

Online Supplement Data

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Exposure to the heated tobacco product IQOS generates apoptosis-mediated pulmonary emphysema in murine lungs

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10 **Supplemental methods**

11 *Microarray analysis*

12 Total RNA from the right lungs was extracted using miRNeasy mini kits (Qiagen,
13 Hilden, Germany). RNA samples were labeled using the Low Input Quick Amp Labeling Kit
14 (Agilent Technologies, Waldbronn, Germany). Labeled RNA was hybridized to an
15 oligonucleotide microarray (SurePrint G3 Mouse GE microarray 8x60K Ver. 2.0, Agilent
16 Technologies) at 60 °C, 10 rpm for 17 h. After washing with the Gene Expression Wash Pack
17 (Agilent Technologies) and drying, the slides were scanned with DNA Microarray Scanner
18 and analyzed with the Feature Extraction software version 10.7.1.1 (Agilent Technologies).
19 Normalization was performed using global normalization methods. Each raw data was
20 flagged as 'Detected,' 'Compromised,' or 'Not detected.' When any one of the probes was
21 judged 'Compromised,' GeneSpring flags of 'Compromised' were attached, and the same is
22 true for 'Not detected.' When there were no flags 'Compromised' and 'Not detected,' the
23 'Detected' flag was attached to the data.

24 Significance tests (Welch's *t*-test) between two groups (IQOS vs. air, CS vs. air) were
25 performed using normalized signal value, and *P*-values were calculated. We selected probes
26 that meet any of the following three conditions; 1) both groups showed the 'Detected' flag,
27 the expression ratio to air was more than 1.5 times or less than 0.67 times and $P < 0.05$, 2)
28 both groups showed the 'Detected' flag, the expression ratio to air was greater than 1 time and
29 less than 1.5 times or less than 1 time and more than 0.67 times and $P < 0.01$, 3) Switch ON:
30 at least one sample of air were 'Not detected' or 'Compromised,' and all of IQOS/CS were
31 'Detected,' the expression ratio to air was greater than 4 times and $P < 0.01$; Switch OFF: at
32 least one sample of IQOS/CS were 'Not detected' or 'Compromised,' and all of the air were
33 'Detected,' the expression ratio to air was less than 0.25 times and $P < 0.01$.

