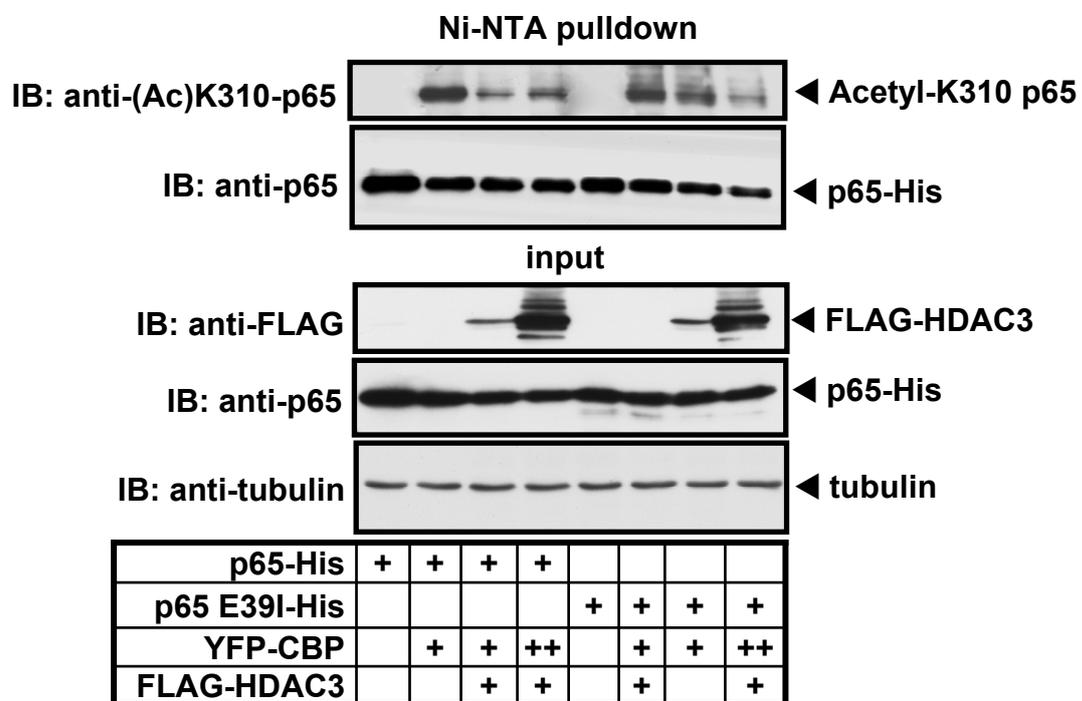
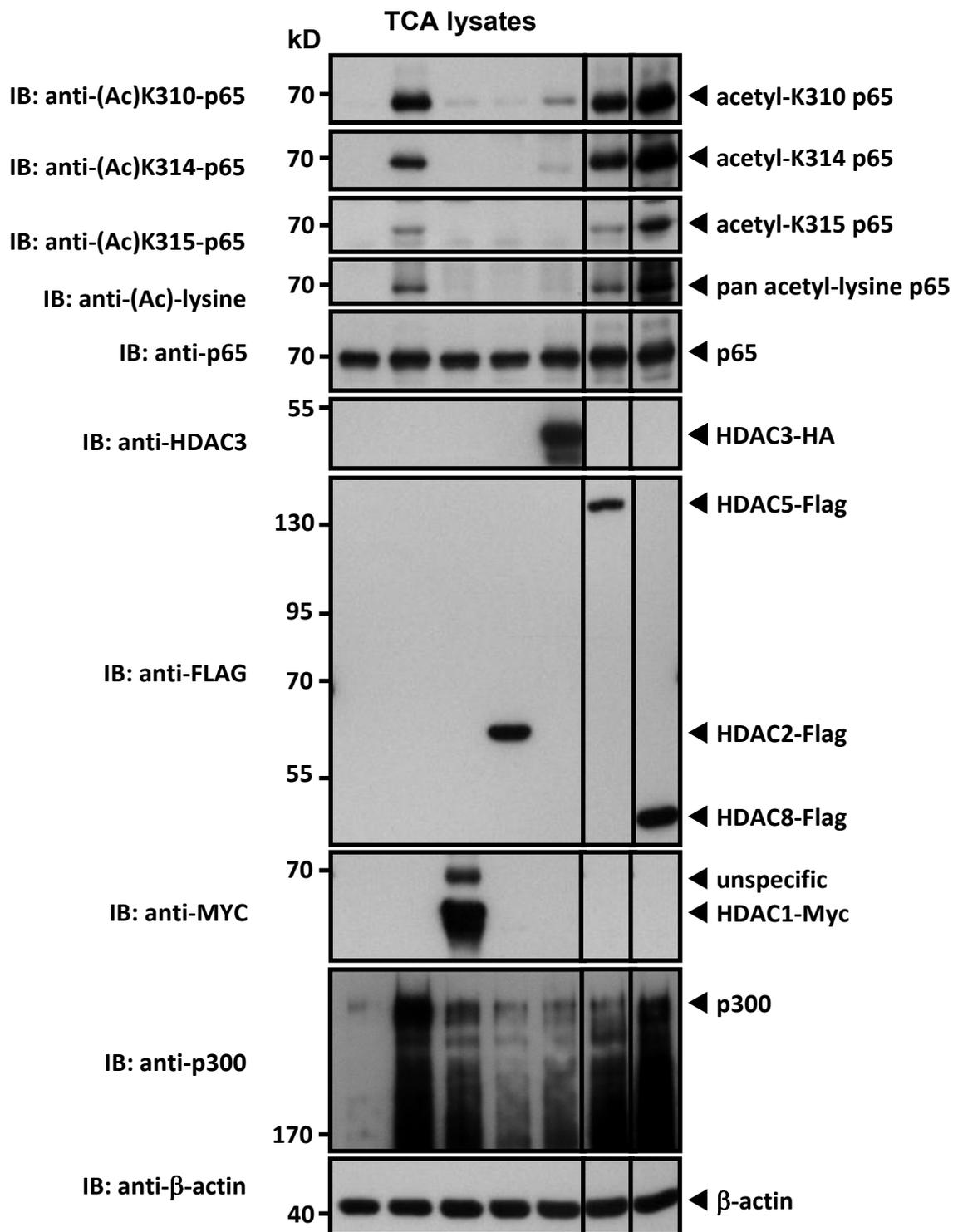


