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Article type : Letter to the Editor

***Has2* deficiency enhances OVA-induced airway inflammation and hyperresponsiveness in mice**

To the Editor,

Although the underlying pathogenesis of asthma is not fully understood, hyaluronan (HA) are thought to be associated with the persistence of symptoms ¹⁻³. Our genome-wide association study revealed that the hyaluronan synthase 2 (HAS2) gene is a candidate for susceptibility to adult asthma ². HAS2 mainly generates high molecular weight hyaluronan (HMW-HA), which has anti-inflammatory effects ¹. Conversely, low molecular weight hyaluronan (LMW-HA) induces the pro-inflammatory response ¹. We hypothesized that the attenuation of *Has2* gene expression decreases HAS2 expression, causes an imbalance between HMW-HA and LMW-HA, and promotes severe airway inflammation and enhanced airway hyperresponsiveness (AHR). To test this hypothesis, we examined the effects of genetic *Has2* dysfunction in eosinophilic airway inflammation condition with *Has2* heterozygous deficient (*Has2*^{+/-}) mice.

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In this study, 6–10-week-old female *Has2*^{+/-} mice and their wild-type (WT) littermates were used. OVA-induced eosinophilic airway inflammation model was used (Fig. 1A). All animal studies were approved by the Institutional Review Board of University of Tsukuba, Japan. For more information, see the Supplementary Methods section.

To confirm the effect of genetic modulation, we first evaluated the levels of *Has2* mRNA expression in WT and *Has2*^{+/-} mice lung using quantitative polymerase chain reaction. The levels of *Has2* mRNA were significantly decreased in OVA-stimulated *Has2*^{+/-} (*Has2*^{+/-}-OVA) mice compared with those in OVA-stimulated WT (WT-OVA) mice (Fig. 1B). Further, CD44 and TLR4 are known as HA-binding proteins and a decrease of CD44 downregulates TGF-β¹. The expression levels of *Cd44*, *Tlr4*, and *Tgfb1* mRNA decreased significantly in the lungs from *Has2*^{+/-}-OVA mice compared with those from WT-OVA mice (Fig. 1C, S1). The genetic modulation of *Has2* did not affect the expression levels of *Has2*, *Cd44*, *Tlr4*, and *Tgfb1* mRNA in the saline control group. These HA-binding proteins may be constitutively expressed without the involvement of *Has2* induction at a steady state. Similar trends of decrease in the mRNA level of HA-binding protein were observed in 4-methylumbelliferone (4-MU)-, which was known as HA synthesis inhibitor, treated WT-OVA mice (Fig. S2B). Furthermore, the analysis of HA size showed that hyaluronidase-sensitive HMW-HA band was less abundant in *Has2*^{+/-}-saline mice lung extracts than in WT-saline mice lung tissue (Fig. 1D). Our results suggested that the expression level of *Has2* mRNA in *Has2*^{+/-} mice was downregulated during eosinophilic airway inflammation, and *Has2* dysfunction impaired HMW-HA expression, affecting the expression of HA-binding protein.

Because inhibition of the CD44 and TGF-β pathway exacerbates airway inflammation and AHR^{4, 5}, we evaluated the severity of lung eosinophilia and AHR. Lung sections from

Has2^{+/-}-OVA mice stained with hematoxylin and eosin (HE) show greater infiltration of inflammatory cells compared with those from WT-OVA mice (Fig. 1E). Furthermore, the numbers of total cell, alveolar macrophages, neutrophils, and eosinophils in the BALF were significantly increased in *Has2*^{+/-}-OVA mice compared with WT-OVA mice (Fig. 1F). Similar results were also observed in 4-MU-treated WT mice (Fig. S2C). Airway resistance in response to aerosolized methacholine was significantly increased in *Has2*^{+/-}-OVA mice compared with WT-OVA mice (Fig. 1G). These results indicated that attenuation of *Has2* expression promotes airway inflammation and AHR by inhibiting the anti-inflammatory pathway mediated by HMW-HA, CD44, and TGF- β .

Next, the levels of inflammatory mediators in BALF were measured. IL-4, IL-5, IL-13, and GM-CSF levels were significantly increased in *Has2*^{+/-}-OVA mice compared with WT-OVA mice (Fig. S3A). Multiplex assays also revealed that G-CSF, IL-6, IL-10, TNF- α , IP-10, KC, MCP-1, MIP-1 α , and MIP-1 β levels were significantly increased in *Has2*^{+/-}-OVA mice (Fig. S3B). However, there were no significant differences in the levels of Th17 and IL-33 related cytokines in the BALF from *Has2*^{+/-}-OVA mice and WT-OVA mice (Fig. S4). In addition, there were no significant differences in the serum levels of total IgE, OVA-specific IgE, OVA-specific IgG1, and drop in core body temperature from *Has2*^{+/-}-OVA mice and WT-OVA mice (Fig. S5-6). Taken together, these data suggest that the attenuation of *Has2* expression significantly increases Th2 cytokines, but it does not cause a systemic allergic response.

To gain insight into the molecular mechanisms, RNA sequencing was performed (Fig. S7). In the top 10 upregulated and downregulated genes, genes related to muscle function were significantly downregulated in OVA-stimulated *Has2*^{+/-} mice compared with that of WT mice (Table S1). Next, we identified 70 genes with significantly different expression between the lungs

of WT mice and *Has2*^{+/-} mice after OVA sensitizations and challenge (Fig. S8). Gene ontology analyses identified significant biological process terms such as “apoptotic signaling pathway,” “cytokine production,” “negative regulation of smooth muscle cell proliferation,” and “muscle contraction” among the downregulated genes (Fig. 2A and Table S2). Furthermore, canonical pathway analyses identified the following significant terms: “Integrin-linked kinase Signaling,” “Tight Junction Signaling,” “Agranulocyte Adhesion and Diapedesis,” “Calcium Signaling,” “Epithelial Adherens Junction Signaling,” and “Actin Cytoskeleton Signaling” (Fig. 2B and Table S3). These signaling pathways are relevant to “airway smooth muscle function” and carry similar genes (Table S3 and Fig. S9). We also identified the significant term “Protein Kinase A Signaling” which is relevant to AHR ⁶. Interestingly, pathway analyses identified “IL-17A Signaling in Fibroblasts,” “NRF2-mediated Oxidative Stress Response,” and “Glucocorticoid Receptor Signaling.” These terms were thought to be related to intractable asthma. We also observed similar results in comparison analysis (Fig. 2C-2E). Taken together, *Has2* suppression affects leading to increased AHR, worsened airway inflammation, and possible treatment resistance.

The limitations of this study are as follows:

1. Because we examined the effects of *Has2* genetic abnormalities on allergic airway inflammation and *Has2*-deficient mice exhibit recessive lethal phenotype, we used heterozygous deficient mice; however, we have not confirmed whether the *Has2* genetic deletion directly affects inflammatory cells and airway smooth muscle. In addition, we have not confirmed *Has2* function by the overexpression or direct injection of *Has2*, or by adoptive transfer experiments.
2. We have not confirmed whether the attenuation of *Has2* expression affects the disease severity in chronic eosinophilic airway inflammation.

In conclusion, we demonstrated that the attenuation of *Has2* expression enhances OVA-induced airway inflammation and AHR and attenuates CD44 and TGF- β signaling in mice. Therefore, modulating HAS2 signaling might be a novel and highly effective therapeutic strategy for treating intractable bronchial asthma.

References

1. Liang J, Jiang D, Noble PW. Hyaluronan as a therapeutic target in human diseases. *Adv Drug Deliv Rev* 2016;**97**:186-203.
2. Yatagai Y, Sakamoto T, Yamada H, Masuko H, Kaneko Y, Iijima H, et al. Genomewide association study identifies HAS2 as a novel susceptibility gene for adult asthma in a Japanese population. *Clin Exp Allergy* 2014;**44**:1327-1334.
3. Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T, et al. Role of hyaluronan and hyaluronan-binding proteins in human asthma. *J Allergy Clin Immunol* 2011;**128**:403-411 e403.
4. Chen W, Ten Dijke P. Immunoregulation by members of the TGFbeta superfamily. *Nat Rev Immunol* 2016;**16**:723-740.
5. Guan H, Nagarkatti PS, Nagarkatti M. Role of CD44 in the differentiation of Th1 and Th2 cells: CD44-deficiency enhances the development of Th2 effectors in response to sheep RBC and chicken ovalbumin. *J Immunol* 2009;**183**:172-180.
6. Morgan SJ, Deshpande DA, Tiegs BC, Misior AM, Yan H, Hershfild AV, et al. beta-Agonist-mediated relaxation of airway smooth muscle is protein kinase A-dependent. *J Biol Chem* 2014;**289**:23065-23074.