34

35 ***Specific primers (5'→3') for RT-qPCR***

36 ***COX-2***

37 Fw: CTGGCGCTCAGCCATACAG

38 Rv: CCGGGTACAATCGCACTTATACT

39 ***IL-6***

40 Fw: ACAAAGCCAGAGTCCTTCAGAGAGATAC

41 Rv: TGAATTGGATGGTCTTGGTCCTTAGCCA

42 ***nfn2l2***

43 Fw: GGTTGCCACATTCCCAAAC

44 Rv: TGATGAGGGGCAGTGAAGAC

45 ***hmox-1***

46 Fw: CACTCTGGAGATGACACCTGAG

47 Rv: GTGTTCCCTCTGTCAGCATCACC

48 ***Ccl2***

49 Fw: GCTACAAGAGGATCACCAGCAG

50 Rv: GTCTGGACCCATTCCTTCTTGG

51 ***Apaf1***

52 Fw: CACGAGTTCGTGGCATATAGGC

53 Rv: GGAAATGGCTGTCGTCCAAGGA

54 ***GAPDH***

55 Fw: AACTTTGGCATTGTGGAAGG

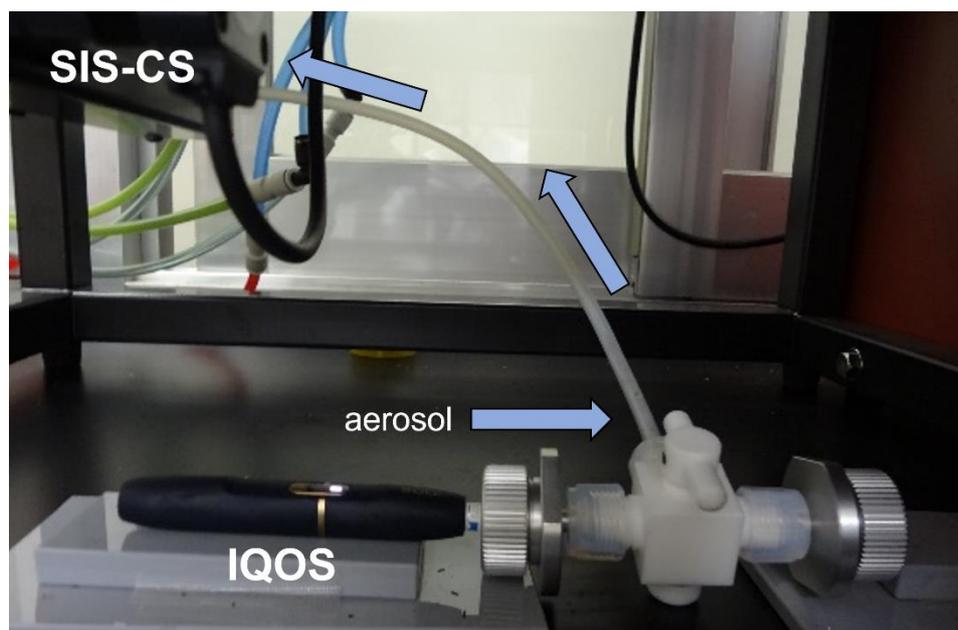