Figure S8



p65-His	+	+	+	+	+	+	+
p300		+	+	+	+	+	+
HDAC1-MYC			+				
HDAC2-FLAG				+			
HDAC3-HA					+		
HDAC5-FLAG						+	
HDAC8-FLAG							+

Figure S9

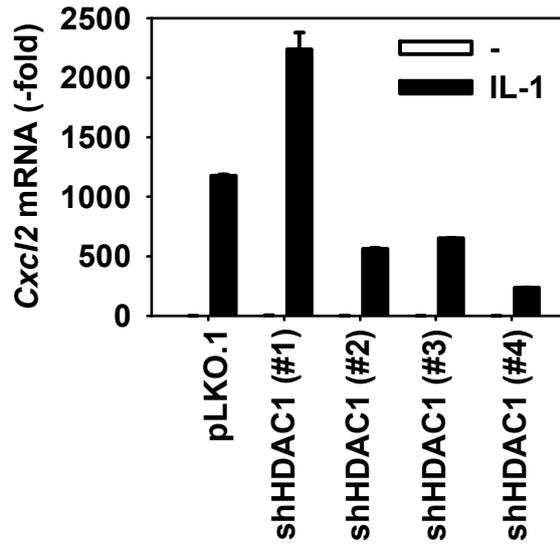
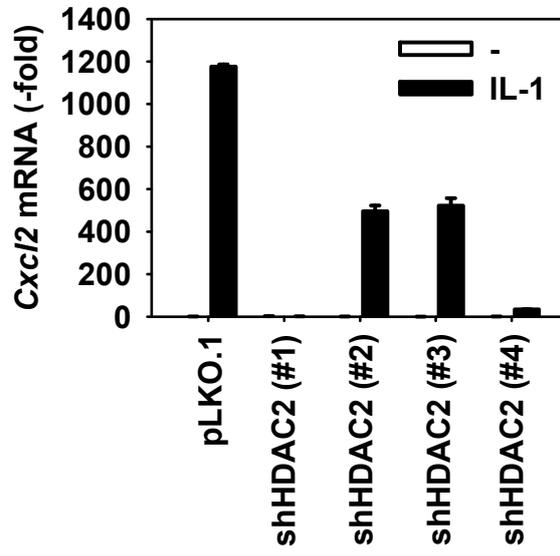
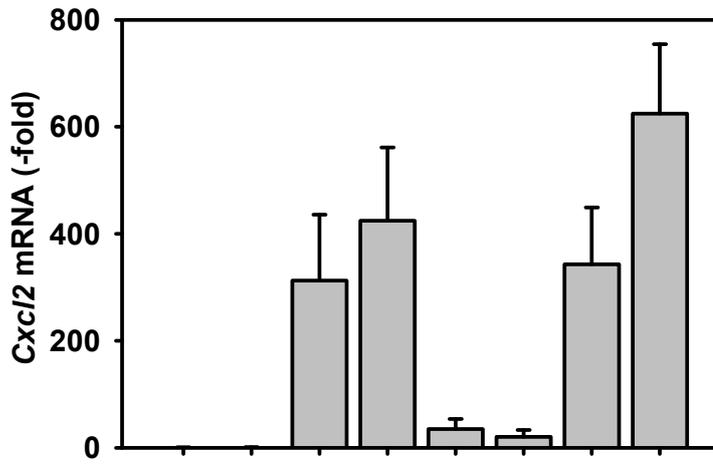
A**B**