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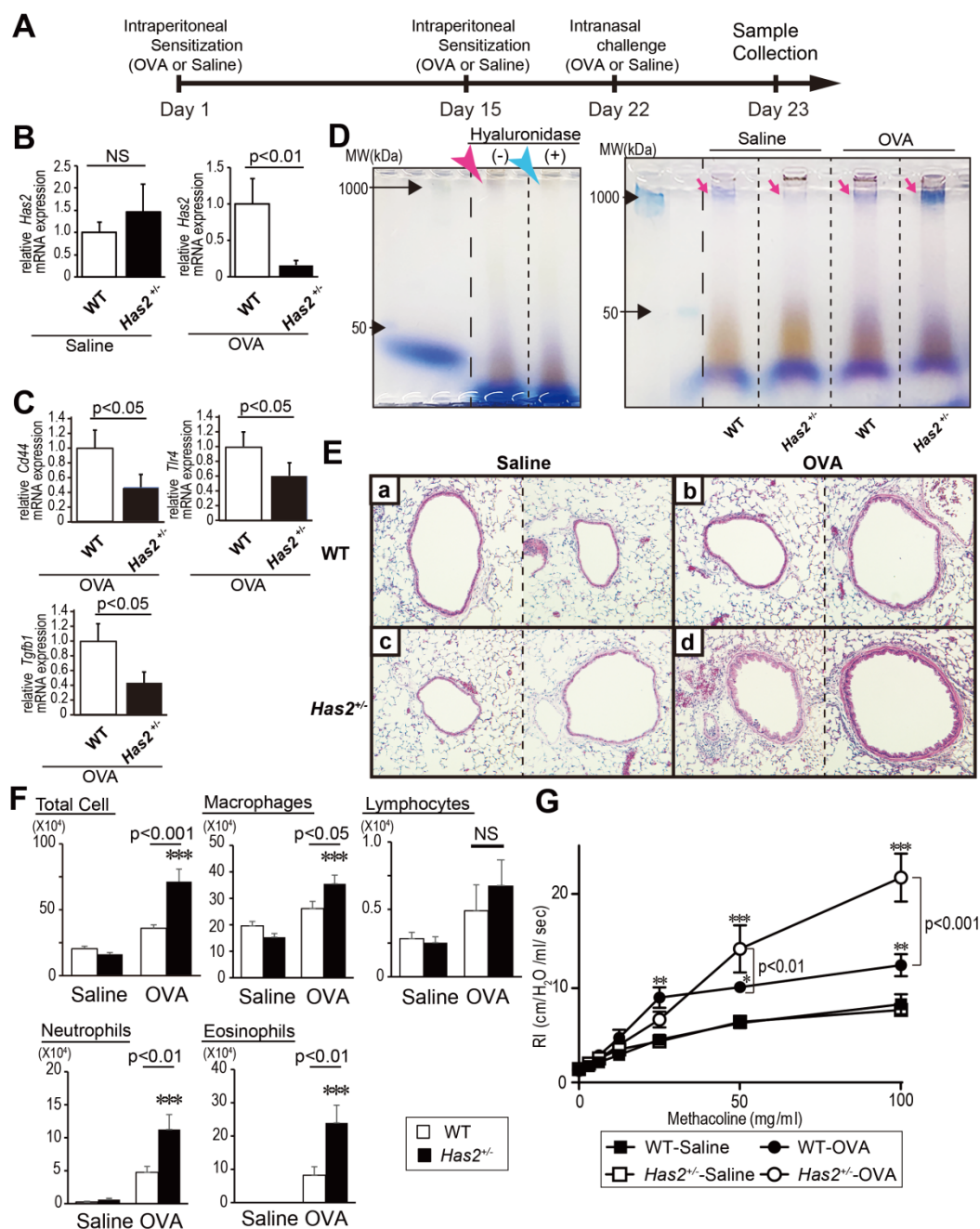
Conflict of interests: The authors have no conflicts of interest to declare.

Keywords: Airway hyperresponsiveness, CD44, eosinophilic airway inflammation, HAS2, TGF- β

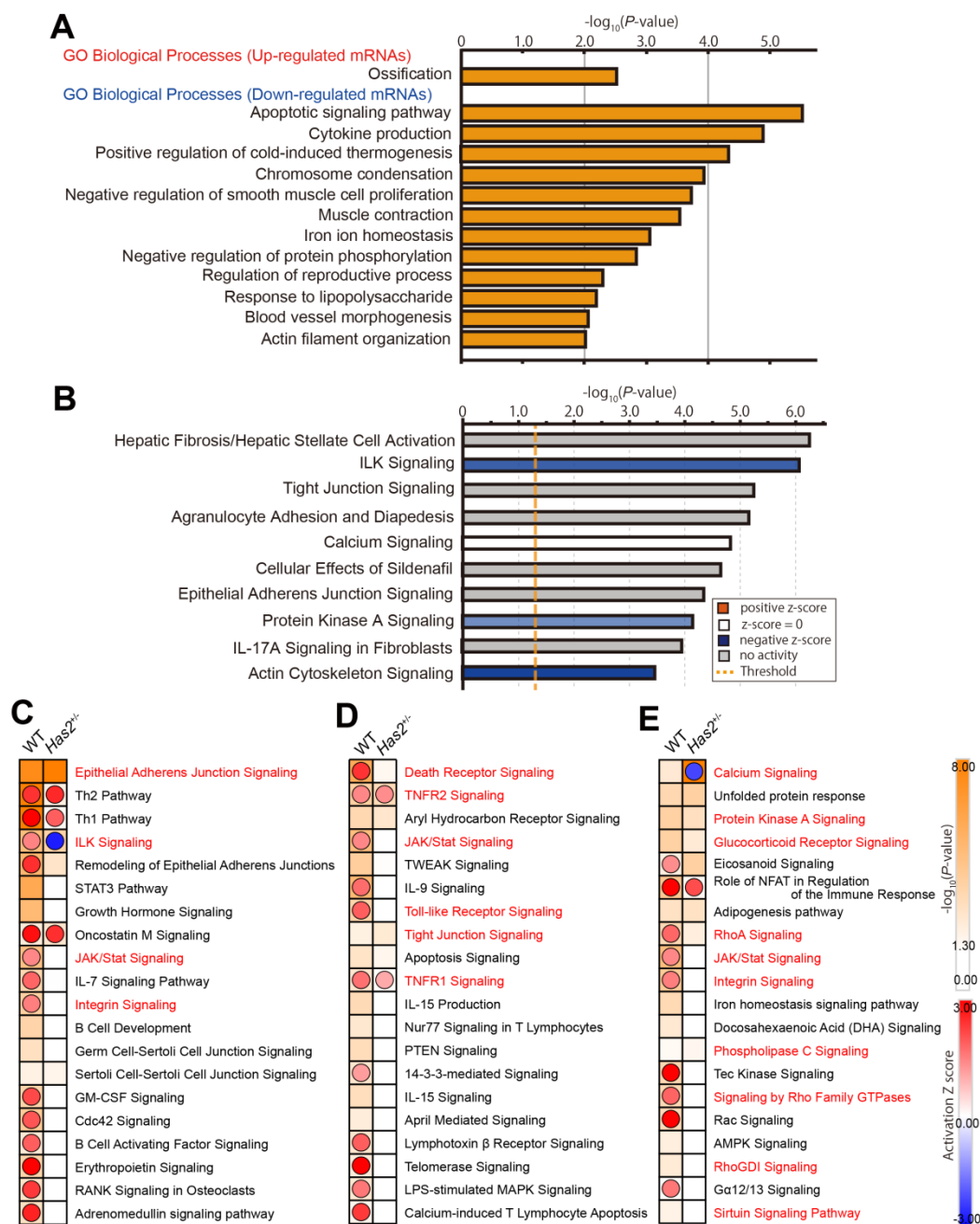
Figure legends

Figure 1. Inflammatory phenotype of *Has2*^{+/-} mice. **A**, Schematic illustration of the experimental design. **B-C**, levels of mRNA transcripts encoding; **(B)** *Has2* (n = 6–9), **(C)** *Cd44*, *Tlr4*, and *Tgfb1* (n = 16–17). **D**, **(left)** HA size analysis with hyaluronidase treatment. **(right)** HA size analysis using WT-saline, *Has2*^{+/-} -saline, WT-OVA, and *Has2*^{+/-} -OVA mouse lung tissue. **E**, lung tissue HE staining. **F**, BALF cell count obtained from WT and *Has2*^{+/-} mice (n = 9–11). **G**, airway hyperresponsiveness to methacholine (n = 7–9). * P < 0.05, ** P < 0.01, *** P < 0.001 relative to the WT-saline mice.

Figure 2. Differentially expressed gene analysis between the lungs from WT-OVA and *Has2*^{+/-}-OVA mice. **A**, significant biological process terms detected by GO analysis. **B**, top 10 significant terms detected by pathways analysis. **C-E**, Comparison analyses between the lungs from WT-OVA or *Has2*^{+/-}-OVA and WT-saline mice. Charts showing the enrichment of functional annotations in the categories of cellular growth proliferation and development (**C**), apoptosis (**D**), and intracellular and second messenger signaling (**E**). Red character terms were also detected as significant terms in pathway analyses between the lungs from WT-OVA and *Has2*^{+/-}-OVA mice.