56 Rv: GGATGCAGGGATGATGTTCT

57

58

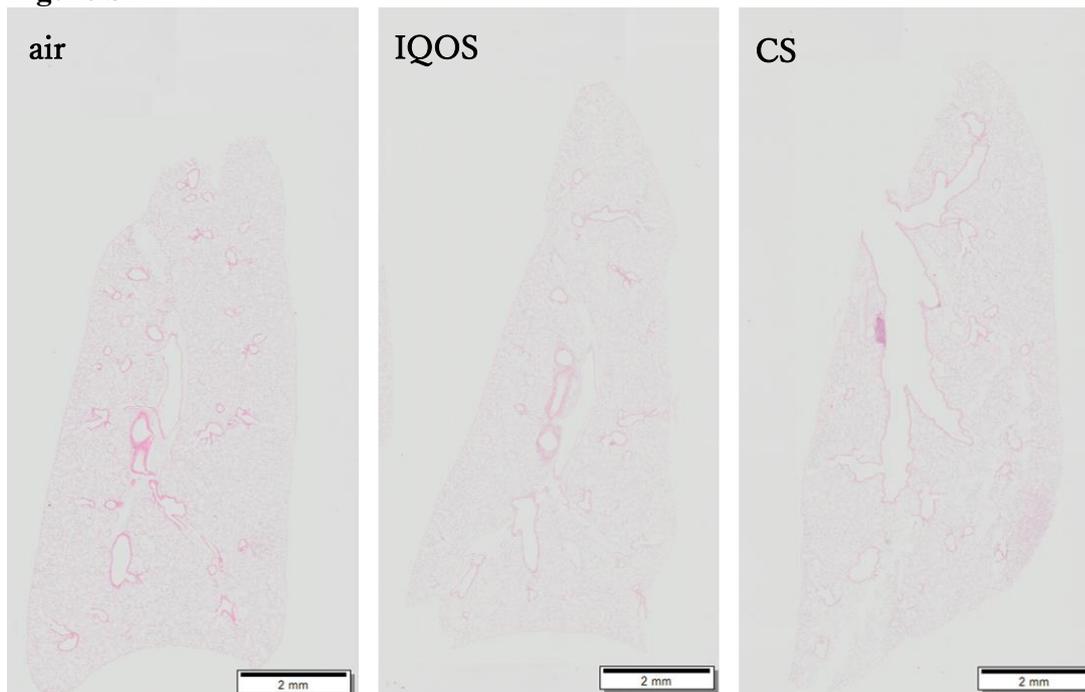
59

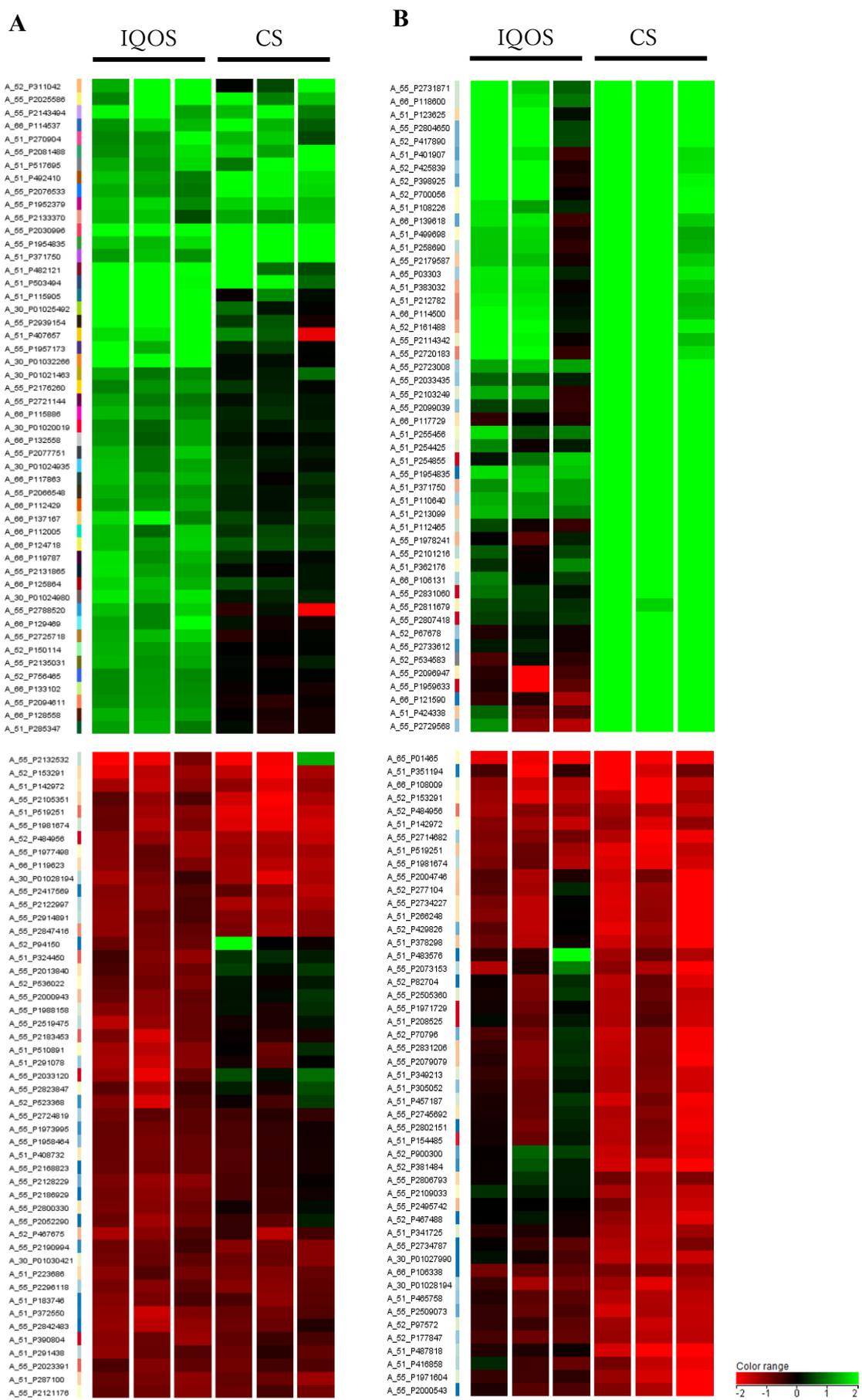
60 **Figure S1**
61



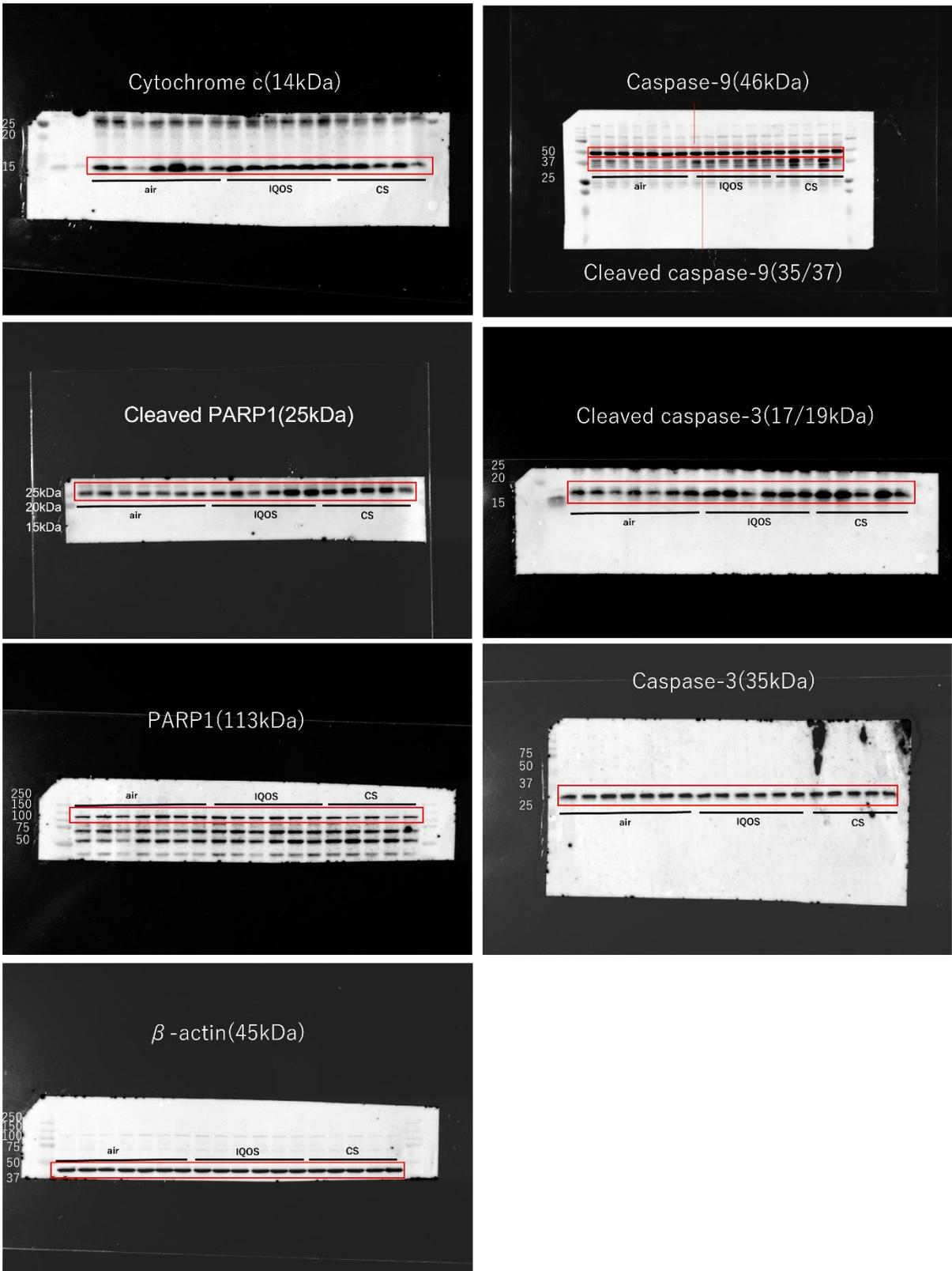
62

63 **Figure S2**
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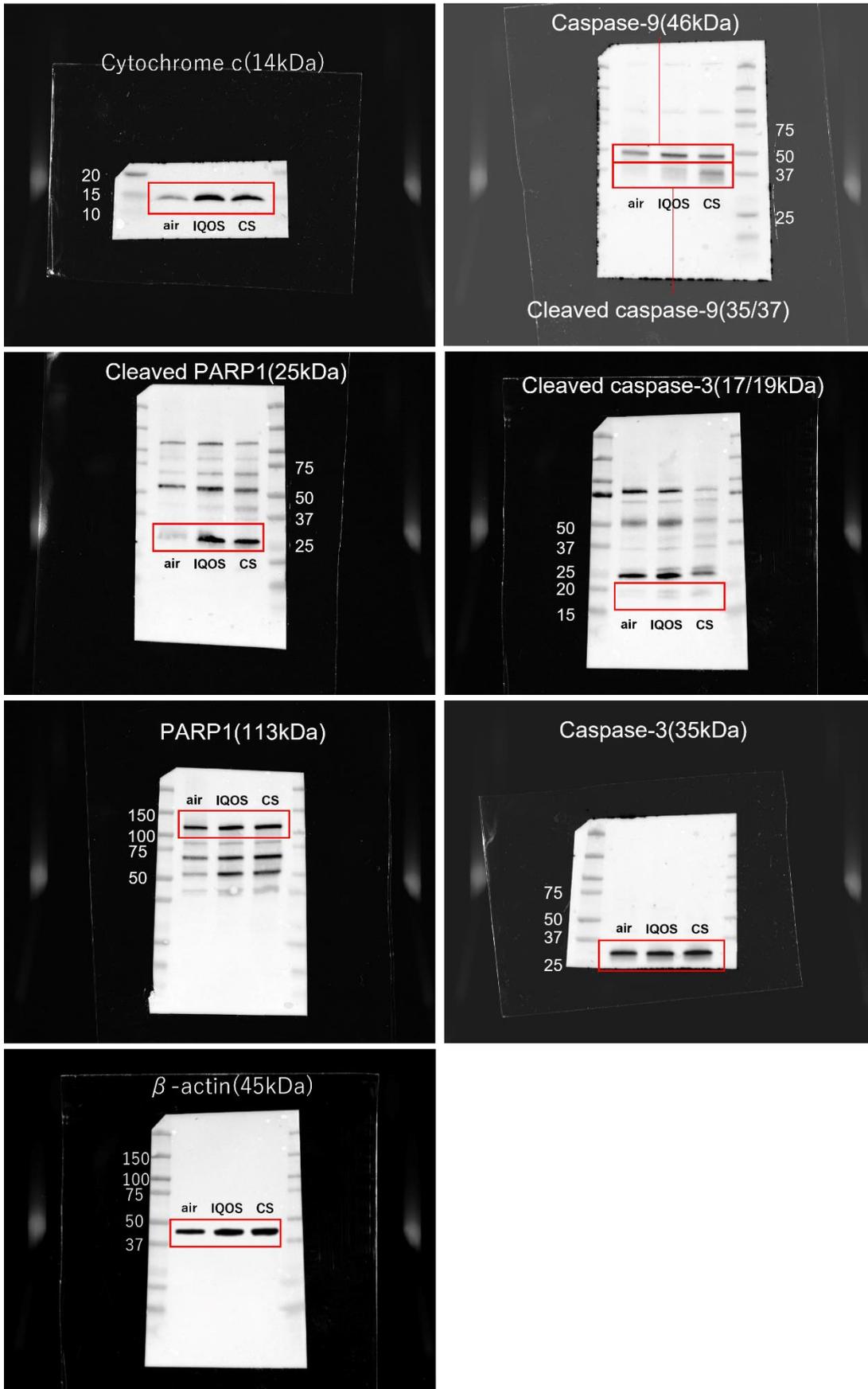