Figure S10

A



tamoxifen					+	+	+	+
TNF			+	+			+	+
<i>Hdac3</i> ^{fl/+} Cre-ER ⁺	+		+		+		+	
<i>Hdac3</i> ^{fl/-} Cre-ER ⁺		+		+		+		+

Figure S11

B

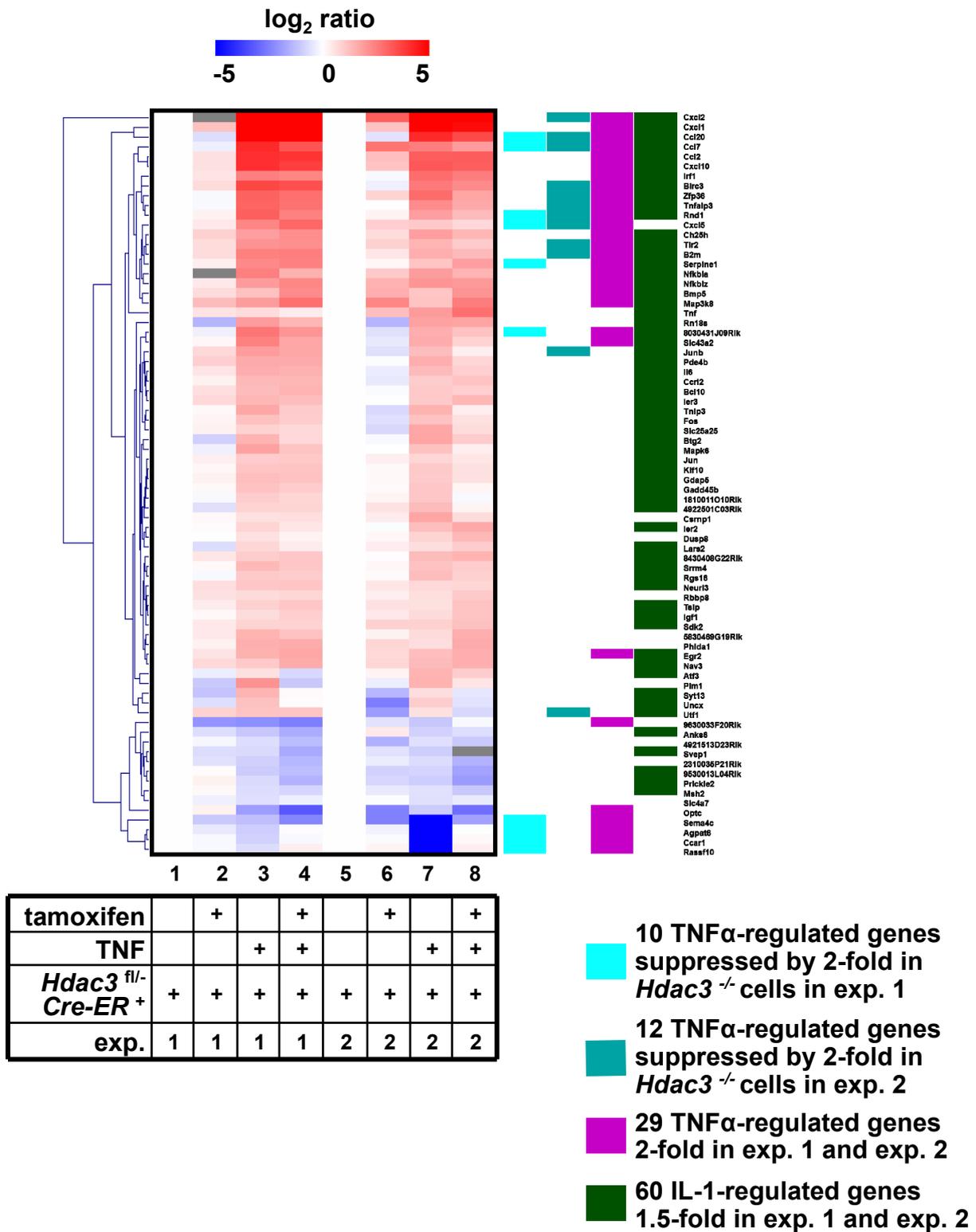


Figure S11

	EntrezGeneID	GenbankAccession	GeneSymbol	expression reduced in HDAC3 -/-	expression suppressed in p65 -/-	IL-1-induced expression suppressed by p65 K4Q	IL-1-induced expression suppressed by K5Q	basal expression suppressed by K4Q	basal expression suppressed by K5Q
1	320897	AK033113	8030431J09Rik	x					
2	213393	NM_145980	8430408G22Rik	x	x				
3	225339	AK030009	Ammecr1l	x					
4	12010	AK086184	B2m	x					
5	11796	BC011338	Birc3	x	x				
6	20296	NM_011333	Ccl2	x	x				
7	20297	NM_016960	Ccl20	x					
8	20306	NM_013654	Ccl7	x	x				
9	54199	NM_017466	Ccl2	x	x	x	x		
10	12642	NM_009890	Ch25h	x	x				
11	14825	NM_008176	Cxcl1	x					
12	15945	NM_021274	Cxcl10	x	x				
13	20310	NM_009140	Cxcl2	x	x	x	x		