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all_14715_f2.tif

Supporting Information

***Has2* deficiency enhances OVA-induced airway inflammation and hyperresponsiveness in mice**

Yoshiya Tsunoda, M.D., Ph.D.^{a,*}, Mingma Thsering Sherpa, M.D.^{a,*}, Takumi Kiwamoto, M.D., Ph.D.^a, Masashi Matsuyama, M.D., Ph.D.^a, Hajime Osawa, M.D.^a, Hirofumi Sakurai, M.D., Ph.D.^a, Shigen Hayashi, M.D., Ph.D.^a, Yosuke Matsuno, M.D., Ph.D.^a, Yuko Morishima, M.D., Ph.D.^a, Yukio Ishii, M.D., Ph.D.^a, Nobuyuki Hizawa M.D., Ph.D.^a

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Supplemental Methods

Animals, ovalbumin sensitization, and airway challenge

Six to 10-week-old female *Has2* heterozygous deficient (*Has2*^{+/-}) mice and their wild-type (WT) littermates were used in this study. Breeding sets of *Has2*^{+/-} mice (Jackson Laboratory, Bar Harbor, ME) were backcrossed to C57BL/6J background for at least six generations¹. Mice were sensitized via intraperitoneal injection with 100 µg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) in 1.6 mg of alum on days 1 and 15 and subsequently challenged with intranasal administration of 10 µg OVA on day 22 to induce allergic airway inflammation (Fig 1A). In some experiments, 4-Methylumbelliferone (4-MU, MilliporeSigma, St. Louis, MO) with 0.04% concentration (50 mg/kg/day) in drinking water was administered to inhibit HA synthesis (Fig S2A). All mice were euthanized 24 h after the final challenge. Control mice were injected and challenged with saline. All animal studies were approved by the Institutional Review Board of the University of Tsukuba (approval number: 15-256, 16-248, 17-142, and 18-089).

Measurement of mRNA levels

Total RNA was extracted from snap-frozen lung tissue using an RNeasy[®] Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. Levels of mRNA expression were quantified by qRT-PCR using the 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA). The sequences for the *Has2*^{+/-} mice specific TaqMan[®]-MGB probes and primers (Thermo Fisher Scientific, Waltham, MA) were as follows:

*Has2*F 5'-TGCTTGACCCTGCCTCATC-3',
*Has2*R 5'-CCCATGAATTCCTGATTGTACCA-3',
MGB probe 5'- TGTCCAGATTTTAAACAAG -3'.

Mouse primers and probes were purchased pre-mixed from Thermo Fisher Scientific: CD44 (*Cd44*; Mm01277163_m1), TLR4 (*Tlr4*; Mm00445273_m1), TGF-β1 (*Tgfb1*; Mm01178820_m1), and GAPDH (*Gapdh*; Mm99999915_g1). All mRNA levels were normalized to levels of *Gapdh* mRNA using the 2^{-ΔΔCT} method.

HA size analysis

Lung tissue samples were digested using proteinase K (MilliporeSigma, St. Louis, MO) at 1 mg/ml in 100mM ammonium acetate (1 ml/25 mg tissue weight) and precipitated. After sample preparation, HA size was analyzed as previously described².

Bronchoalveolar lavage cell counting

Mouse lungs were lavaged using five repeated instillations of 0.6 mL of saline each through the tracheal cannula. The first 1.2 mL of bronchoalveolar lavage fluid (BALF) was

centrifuged, and the supernatant was used for the measurements of various cytokines and chemokines as previously described ³. Centrifuged cells were redissolved in the remaining BALF sample. The total cell count was determined using a hemocytometer. Differential cell counts were obtained based on count of 300 cells, which morphologically classified the cells as macrophages, lymphocytes, neutrophils and eosinophils based on staining with Diff-Quik (Polysciences, Inc., Warrington, PA), using standard light microscopic techniques.

Assessing airway responsiveness

Mice were anesthetized, intubated, and connected to a mechanical ventilator (Fine-Pointe; Buxco, Wilmington, NC). Aerosolized methacholine (Sigma-Aldrich Japan, Tokyo, Japan) was administered at increasing doses (0–100 mg/mL). The peak value of respiratory resistance for each dose was recorded.

Measurement of cytokines and chemokines

The concentrations of cytokines and chemokines in BALF were determined using a MILLIPLEX MAP Kit (EMD Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. Since the concentrations of cytokines and chemokines in BALF were very low ³, twice the recommended sample volume was used for testing. The concentration of each sample was calculated by dividing the measured value by two.

Measurement of immunoglobulin levels

Serum levels of total IgE, OVA-specific IgE, and OVA-specific IgG1 were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits for total IgE and OVA-specific IgE were purchased from R&D Systems (Minneapolis, MN), and the OVA-specific IgG1 ELISA kit was purchased from Cayman Chemical (Ann Arbor, MI).

Measurement of passive systemic anaphylaxis

Passive systemic anaphylaxis assay was performed after intranasal administration of OVA. Changes in rectal temperature were assessed at 5-min intervals for 60 min using a digital thermometer (A&D, Japan).

RNA sequencing and bioinformatic analysis

For the RNA sequencing analysis, total RNA was extracted from mouse lungs using TRIZOL[®] reagent (Thermo Fisher Scientific) according to the manufacturer's instructions (n = 3, each group, Fig. S4). The concentration and purity of the RNA samples were determined by automated optical density evaluation ($OD_{260}/OD_{280} \geq 1.8$ and $OD_{260}/OD_{230} \geq 1.8$) using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA sequencing libraries

were prepared using a NEBNext rRNA Depletion Kit (New England Biolabs, Ipswich, MA) and an ENBNext Ultra Directional RNA Library Prep Kit (New England Biolabs) according to the manufacturer's instructions using 500 ng of total RNA samples. Next, 2 × 36 base paired-end sequencing was performed using a NextSeq 500 sequencer (Illumina, San Diego, CA) by Tsukuba i-Laboratory LLP (Tsukuba, Japan). Sequences were mapped to the mm10 mouse genome and quantified using CLC Genomics Workbench version 10.1.1 (QIAGEN). An adjusted P value < 0.05 (Benjamini–Hochberg FDR method for multiple testing corrections) was used as the cutoff criterion. Upregulation and downregulation were defined as relative changes in transcription levels > 1.2-fold. The data are available under GEO series accession number GSE148161.

Functional Enrichment Analysis and Pathway Analysis of DE Genes

GO terms enriched in the DE genes were identified using Metascape (<http://metascape.org>). Biological pathways enriched in the data were identified with Ingenuity Pathway Analysis (IPA) software (QIAGEN, Venlo, Netherlands) using Fisher's exact test (P < 0.05 indicates statistical significance). A comparison analysis was performed according to previously described methods ⁴. A heat map was generated using Morpheus (<https://software.broadinstitute.org/morpheus>).

Statistical analysis

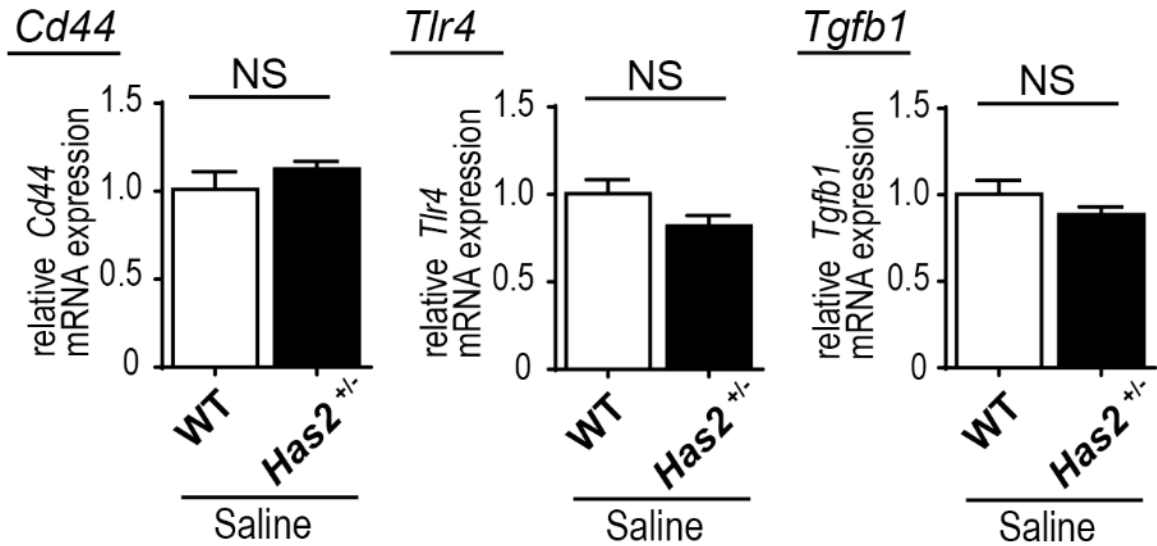
Data are shown as means ± SEMs or individual dot plots with means ± SEMs. Statistical significance between groups was evaluated using Mann–Whitney U test (Fig. 1B–1C, S1–2, and S6) or ANOVA with Tukey's multiple comparison test (Fig. 1F–1G and S3–5). P values < 0.05 were considered statistically significant.

