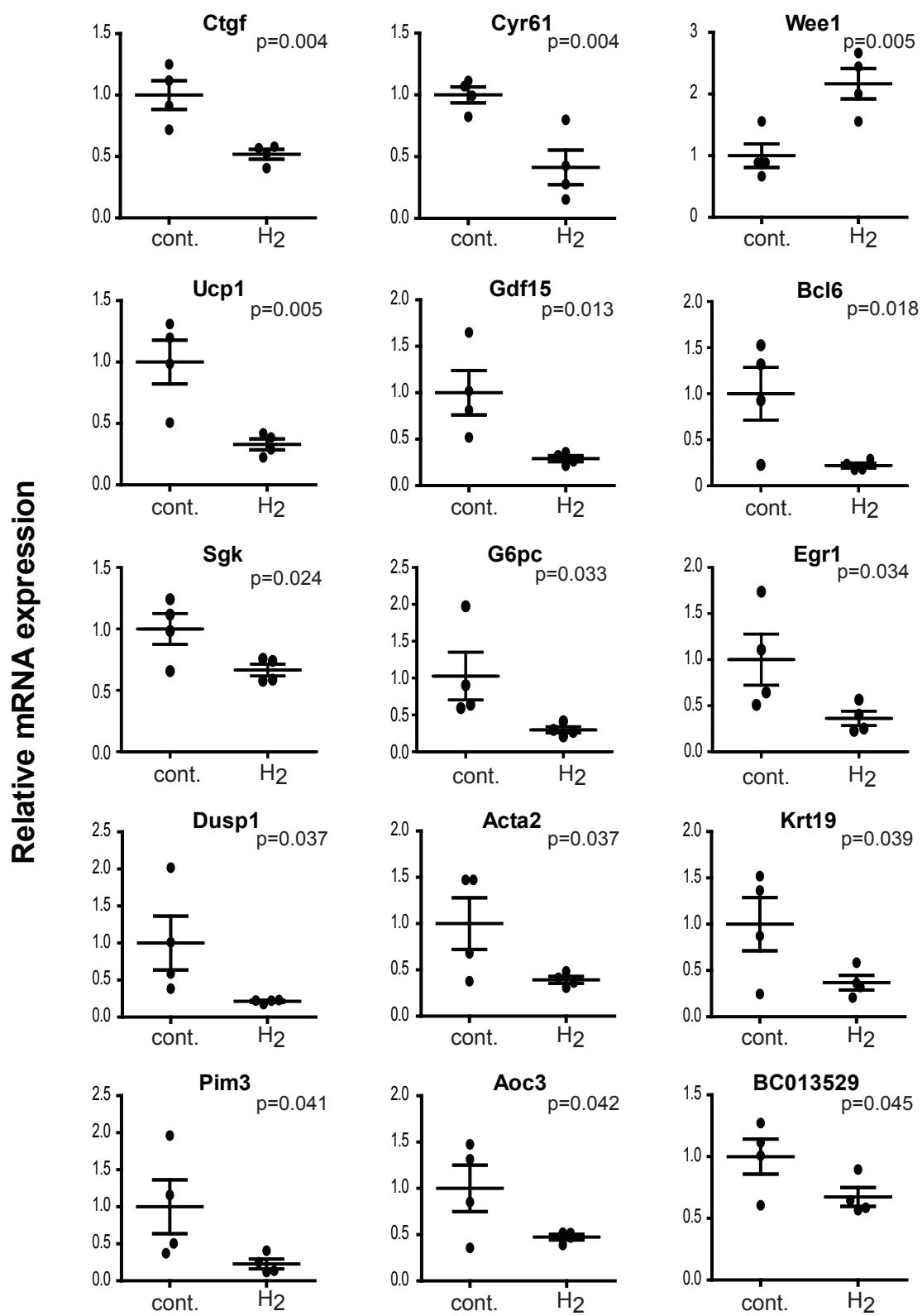
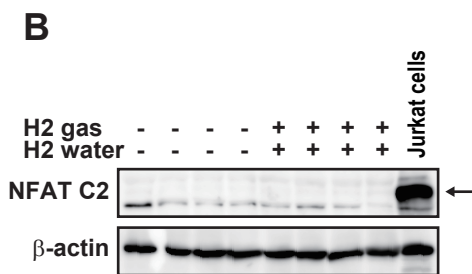