14	20311	NM_009141	Cxcl5	x	x				
15	13537	NM_010090	Dusp2	x					
16	18218	NM_008748	Dusp8	x					
17	13654	NM_010118	Egr2	x	x	x			
18	13710	NM_007921	Elf3	x					
19	14281	NM_010234	Fos	x					
20	15894	NM_010493	Icam1	x		x	x		
21	16000	AK050118	Igf1	x					
22	16362	NM_008390	Irf1	x	x	x	x		
23	16410	AK171739	Irgav	x					
24	16476	NM_010591	Jun	x					
25	50772	AK011505	Mapk6	x					
26	214854	NM_153408	Neurl3	x	x				
27	18035	NM_010907	Nfkbia	x	x				
28	80859	NM_030612	Nfkbiz	x		x	x		
29	18578	AK171700	Pde4b	x	x		x		
30	18712	NM_008842	Pim1	x		x	x		
31	225182	AK087007	Rbbp8	x					
32	223881	NM_172612	Rnd1	x	x	x	x		
33	18787	AK040662	Serpine1	x		x	x		
34	215113	AK090207	Slc43a2	x	x				
35	24088	NM_011905	Tlr2	x	x	x	x		
36	21926	NM_013693	Tnf	x		x	x		
37	21929	NM_009397	Tnfaip3	x	x	x	x	x	x
38	53603	NM_021367	Tslp	x		x	x		
39	22329	NM_011693	Vcam1	x	x	x	x	x	x
40	22695	NM_011756	Zfp36	x		x			
				40	19	15	14	2	2

Table S1