References for the supplemental methods

1. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A, Jr., et al. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 2000;**106**:349-360.
2. Matuska B, Comhair S, Farver C, Chmiel J, Midura RJ, Bonfield T, et al. Pathological Hyaluronan Matrices in Cystic Fibrosis Airways and Secretions. *Am J Respir Cell Mol Biol* 2016;**55**:576-585.
3. Kiwamoto T, Brummet ME, Wu F, Motari MG, Smith DF, Schnaar RL, et al. Mice deficient in the St3gal3 gene product alpha2,3 sialyltransferase (ST3Gal-III) exhibit enhanced allergic eosinophilic airway inflammation. *J Allergy Clin Immunol* 2014;**133**:240-247 e241-243.
4. Feld M, Garcia R, Buddenkotte J, Katayama S, Lewis K, Muirhead G, et al. The pruritus- and TH2-associated cytokine IL-31 promotes growth of sensory nerves. *J Allergy Clin Immunol* 2016;**138**:500-508 e524.

Supplemental Figure S1.

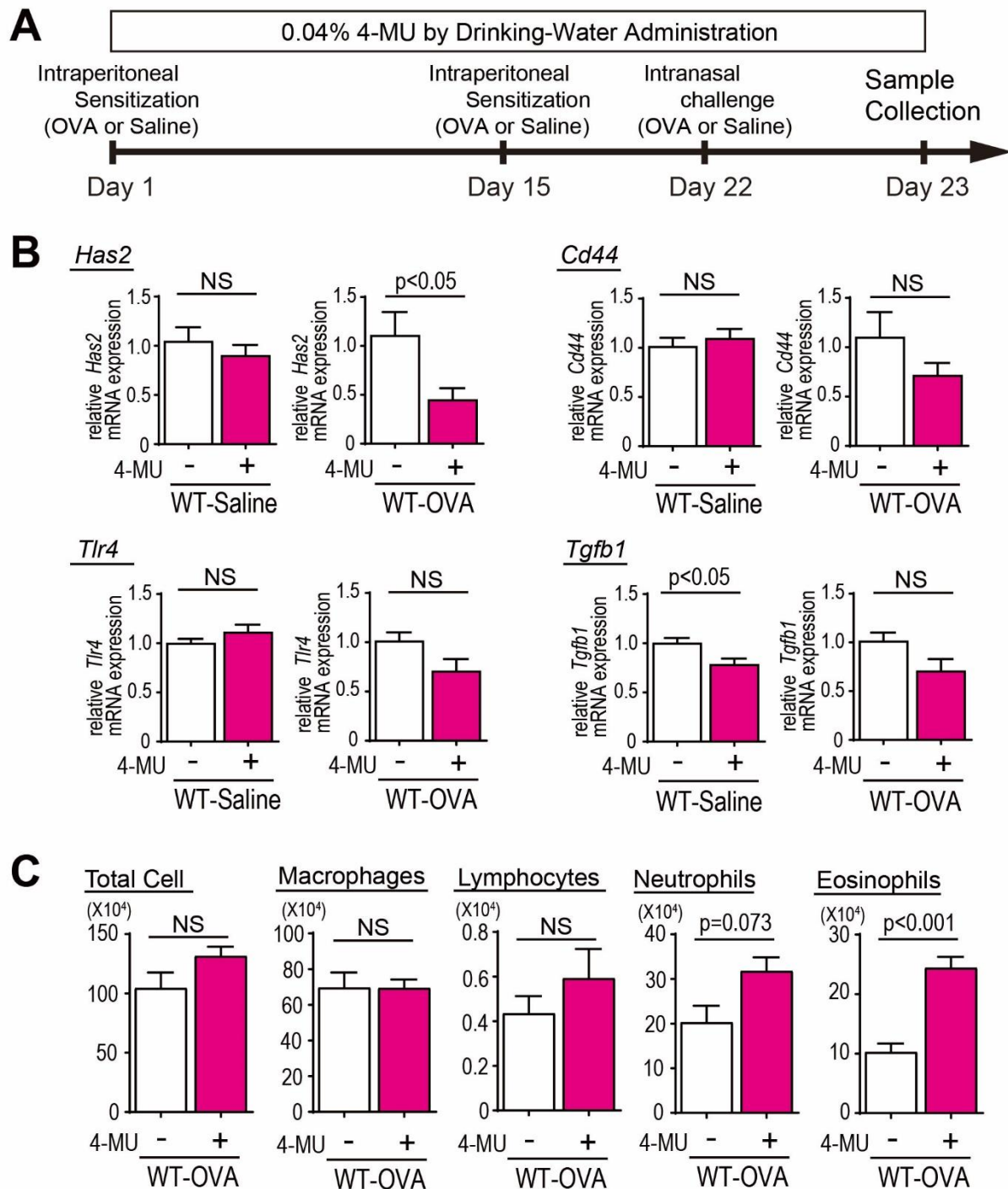
Effect of *Has2* attenuation on the on the mRNA expression levels of HA-related molecules in saline control mice lung.



Levels of the encoding mRNA transcripts; *Cd44*, *Tlr4*, and *Tgfb1* (n = 7). Horizontal bars indicate direct statistical comparisons using Mann–Whitney U test. *NS*, not significant.

Supplemental Figure S2.

Effect of Has2 and HA synthesis inhibition by 4-MU on the severity of OVA-induced airway inflammation and on the mRNA expression levels of HA-related molecules in WT mice lung.

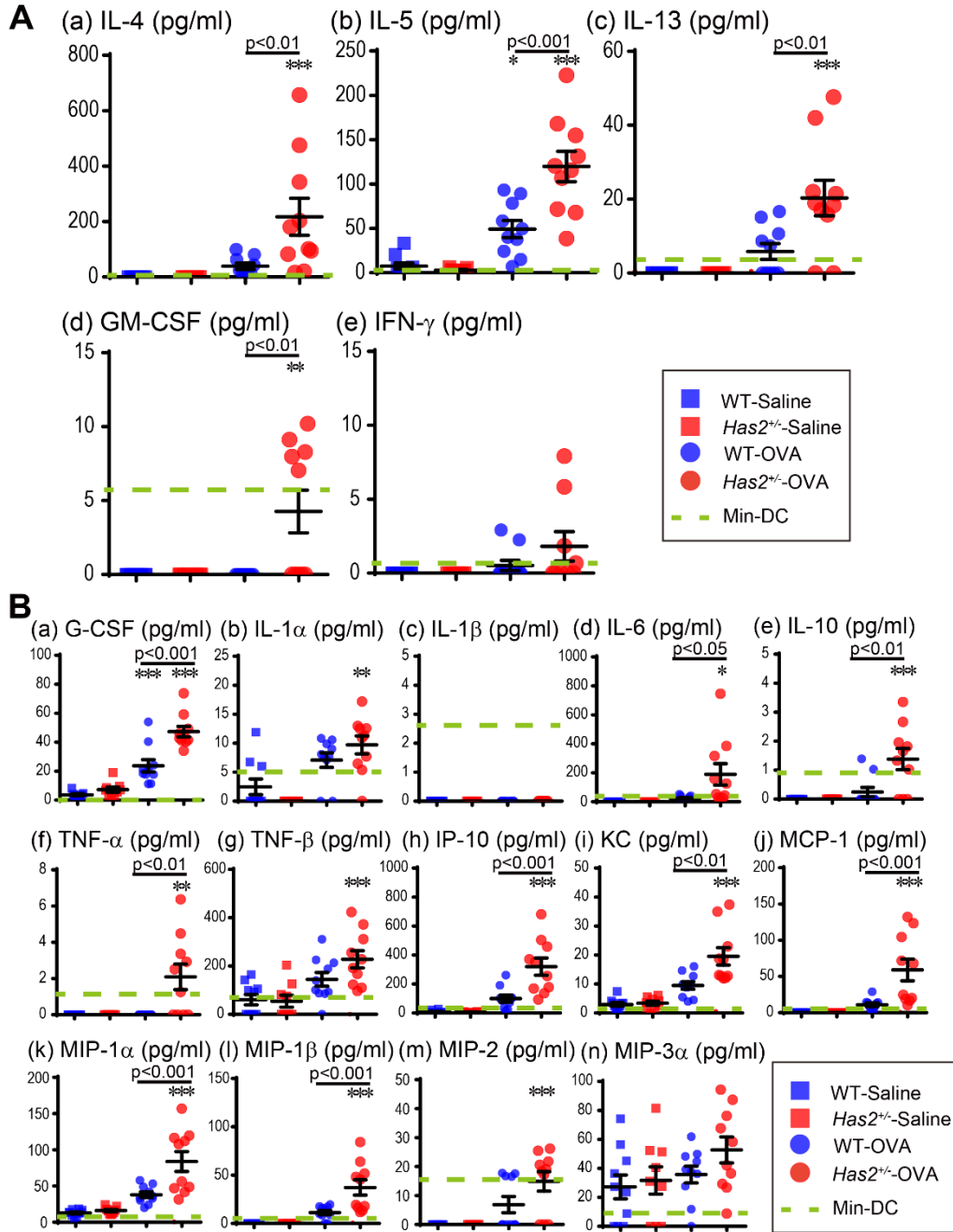


To confirm whether Has2 and HA synthesis inhibition affect OVA-induced airway inflammation, 4-MU with 0.04% concentration in drinking water was administrated.