67 **Figure S4**
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71 **Figure S5**
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74 **Supplemental Figure Legends**

75 **Figure S1.** The system for connecting the IQOS cartridge and the cigarette smoke exposure
76 system (Model SIS-CS). IQOS aerosol is aspirated under computer control and sent to SIS-CS
77 in the direction of the arrow.

78

79 **Figure S2.** The representative full scan images of the hematoxylin–eosin-stained lung
80 exposed to air, IQOS, and CS. Scale bars: 2 mm.

81

82 **Figure S3.** (A) Heatmap displaying the top 50 up- and down-regulated genes in the IQOS
83 group compared to the air group and expression of those genes in the CS group. (B) Heatmap
84 displaying the top 50 up- and down-regulated genes in the CS group compared to the air
85 group and expression of those genes in the IQOS group. CS = cigarette smoke. $n = 3$ for each
86 group.

87

88 **Figure S4.** The western blot images of apoptosis-associated proteins in all samples.

89

90 **Figure S5.** Full blot images of apoptosis-associated proteins in Figure 7A.

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92

93 **Table S1. Weight gain suppression compared to the air group**

| Month after starting exposure | | Mean difference (g) | 95% CI (g) | P-value |
|-------------------------------|------------|---------------------|-------------------|---------|
| 1 month | | | | |
| | IQOS group | -0.605 | 0.8851 to -2.095 | 0.56 |
| | CS group | -1.935 | -0.4452 to -3.425 | 0.084 |
| 2 months | | | | |
| | IQOS group | -1.084 | 0.4064 to -2.574 | 0.18 |
| | CS group | -2.543 | -1.053 to -4.033 | < 0.001 |
| 3 months | | | | |
| | IQOS group | -1.87 | -0.3802 to -3.361 | 0.011 |
| | CS group | -2.141 | -0.6506 to -3.631 | 0.0033 |
| 4 months | | | | |
| | IQOS group | -2.417 | -0.9805 to -3.961 | < 0.001 |
| | CS group | -3.463 | -1.895 to -5.032 | < 0.001 |
| 5 months | | | | |
| | IQOS group | -3.263 | -1.773 to -4.753 | < 0.001 |
| | CS group | -4.664 | -3.096 to -6.232 | < 0.001 |
| 6 months | | | | |
| | IQOS group | -4.93 | -3.44 to -6.42 | < 0.001 |
| | CS group | -5.504 | -3.936 to -7.072 | < 0.001 |