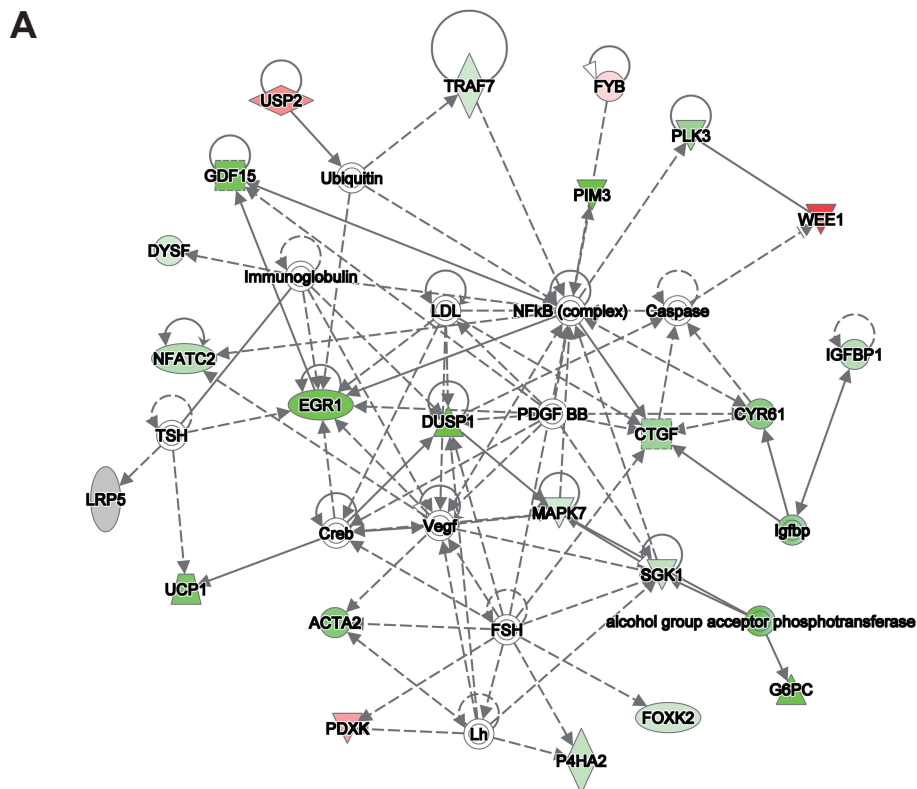