(Panel A) Schematic illustration of the experimental design. **(Panel B)** Levels of encoding mRNA transcripts; *Has2*, *Cd44*, *Tlr4*, and *Tgfb1* ($n = 7$). **(Panel C)** BALF cell count obtained from WT mice with or without 4-MU administration ($n = 7$). White bars indicate WT mice group without 4-MU administration. Pink bars indicate WT mice group with 4-MU administration. Horizontal bars indicate direct statistical comparisons using Mann–Whitney U test. 4-MU, 4-Methylumbelliferone; *NS*, not significant.

Supplemental Figure S3.

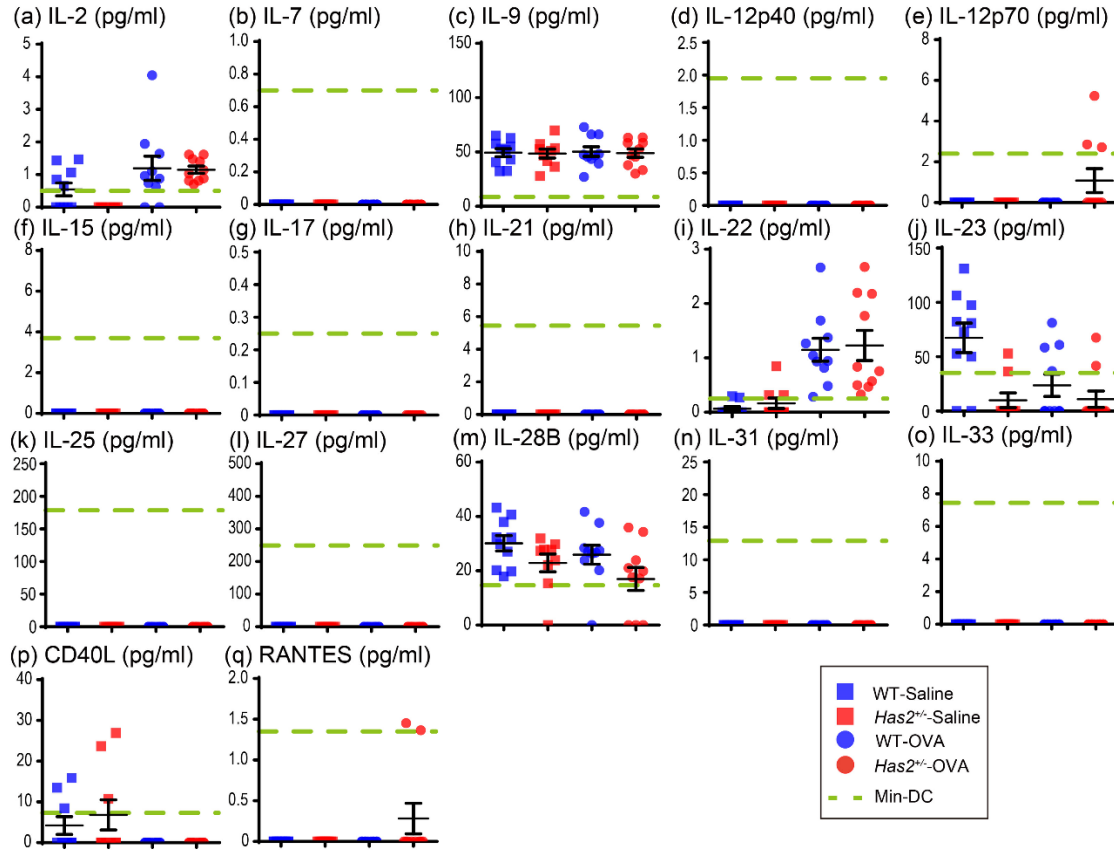
Effect of *Has2* attenuation on the levels of the indicated Th1/Th2 related or inflammatory cytokines and chemokines in BALF.



Levels of the indicated Th1/Th2 related cytokines (**Panel A**) and inflammatory cytokines and chemokines (**Panel B**) were quantified in BALF obtained 24 h after saline or OVA final challenge (n = 9–10). *P < 0.05, **P < 0.01 and ***P < 0.001 relative to WT-saline mice as determined using Tukey's multiple comparison test. Horizontal bars indicate direct statistical comparisons between WT-OVA and *Has2*^{+/-}-OVA mice. Dashed green lines indicate the level of detection. NS, not significant.

Supplemental Figure S4.

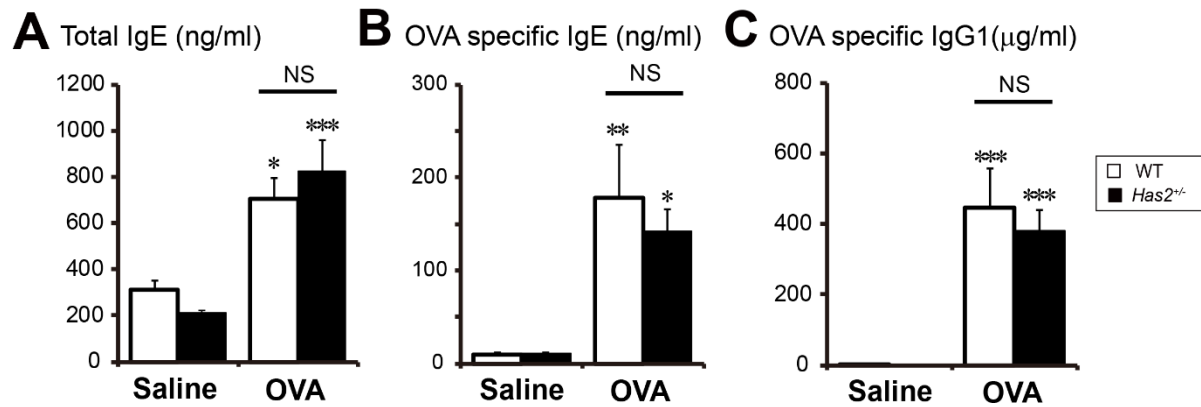
Effect of *Has2* attenuation on the levels of various cytokines and chemokines in BALF.



Levels of the indicated mediators were quantified in BALF that was obtained 24 h after saline or OVA final challenge (n = 9–10). *P < 0.05, **P < 0.01, and ***P < 0.001 relative to WT-saline mice, determined using Tukey's multiple comparison test. Horizontal bars indicate direct statistical comparisons between WT-OVA and *Has2*^{+/-}-OVA mice. Dashed green lines indicate the level of detection. NS, not significant.

Supplemental Figure S5.

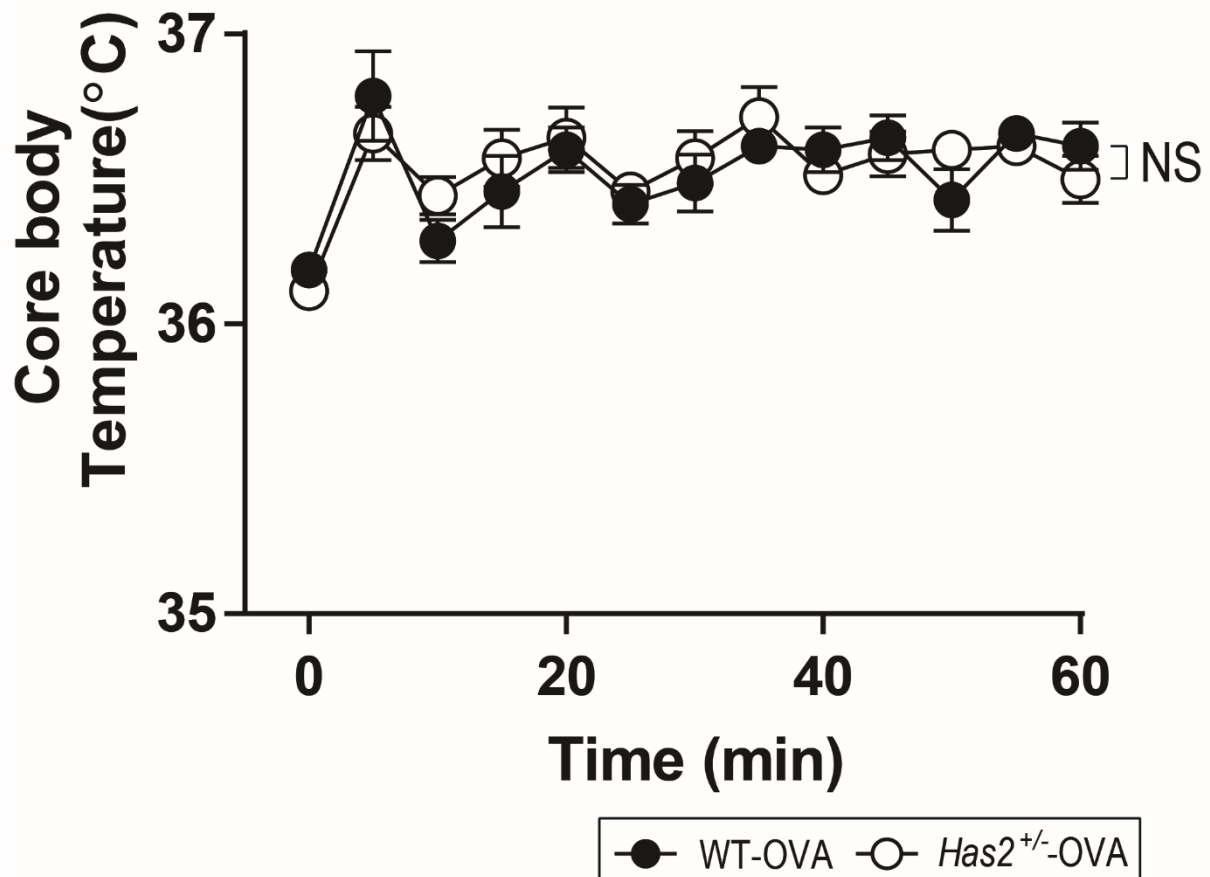
Effect of Has2 attenuation on OVA-induced serological markers associated with allergic sensitization.