Supplemental Fig. 1

Measurement of H₂ concentration using gas chromatography. (A) Following gradual dilution of H₂-saturated water, the actual H₂-concentrations in these waters were measured using gas chromatography. (B) After oral administration of H₂ rich water, H₂ concentrations in the exhaled gas were sequentially measured.



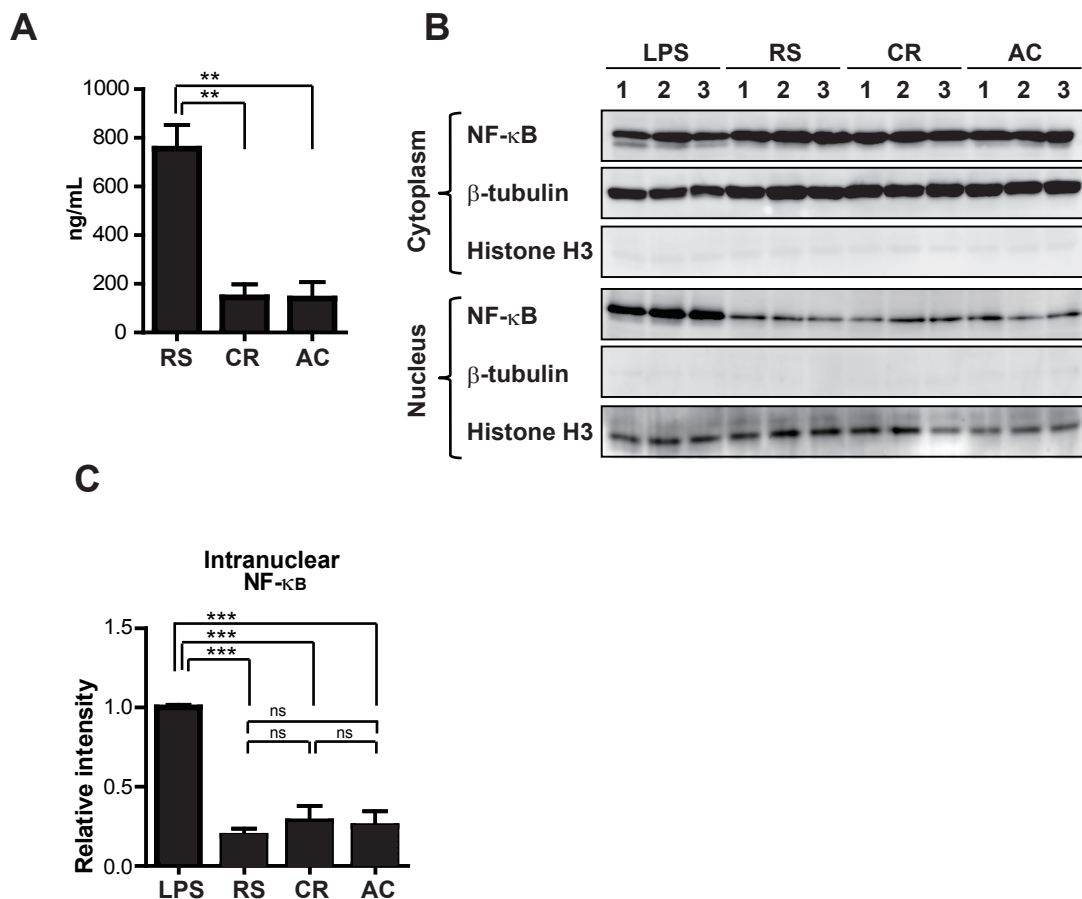
Supplemental Fig. 2
Relative gene expression for genes showing statistically significant up- or down-regulation after H2 administration.



Supplemental Fig. 3.

Ingenuity Pathway Analysis based on actual gene expression levels determined by qPCR, and NFAT expression in liver with or without H2 administration.

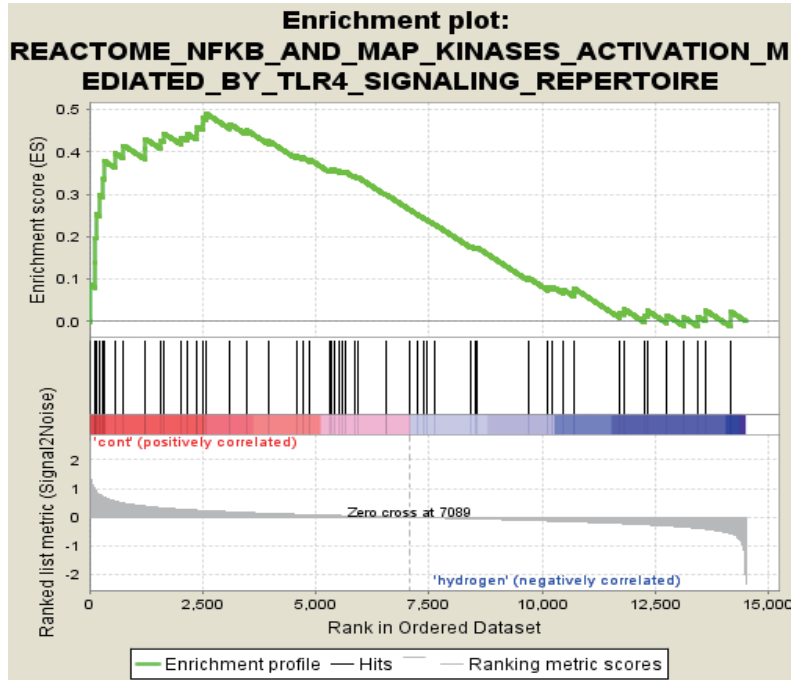
(A) Actual gene expression levels determined by qPCR in Table S1 were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems), and the network was graphically generated. Green (down-regulated genes) and red (up-regulated genes) nodes indicate input genes. The biological relationship between two nodes is represented as a line. Bold lines indicate a direct interaction, and dotted lines indicate an indirect interaction. (B) Liver lysates were subjected to western blot analysis for NFAT C2 expression with or without H2 administration. Lysate from Jurkat cells was used as positive control.



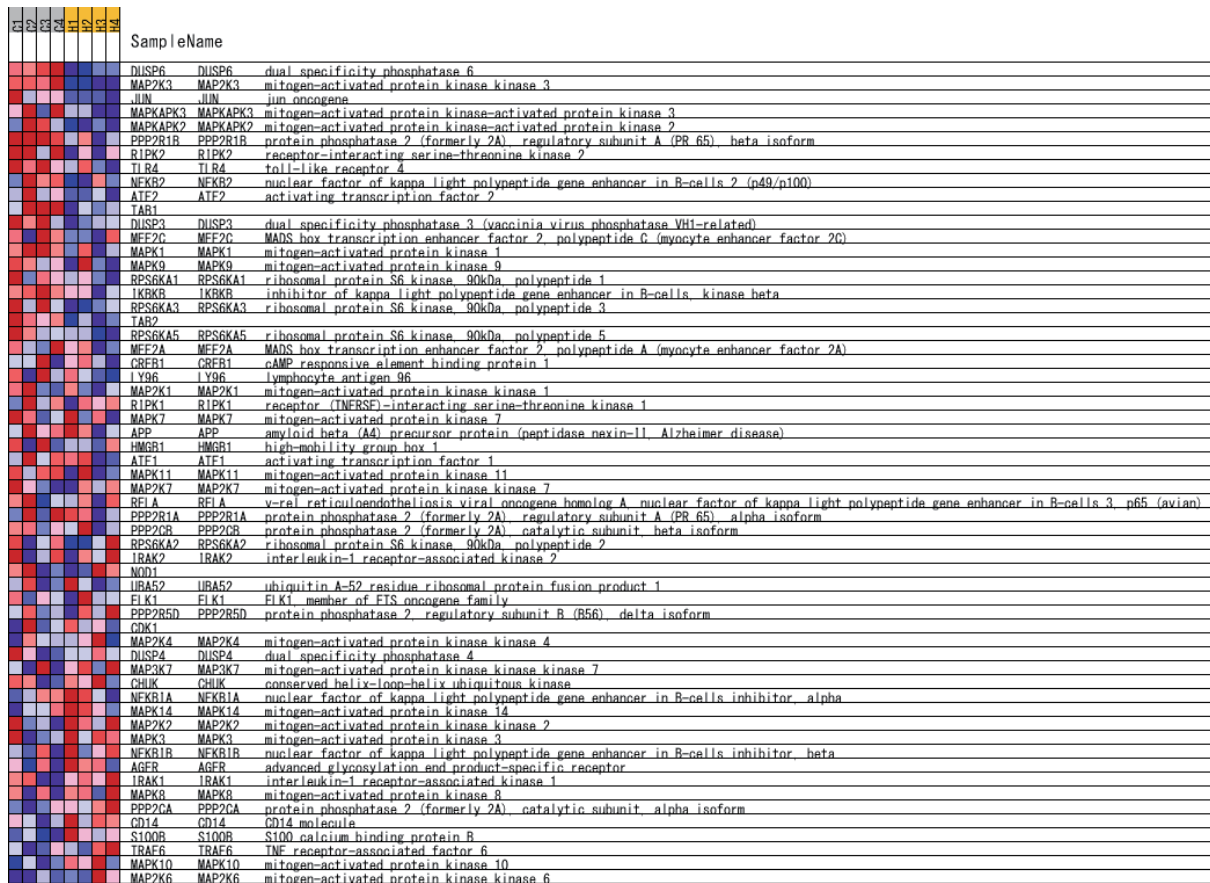
Supplemental Fig. 4.