Levels of total IgE (**Panel A**), OVA-specific IgE (**Panel B**), and OVA-specific IgG1 (**Panel C**) in the serum were obtained 24 h after the final challenge with saline or OVA (n = 9–10). *P < 0.05; **P < 0.01; ***P < 0.001 relative to the WT-saline mice group as determined using Tukey's multiple comparison test. Horizontal bars indicate direct statistical comparisons between WT-OVA and *Has2*^{+/-}-OVA mice. NS, not significant.

Supplemental Figure S6.

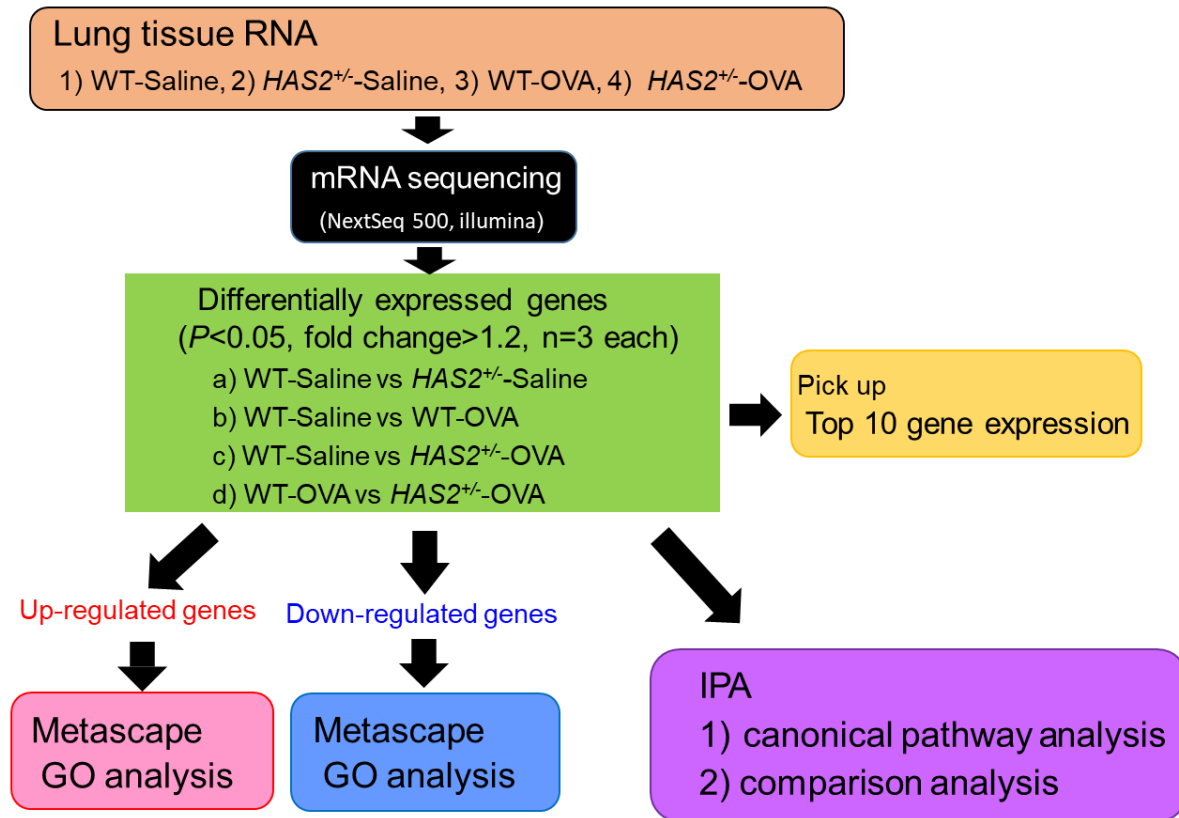
Effect of Has2 attenuation on OVA-induced passive systemic anaphylaxis associated with allergic sensitization and challenge.



Rectal temperature was measured and is shown as a function of time; data are represented as means $\pm$ SEMs of experiments with seven mice per group. *NS*, not significant.

Supplemental Figure S7.

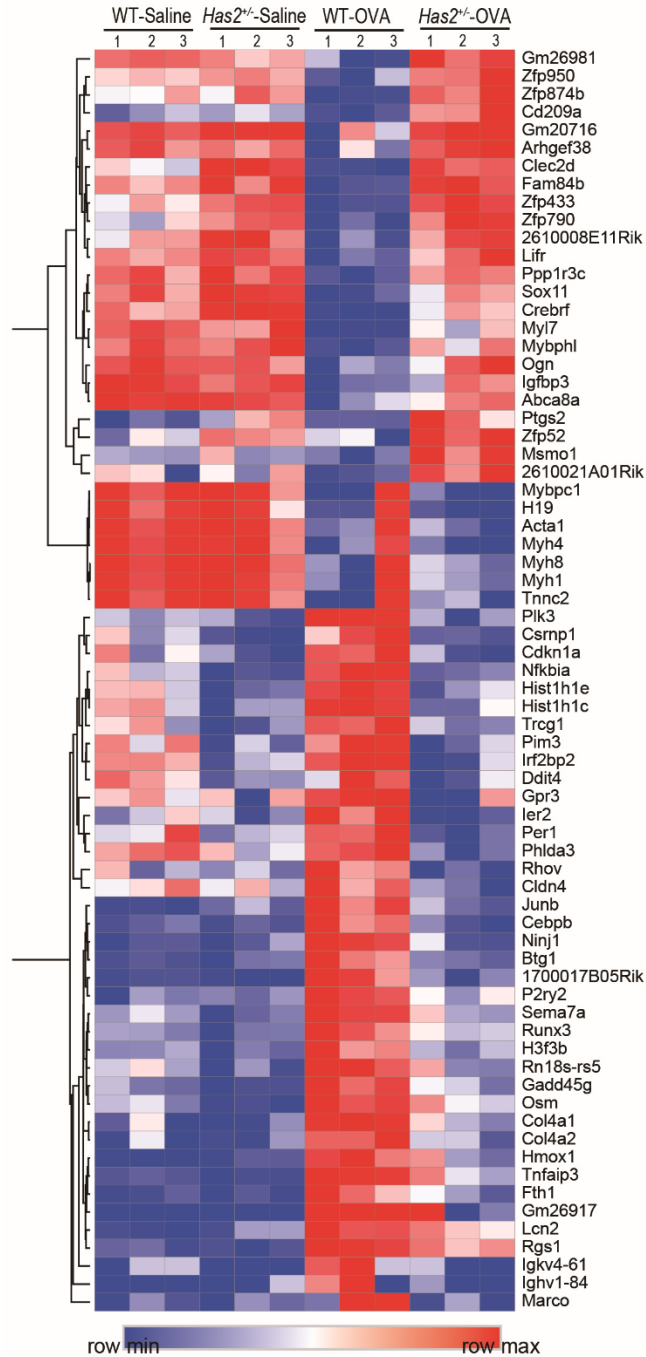
Schematic workflow for the analysis of gene lists generated from the transcriptome profiling experiments.



Gene lists represent gene products significantly upregulated and downregulated in the lungs under the following four conditions: 1) WT-saline mice compared with *Has2*^{+/-}-saline mice, 2) WT-saline mice compared with WT-OVA mice, 3) WT-saline mice compared with *Has2*^{+/-}-OVA mice, and 4) WT-OVA mice compared with *Has2*^{+/-}-OVA mice (P < 0.05, fold change > 1.2, n = 3 each). Lists of differentially expressed genes were analyzed using two types of software: Metascape (<http://metascape.org>) and ingenuity pathways analysis (IPA) software. Important representative genes were also manually picked up. GO, gene ontology.

Supplemental Figure S8.