Stress hormone levels and NF-κB activation in mice housed in air-tight chambers used for continuous hydrogen gas treatment. (A) Serum corticosterone was measured using ELISAs (Corticosterone EIA, Yanaihara Institute Inc., Fujinomiya, Japan) in mice housed for 3 weeks in a conventional specific-pathogen-free rack of cages or in air-tight chambers supplied with 10 L/min air without H₂, as well as in mice under 20-hour restraint stress. (B) Cytosolic and nuclear proteins were subjected to western blot analysis for the indicated proteins from mouse livers in four different conditions (n=4 per condition). (C) Band intensities for intranuclear NF-κB were quantified. RS, mice under restraint stress for 20 hours (positive control for serum corticosterone); CR, mice in a conventional clean rack; AC, mice in an air-tight chamber for continuous H₂ gas treatment; LPS, mice after peritoneal injection of LPS (positive control for NF-κB activation). Asterisks indicate statistical significance as determined by one-way ANOVA and Tukey's multiple-comparison test (**p < 0.01, ***p < 0.001).

A



B



Supplemental Fig. 5.

Enrichment plot and heat map for the gene set of the NF- κ B and MAP kinases activation Reactome mediated by the Toll-like receptor (TLR) 4 signaling repertoire, generated by Gene Set Enrichment Analysis (GSEA).

(A) Microarray results by Nakai et al. (Nakai et al. Biosci Biotechnol Biochem, 2011) were subjected to GSEA using Gene Set of Reactome (Reactome gene sets in C2 curated gene sets of default genes). Enrichment plot (A) and Heat map of the genes (B) within the gene set of the NF- κ B and MAP kinases activation Reactome mediated by the TLR 4 signaling repertoire are shown. Normalized enrichment score (NES), normalized p-value and FDR q-value were 1.94, 0.0 and 0.033, respectively.

Table S2. Sequences of primers and probes used in quantitative real-time PCR.

CYBR Green method		
Gene Symbol	Forward primers	Reverse primers
Acta2	5'-TGCCGAGCGTGAGATTGTCC-3'	5'-GGTGATCACCTGCCCGTCAG-3'
Actb	5'-CCCTGAAAAGATGACCCAGA-3'	5'-GTCTCCGGAGTCCATCACAA-3'
Aoc3	5'-TTGTCCCACGATTGTACCTT-3'	5'-TGGCCAGGTACAGGTAGCG-3'
BC013529	5'-AAAAGCGCTCTGTAGGTTCTG-3'	5'-GAGCCGTCTGGGTATAAGATG-3'
Bcl6	5'-GGCACTCGTTCGGGCACCT-3'	5'-CCGACACGCGGTATTGCACCTT-3'
Ctgf	5'-CACACCGCACAGAACCACCA-3'	5'-GGCAGGCACAGGTCTTGATGA-3'
Cygb	5'-GCGGAGAGGAAGGCGTTTCAG-3'	5'-ATGACCCGGCAGGCGTGCTT-3'
Cyp4a14	5'-GACCCCTCTAGATTTGCACC-3'	5'-TGGATCCCATTCTTGACTT-3'
Cyr61	5'-CCTTCAGGACGCTCGTGTC-3'	5'-GTCTGCCTTCTGACTGAGCTCTG-3'
Dusp1	5'-CCTCTCTACGATCAGGGGG-3'	5'-CTGCCTGTGGTTGTCTC-3'
Dysf	5'-TGTGAACCCCGTGTGGAATG-3'	5'-CCTGGGGGTGGCGTGTAG-3'
Eil3	5'-AGCCTCTCAGCTCCATCAGCC-3'	5'-CCTGTTGCTGCTCTGTGCTGTG-3'
Etv5	5'-TCCCCACCTCAGGCAACTC-3'	5'-ACATCTGCCGGTGGTAGCTGG-3'
Fbln5	5'-TTCACCCTCAACGACGATGG-3'	5'-TCCTGGCAGCTTCGGTTATC-3'
Fgf21	5'-CAGCACACCGCAGTCCAG-3'	5'-TCAGGATCAAAGTGAGGCGA-3'
Fkbp14	5'-GCTCCCTGTTTCATTCCAATC-3'	5'-TTTTACCTTTTCTTTTCC-3'
Foxk2	5'-GTGTTTCGTGGACGGGGTGT-3'	5'-GAGGTGGGCCATCGTGTCTG-3'
Fyb	5'-CCACCACCTCCGCCTACA-3'	5'-CCCGTTTCTGTCTCGTTCC-3'
G6pc	5'-AAGAGACTGTGGGCATCAA-3'	5'-AACACCGGAATCCATACG-3'
Gdf15	5'-ACTCAACGCCGACGAGCTAC-3'	5'-GTGGGACCCCAATCTCACCT-3'
Grap	5'-AGGATCTCTCGACAGCTGG-3'	5'-GCACCTGGTCACCATAATTC-3'
Hif1a	5'-CAAGATCTCGGCGAAGCAA-3'	5'-GTCTAGACCACCGGCATCCA-3'
Igfbp1	5'-ACCCGTGCCCGCTTCGTG-3'	5'-CCTGGGCGCAGCGAGCAG-3'
Krt19	5'-CCCTCCCAGATTACAACCACT-3'	5'-TGCAGCCAGGCGAGCATT-3'
Lrp5	5'-TGGTCCCCGCCGCGCCT-3'	5'-CAGCAGCTGCATCCTCCAGGCCAC
Mapk7	5'-AAGCCTACTGTGCCCTATGGAGA-3'	5'-TATTTGAGGCCCGAAGCAG-3'
Nfatc2	5'-GCGGAAGGGCGAGACGGACA-3'	5'-CGCAGCCTGCAGGGAGACGA-3'
Nle1	5'-GCGGCCGTGTACCAGATTGC-3'	5'-TCTTTCCCGCCGCTTGCTAC-3'
P4ha2	5'-GCGGGCGGAGACTGTGACGA-3'	5'-GCACCCAGCTCAGGACACCGAA-3'
Pdxk	5'-TCCTCACAGGGCAGCGACTA-3'	5'-AGAAGCATGGCAGCGAAGAG-3'
Pfkfb3	5'-TCCTCCGAGACTCCGCACAA-3'	5'-CACGGTTGGGGAGTTGGTCA-3'
Pim3	5'-AGTGGGGCAGTCTCGGCG-3'	5'-GGGCCGCTCGAACCAAGTC-3'
Pkdcc	5'-CCGCCCTCCCTCCGACCTCT-3'	5'-GCCCGCGTGGTATAGGATG-3'
Plk3	5'-GCTGGGCATCCTGCGGTATTT-3'	5'-GATTGCGGGCCACGAAGGTC-3'
Sgk	5'-GAAGGATGGGCTGAACGAT-3'	5'-GTTTGGCGTGAGGGTTGGA-3'
Sh3bp1	5'-CACGGGCAGCTCGGGACGCA-3'	5'-CCCCGCTTTGGCCCTGCAGAC-3'
Sh3bp5l	5'-GCAGGGGGAACCTTCGGTC-3'	5'-GGTGGTCCTGGCCTCGTC-3'
Smad7	5'-CCGGGGGAACGAATTATCTG-3'	5'-CCGATCTTGCTCCGCACTTT-3'
Tnfsf8	5'-ACCTCAGCACCACCGCACT-3'	5'-TGGTGCCATCTTCGTTCCAT-3'
Traf3	5'-GTAGGCCAGCAGCATCGTGT-3'	5'-AGGGCCACCTCGCATAGGAA-3'
Traf7	5'-CTTCCGGCTCCTGTATCCTG-3'	5'-AAAGGTAGGCCCAAAGTTGT-3'
Ucp1	5'-TCTGGGCTTAACGGGTCTCTC-3'	5'-TCGGAAGTTGTCGGGTTACAC-3'
Usp2	5'-ACCGACCAAGTGGCCGTTAC-3'	5'-GCCGAGGTCCCGCATGTA-3'
Wee1	5'-CCACGTCGTTTCGCTATTT-3'	5'-TTTGGGATTGAGGTTGAGA-3'
Zfp672	5'-TGGTGGCGACAAGCGTTTCT-3'	5'-CAGCAGCGCAGGGACATGAG-3'