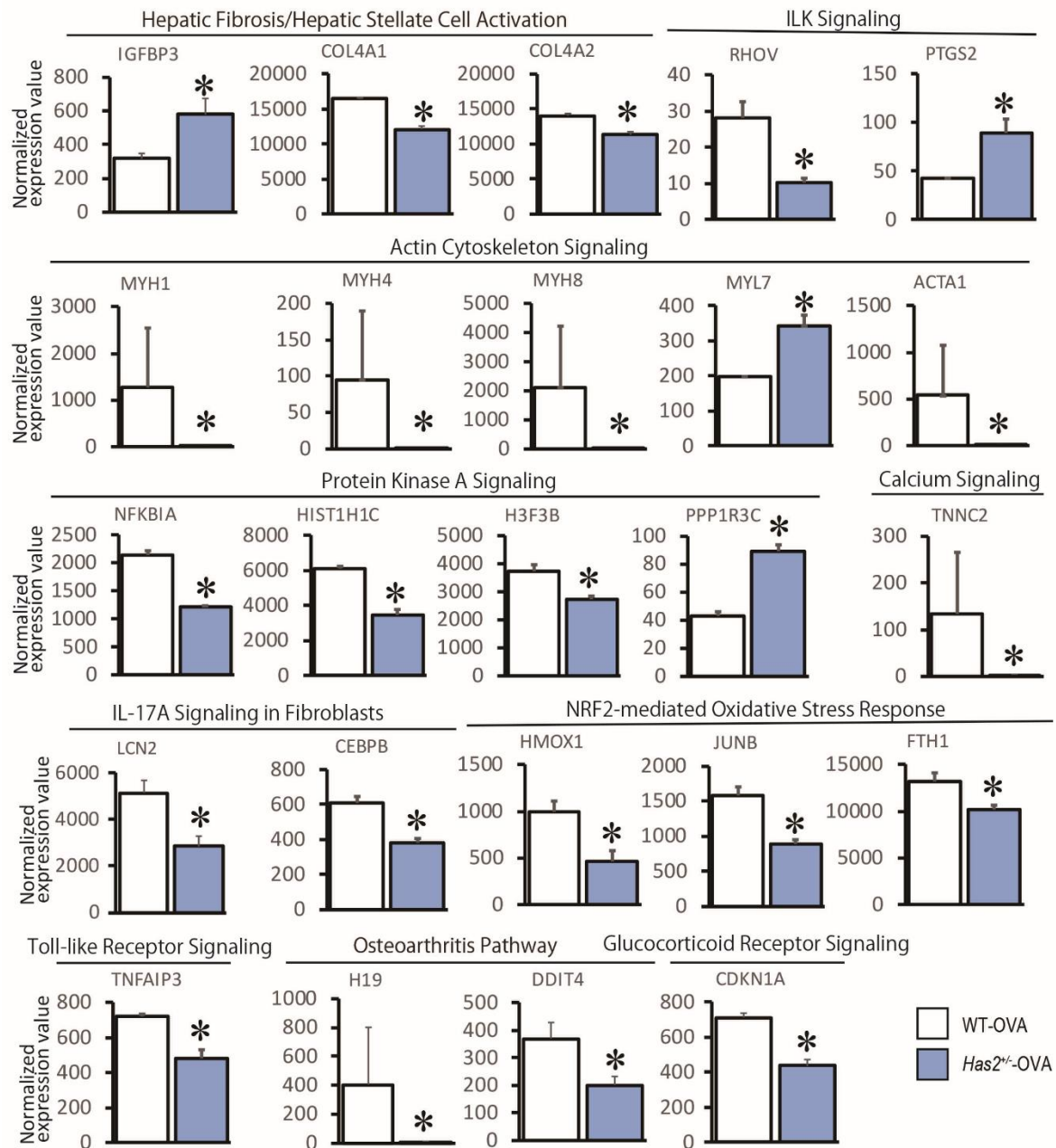
Heat map of differentially expressed genes between the lungs from WT-OVA mice and *Has2*^{+/-}-OVA mice.



Heat map of differentially expressed genes between the lungs from WT-OVA mice and *Has2*^{+/-}-OVA mice ($P < 0.05$, fold change > 1.2 , $n = 3$ each). Hierarchical clustering was performed based on the mean log₂ fold change. Heat maps were visualized using a relative color scheme by Morpheus.

Supplemental Figure S9.

Normalized expression value of differentially expressed genes between the lungs from WT-OVA mice and *Has2*^{+/-}-OVA mice.



Representative genes in significant terms were detected using the canonical analysis of the IPA software. *P < 0.05

Supplemental Table S1.

Top 10 upregulated and downregulated differentially expressed gene lists.

WT-Saline vs <i>Has2</i> ^{+/-} -Saline			WT-Saline vs WT-OVA			WT-Saline vs <i>Has2</i> ^{+/-} -OVA			WT-OVA vs <i>Has2</i> ^{+/-} -OVA		
Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj
<i>Hamp2</i>	6.34	5.7E-35	<i>Ighv1-84</i>	8.75	8.5E-04	<i>Ighg2c</i>	6.55	8.1E-03	<i>Gm20716</i>	3.53	9.3E-08
<i>Fos</i>	1.49	8.9E-05	<i>Igkv1-88</i>	6.93	4.6E-02	<i>Chil4</i>	6.36	1.9E-13	<i>Cd209a</i>	1.26	1.1E-02
<i>Egr1</i>	1.03	1.7E-04	<i>Ighg1</i>	6.50	2.1E-05	<i>Clca1</i>	6.07	1.3E-08	<i>Zfp433</i>	1.14	1.1E-04
<i>Clec2d</i>	0.61	5.5E-04	<i>Ighg2c</i>	6.40	4.8E-03	<i>Ighv14-2</i>	5.73	1.3E-02	<i>Mybphl</i>	1.13	1.8E-02
N/A	N/A	N/A	<i>Cxcl10</i>	5.99	1.4E-16	<i>Ighg1</i>	5.70	5.1E-04	<i>Ptgs2</i>	1.12	1.2E-02
N/A	N/A	N/A	<i>Ighv2-7</i>	5.93	3.6E-02	<i>Mrgprg</i>	5.42	4.4E-02	<i>Ppp1r3c</i>	1.08	1.0E-03
N/A	N/A	N/A	<i>Ighv2-9-1</i>	5.89	6.7E-03	<i>Cxcl10</i>	5.21	2.7E-12	<i>Zfp52</i>	1.07	1.5E-02
N/A	N/A	N/A	<i>Ighv4-1</i>	5.72	1.6E-04	<i>Retnla</i>	4.79	1.4E-22	<i>Gm26981</i>	1.07	5.5E-03
N/A	N/A	N/A	<i>Gzmc</i>	5.55	2.4E-02	<i>Fer1l6</i>	4.69	7.1E-12	<i>Zfp874b</i>	1.03	5.1E-03
N/A	N/A	N/A	<i>Ighv2-6</i>	5.47	1.9E-02	<i>Capn9</i>	4.51	1.8E-02	<i>Clec2d</i>	1.01	3.3E-14
<i>Ngp</i>	-2.44	1.4E-02	<i>Cplx1</i>	-5.33	6.6E-03	<i>Kprp</i>	-10.15	3.4E-03	<i>Myh8</i>	-8.34	1.9E-02
<i>Cfd</i>	-1.83	3.5E-03	<i>Lep</i>	-5.14	1.8E-06	<i>Myh4</i>	-9.94	1.6E-04	<i>Mybpc1</i>	-8.15	1.5E-02
<i>Cyp2e1</i>	-1.31	2.1E-03	<i>Slitrk2</i>	-5.04	4.6E-02	<i>Lce3a</i>	-9.85	4.8E-03	<i>Myh1</i>	-8.06	2.6E-02
N/A	N/A	N/A	<i>Vat1l</i>	-4.29	2.4E-04	<i>Lce3f</i>	-9.71	5.8E-03	<i>Acta1</i>	-7.87	3.7E-02
N/A	N/A	N/A	<i>Adipoq</i>	-4.18	2.4E-10	<i>Myh8</i>	-9.62	6.0E-04	<i>Myh4</i>	-7.77	2.8E-02
N/A	N/A	N/A	<i>Retn</i>	-3.78	1.0E-09	<i>Defb4</i>	-9.42	8.1E-03	<i>Ighv1-84</i>	-6.92	3.4E-02
N/A	N/A	N/A	<i>A530016L24Rik</i>	-3.69	3.3E-09	<i>Mybpc1</i>	-9.41	3.2E-04	<i>Tnnc2</i>	-6.91	3.3E-02
N/A	N/A	N/A	<i>Tshr</i>	-3.60	1.4E-06	<i>Lce3c</i>	-9.34	8.8E-03	<i>H19</i>	-5.56	2.6E-02
N/A	N/A	N/A	<i>Thrsp</i>	-3.52	6.2E-08	<i>Acta1</i>	-9.05	1.6E-03	<i>Igkv4-61</i>	-5.14	3.2E-02
N/A	N/A	N/A	<i>Pck1</i>	-3.50	8.7E-09	<i>Myh1</i>	-8.91	1.5E-03	<i>Gpr3</i>	-3.60	1.0E-03

Definition of abbreviations: FC, fold change; Padj, adjusted *P* value; N/A, not applicable.

Supplemental Table S2.

Significant biological process terms detected by gene ontology (GO) analysis with Metascape in the lungs of *Has2*^{+/-} mice compared with WT mice after OVA stimulation.