Taqman probe method	
Gene Symbol	Assay ID
Actb	Mm00607939_s1
Bcor	Mm00551516_m1
Egr1	Mm00656724_m1
Ins2	Mm00731595_gH
Itfg2	Mm00505801_m1
Ms4a5	Mm00806060_m1
Wnt3	Mm00437336_m1

Table S3. DAVID Gene Ontology Analysis for down-regulated genes by H2 treatment

Clustered annotations						
Annotation Cluster 1		Enrichment Score: 2.37				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
INTERPRO	Thrombospondin, type 1 repeat	4	0.36	3.65E-03	12.81	4.66
PFAM	TSP_1	4	0.36	3.81E-03	12.62	4.75
UP_SEQ_FEATURE	domain:TSP type-1	3	0.27	3.87E-03	31.62	5.38
SMART	TSP1	4	0.36	6.15E-03	10.49	6.05
Annotation Cluster 2		Enrichment Score: 1.97				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
PANTHER_PATHWAY	PI3 kinase pathway	5	0.44	3.76E-03	6.99	3.21
PANTHER_PATHWAY	Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	4	0.36	5.01E-03	10.47	4.26
PANTHER_PATHWAY	Interleukin signaling pathway	4	0.36	6.42E-02	4.01	43.72
Annotation Cluster 3		Enrichment Score: 1.90				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
INTERPRO	Insulin-like growth factor binding protein, N-terminal	3	0.27	1.45E-03	51.54	1.87
INTERPRO	Insulin-like growth factor-binding protein, IGFBP	3	0.27	4.38E-03	29.84	5.55
PFAM	IGFBP	3	0.27	4.50E-03	29.40	5.59
UP_SEQ_FEATURE	domain:IGFBP N-terminal	3	0.27	4.90E-03	28.11	6.76
SMART	IB	3	0.27	6.33E-03	24.44	6.22
GOTERM_MF_ALL	insulin-like growth factor binding	3	0.27	6.77E-03	23.87	8.40
GOTERM_MF_ALL	growth factor binding	3	0.27	6.25E-02	7.29	56.58
GOTERM_BP_ALL	regulation of cell growth	3	0.27	1.05E-01	5.39	82.15
GOTERM_BP_ALL	regulation of growth	4	0.36	1.97E-01	2.58	96.73
Annotation Cluster 4		Enrichment Score: 1.59				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
GOTERM_BP_ALL	lipid metabolic process	13	1.15	8.49E-04	3.09	1.31
GOTERM_BP_ALL	oxoacid metabolic process	9	0.80	7.79E-03	3.11	11.47
GOTERM_BP_ALL	carboxylic acid metabolic process	9	0.80	7.79E-03	3.11	11.47
GOTERM_BP_ALL	organic acid metabolic process	9	0.80	7.89E-03	3.10	11.60
GOTERM_BP_ALL	cellular lipid metabolic process	9	0.80	8.08E-03	3.09	11.86
GOTERM_BP_ALL	cellular ketone metabolic process	9	0.80	8.98E-03	3.03	13.10
GOTERM_BP_ALL	monocarboxylic acid metabolic process	6	0.53	2.22E-02	3.70	29.45
GOTERM_BP_ALL	fatty acid metabolic process	5	0.44	2.44E-02	4.49	31.94
GOTERM_BP_ALL	fatty acid biosynthetic process	3	0.27	8.45E-02	6.12	74.71
GOTERM_MF_ALL	ligase activity	5	0.44	1.44E-01	2.44	86.64
GOTERM_BP_ALL	carboxylic acid biosynthetic process	3	0.27	2.06E-01	3.52	97.25
GOTERM_BP_ALL	organic acid biosynthetic process	3	0.27	2.06E-01	3.52	97.25
GOTERM_BP_ALL	lipid biosynthetic process	4	0.36	2.43E-01	2.32	98.68