Up regulated gene			
GO Term		-log ₁₀ (<i>p</i> -value)	Symbols
GO:0001503	Ossification	2.54	<i>Igfbp3,Ptgs2,Sox11</i>
Down regulated gene			
GO Term		-log ₁₀ (<i>p</i> -value)	Symbols
GO:0097190	apoptotic signaling pathway	5.51	<i>Runx3,Cdkn1a,Cebpb,Hmox1,Lcn2,Osm,Tnfaip3,Phlda3,Ddit4</i>
GO:0001816	cytokine production	4.88	<i>Runx3,Cebpb,Hmox1,Osm,P2ry2,Per1,Sema7a,Tnfaip3,Gadd45g</i>
GO:0120162	positive regulation of cold-induced thermogenesis	4.35	<i>Cebpb,Gpr3,Lcn2,Gadd45g</i>
GO:0030261	chromosome condensation	3.95	<i>H3f3b,Hist1h1c,Hist1h1e</i>
GO:0048662	negative regulation of smooth muscle cell proliferation	3.74	<i>Cdkn1a,Hmox1,Tnfaip3</i>
GO:0006936	muscle contraction	3.56	<i>Myh1,Myh4,Myh8,Tnnc2,Mybpc1</i>
GO:0055072	iron ion homeostasis	3.06	<i>Fth1,Hmox1,Lcn2</i>
GO:0001933	negative regulation of protein phosphorylation	2.85	<i>Cdkn1a,Per1,Tnfaip3,Gadd45g,Ddit4</i>
GO:2000241	regulation of reproductive process	2.29	<i>Gpr3,Osm,P2ry2</i>
GO:0032496	response to lipopolysaccharide	2.20	<i>Cebpb,Junb,Nfkb1a,Tnfaip3</i>
GO:0048514	blood vessel morphogenesis	2.06	<i>Btg1,Col4a1,Col4a2,Hmox1,Junb</i>
GO:0007015	actin filament organization	2.03	<i>Acta1,Nckap1,Mybpc1,Rhov</i>

Supplemental Table S3.

Significant terms detected using canonical pathway analysis with IPA in the lungs of *Has2*^{+/-} mice compared with that in the lungs of WT mice after OVA stimulation.

Canonical Pathways	$-\log_{10}(p\text{-value})$	z-score	Molecules
Hepatic Fibrosis / Hepatic Stellate Cell Activation	6.25	NaN	<i>Col4a1, Col4a2, Igfbp3, Myh1, Myh4, Myh8, Myl7</i>
ILK Signaling	6.08	-1.134	<i>Acta1, Myh1, Myh4, Myh8, Myl7, Ptgs2, Rhov</i>
Tight Junction Signaling	5.24	NaN	<i>Acta1, Cldn4, Myh1, Myh4, Myh8, Myl7</i>
Agranulocyte Adhesion and Diapedesis	5.11	NaN	<i>Acta1, Cldn4, Myh1, Myh4, Myh8, Myl7</i>
Calcium Signaling	4.82	NaN	<i>Acta1, Myh1, Myh4, Myh8, Myl7, Tnnc2</i>
Cellular Effects of Sildenafil (Viagra)	4.65	NaN	<i>Acta1, Myh1, Myh4, Myh8, Myl7</i>
Epithelial Adherens Junction Signaling	4.34	NaN	<i>Acta1, Myh1, Myh4, Myh8, Myl7</i>
Protein Kinase A Signaling	4.14	-0.816	<i>H1-2, H3-3a/H3-3b, Myh4, Myl7, Nfkb1a, Ppp1r3c, Ptgs2</i>
IL-17A Signaling in Fibroblasts	3.94	NaN	<i>Cebpb, Lcn2, Nfkb1a</i>
Actin Cytoskeleton Signaling	3.46	-1.342	<i>Acta1, Myh1, Myh4, Myh8, Myl7</i>
VDR/RXR Activation	2.93	NaN	<i>Cdkn1a, Cebpb, Igfbp3</i>
GADD45 Signaling	2.93	NaN	<i>Cdkn1a, Gadd45g</i>
Acute Phase Response Signaling	2.90	NaN	<i>Cebpb, Hmox1, Nfkb1a, Osm</i>
CD40 Signaling	2.88	NaN	<i>Nfkb1a, Ptgs2, Tnfaip3</i>
Regulation of Actin-based Motility by Rho	2.81	NaN	<i>Acta1, Myl7, Rhov</i>
NRF2-mediated Oxidative Stress Response	2.69	NaN	<i>Acta1, Fth1, Hmox1, Junb</i>
IL-8 Signaling	2.66	0	<i>Hmox1, Myl7, Ptgs2, Rhov</i>
ATM Signaling	2.65	NaN	<i>Cdkn1a, Gadd45g, nfkb1a</i>
Osteoarthritis Pathway	2.65	1	<i>Cebpb, Ddit4, H19, Ptgs2</i>
TNFR2 Signaling	2.56	NaN	<i>Nfkb1a, Tnfaip3</i>
MIF-mediated Glucocorticoid Regulation	2.42	NaN	<i>Nfkb1a, Ptgs2</i>
Pancreatic Adenocarcinoma Signaling	2.36	NaN	<i>Cdkn1a, Hmox1, Ptgs2</i>

PI3K/AKT Signaling	2.31	NaN	<i>Cdkn1a, Nfkbia, Ptgs2</i>
MIF Regulation of Innate Immunity	2.24	NaN	<i>Nfkbia, Ptgs2</i>
TNFR1 Signaling	2.13	NaN	<i>Nfkbia, Tnfaip3</i>
Semaphorin Signaling in Neurons	2.06	NaN	<i>Rhov, Sema7a</i>
Gaq Signaling	2.04	NaN	<i>Hmox1, Nfkbia, Rhov</i>
Heme Degradation	1.97	NaN	<i>Hmox1</i>
RhoGDI Signaling	1.94	NaN	<i>Acta1, Myl7, Rhov</i>
Glucocorticoid Receptor Signaling	1.86	NaN	<i>Cdkn1a, Cebpb, Nfkbia, Ptgs2</i>
IL-10 Signaling	1.83	NaN	<i>Hmox1, Nfkbia</i>
Role of IL-17A in Arthritis	1.82	NaN	<i>Nfkbia, Ptgs2</i>
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.80	NaN	<i>Hmox1, Ppp1r3c, Rhov</i>
Zymosterol Biosynthesis	1.79	NaN	<i>Msmo1</i>
Toll-like Receptor Signaling	1.76	NaN	<i>Nfkbia, Tnfaip3</i>
mTOR Signaling	1.75	NaN	<i>Ddit4, Hmox1, Rhov</i>
Integrin Signaling	1.70	NaN	<i>Acta1, Myl7, Rhov</i>
Small Cell Lung Cancer Signaling	1.64	NaN	<i>Nfkbia, Ptgs2</i>
Prostanoid Biosynthesis	1.62	NaN	<i>Ptgs2</i>
JAK/Stat Signaling	1.60	NaN	<i>Cdkn1a, Cebpb</i>
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	1.59	NaN	<i>Acta1, Hmox1</i>
Death Receptor Signaling	1.59	NaN	<i>Acta1, Nfkbia</i>
Phospholipase C Signaling	1.59	NaN	<i>Hmox1, Myl7, Rhov</i>
IL-17 Signaling	1.58	NaN	<i>Cebpb, Ptgs2</i>
Sumoylation Pathway	1.56	NaN	<i>Nfkbia, Rhov</i>
PPAR Signaling	1.54	NaN	<i>Nfkbia, Ptgs2</i>
Signaling by Rho Family GTPases	1.52	NaN	<i>Acta1, Myl7, Rhov</i>

Prostate Cancer Signaling	1.52	NaN	<i>Cdkn1a, Nfkbia</i>
Antioxidant Action of Vitamin C	1.49	NaN	<i>Hmox1, Nfkbia</i>
Cholecystokinin/Gastrin-mediated Signaling	1.47	NaN	<i>Ptgs2, Rhov</i>
Choline Biosynthesis III	1.46	NaN	<i>Hmox1</i>
Cholesterol Biosynthesis I	1.46	NaN	<i>Msmo1</i>
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	1.46	NaN	<i>Msmo1</i>
Cholesterol Biosynthesis III (via Desmosterol)	1.46	NaN	<i>Msmo1</i>
p53 Signaling	1.43	NaN	<i>Cdkn1a, Gadd45g</i>
Sirtuin Signaling Pathway	1.39	NaN	<i>Gadd45g, H1-2, H3-3a/H3-3b</i>
HGF Signaling	1.38	NaN	<i>Cdkn1a, Ptgs2</i>
RhoA Signaling	1.37	NaN	<i>Acta1, Myl7</i>
Phagosome Formation	1.36	NaN	<i>Marco, Rhov</i>
Granzyme A Signaling	1.35	NaN	<i>H1-2</i>
Fatty Acid α -oxidation	1.32	NaN	<i>Ptgs2</i>
Iron homeostasis signaling pathway	1.31	NaN	<i>Fth1, Hmox1</i>
GP6 Signaling Pathway	1.31	NaN	<i>Col4a1, Col4a2</i>