Annotation Cluster 5		Enrichment Score: 1.32				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
GOTERM_CC_ALL	cytoplasmic vesicle	8	0.71	1.81E-02	2.94	19.71
GOTERM_CC_ALL	vesicle	8	0.71	2.01E-02	2.88	21.65
GOTERM_CC_ALL	cytoplasmic membrane-bounded vesicle	6	0.53	6.87E-02	2.70	57.54
GOTERM_CC_ALL	membrane-bounded vesicle	6	0.53	7.21E-02	2.66	59.36
SP_PIR_KEYWORDS	cytoplasmic vesicle	4	0.36	1.35E-01	3.11	82.87
Annotation Cluster 6		Enrichment Score: 1.31				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
GOTERM_BP_ALL	embryonic development	11	0.98	6.51E-03	2.71	9.67
GOTERM_BP_ALL	central nervous system development	8	0.71	6.53E-03	3.58	9.69
GOTERM_BP_ALL	cell differentiation	17	1.51	2.24E-02	1.79	29.73
GOTERM_BP_ALL	nervous system development	11	0.98	3.07E-02	2.13	38.49
GOTERM_BP_ALL	cellular developmental process	17	1.51	3.17E-02	1.72	39.40
GOTERM_BP_ALL	developmental process	24	2.13	5.04E-02	1.45	55.31
GOTERM_BP_ALL	anatomical structure development	20	1.78	5.24E-02	1.52	56.70
GOTERM_BP_ALL	system development	18	1.60	9.03E-02	1.47	77.07
GOTERM_BP_ALL	multicellular organismal development	21	1.87	1.06E-01	1.38	82.55
GOTERM_BP_ALL	organ development	13	1.15	2.88E-01	1.29	99.50
GOTERM_BP_ALL	multicellular organismal process	27	2.40	5.30E-01	1.03	100.00
Annotation Cluster 7		Enrichment Score: 1.31				
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
GOTERM_BP_ALL	embryonic development	11	0.98	6.51E-03	2.71	9.67
GOTERM_BP_ALL	in utero embryonic development	5	0.44	7.59E-02	3.10	70.71
GOTERM_BP_ALL	chordate embryonic development	6	0.53	1.07E-01	2.36	82.95
GOTERM_BP_ALL	embryonic development ending in birth or egg hatching	6	0.53	1.11E-01	2.33	83.88
Non-clustered annotations						
Category	Term	Count	%	PValue	Fold Enrichment	FDR (%)
GOTERM_MF_ALL	protein binding	48	47.52	1.66E-03	1.44	2.12
BIOCARTA	CD40L Signaling Pathway	3	2.97	2.32E-03	33.78	1.67
BIOCARTA	TNFR2 Signaling Pathway	3	2.97	3.99E-03	25.83	2.86
GOTERM_BP_ALL	cellular process	68	67.33	4.15E-03	1.22	6.27
BIOCARTA	Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK)	3	2.97	4.99E-03	23.11	3.56
GOTERM_MF_ALL	binding	76	75.25	5.32E-03	1.16	6.66
GOTERM_BP_ALL	response to protein stimulus	4	3.96	1.52E-02	7.60	21.26
GOTERM_MF_ALL	transcription factor binding	6	5.94	2.35E-02	3.65	26.49
GOTERM_CC_ALL	cytosol	8	7.92	2.63E-02	2.72	27.43
KEGG_PATHWAY	Adipocytokine signaling pathway	3	2.97	6.24E-02	7.14	48.79
GOTERM_BP_ALL	steroid metabolic process	4	3.96	7.19E-02	4.11	68.71
KEGG_PATHWAY	MAPK signaling pathway	5	4.95	7.59E-02	3.01	55.91
KEGG_PATHWAY	PPAR signaling pathway	3	2.97	8.32E-02	6.05	59.40
PANTHER_PATHWAY	Insulin/IGF pathway-protein kinase B signaling cascade	3	2.97	8.93E-02	5.60	55.53