95 **Table S2. Functional analysis of upregulated genes in IQOS and CS groups compared to**
 96 **the air group.**

| IQOS | | |
|---|---------------------|-------------|
| Category Name | P-value range | # Molecules |
| Disease and Disorders | | |
| Organismal Injury and Abnormalities | 2.88E-02 – 1.90E-04 | 445 |
| Skeletal and Muscular Disorder | 2.88E-02 – 1.90E-04 | 20 |
| Infectious Disease | 2.73E-02 – 4.32E-04 | 39 |
| Renal and Urological Disease | 2.73E-02 – 5.21E-04 | 73 |
| Cancer | 2.88E-02 – 8.25E-04 | 443 |
| Molecular and Cellular Function | | |
| Cellular Function and Maintenance | 2.88E-02 – 2.65E-05 | 119 |
| Cell Death and Survival | 2.88E-02 – 1.90E-04 | 59 |
| Gene Expression | 2.83E-02 – 6.93E-04 | 113 |
| Cellular Growth and Proliferation | 2.88E-02 – 7.43E-04 | 57 |
| Carbohydrate Metabolism | 2.88E-02 – 8.25E-04 | 22 |
| Physiological System Development and Function | | |
| Hematological System Development and Function | 2.88E-02 – 2.65E-05 | 68 |
| Behavior | 2.60E-02 – 4.43E-04 | 39 |
| Connective Tissue Development and Function | 2.06E-02 – 6.36E-04 | 27 |
| Skeletal and Muscular System Development and Function | 2.88E-02 – 5.21E-04 | 23 |
| Hair and Skin Development and Function | 2.88E-02 – 7.59E-04 | 6 |
| CS* | | |
| Category Name | P-value range | # Molecules |
| Disease and Disorders | | |
| Inflammatory Response | 2.78E-07 – 1.80E-34 | 414 |
| Organismal Injury and Abnormalities | 3.88E-07 – 5.66E-32 | 506 |
| Immunological Disease | 2.18E-07 – 2.01E-22 | 277 |
| Inflammatory Disease | 2.28E-07 – 9.36E-22 | 296 |
| Respiratory Disease | 7.59E-08 – 9.36E-22 | 129 |
| Molecular and Cellular Function | | |
| Cellular Movement | 3.14E-07 – 9.01E-39 | 352 |
| Cellular Compromise | 2.76E-08 – 6.86E-31 | 130 |
| Cellular Function and Maintenance | 3.14E-07 – 7.78E-28 | 287 |
| Cell-To-Cell Signaling and Infection | 3.13E-07 – 4.26E-24 | 261 |
| Cell Death and Survival | 4.05E-07 – 1.35E-21 | 410 |
| Physiological System Development and Function | | |
| Immune Cell Trafficking | 2.95E-07 – 2.82E-38 | 229 |
| Hematological System Development and Function | 3.32E-07 – 6.44E-31 | 326 |
| Tissue Morphology | 1.54E-07 – 6.87E-23 | 302 |
| Lymphoid Tissue Structure and Development | 3.32E-07 – 3.60E-19 | 217 |
| Tissue Development | 3.32E-07 – 7.50E-19 | 173 |

97

98 *CS = cigarette smoke.

99

100 **Table S3. Functional analysis of down-regulated genes in IQOS and CS groups**
 101 **compared to the air group.**

| IQOS | | | |
|--|--|-----------------------|-------------|
| Category | Name | <i>P</i> -value range | # Molecules |
| Disease and Disorders | | | |
| | Cancer | 1.34E-02 – 6.35E-08 | 521 |
| | Organismal Injury and Abnormalities | 1.34E-02 – 6.35E-08 | 527 |
| | Hematological Disease | 1.34E-02 – 1.21E-06 | 171 |
| | Immunological Disease | 1.34E-02 – 1.21E-06 | 158 |
| | Reproductive System Disease | 1.34E-02 – 1.49E-05 | 326 |
| Molecular and Cellular Function | | | |
| | Post-Translational Modification | 1.08E-02 – 1.47E-05 | 48 |
| | Cell Morphology | 1.34E-02 – 1.72E-05 | 80 |
| | Cellular Compromise | 1.34E-02 – 1.72E-05 | 25 |
| | RNA Post-Transcriptional Modification | 1.02E-02 – 6.79E-05 | 38 |
| | Cellular Development | 1.34E-02 – 9.30E-05 | 150 |
| Physiological System Development and Function | | | |
| | Nervous System Development and Function | 1.34E-02 – 3.49E-04 | 80 |
| | Organismal Development | 1.34E-02 – 3.49E-04 | 101 |
| | Tissue Development | 1.34E-02 – 3.49E-04 | 90 |
| | Cardiovascular System Development and Function | 1.34E-02 – 3.78E-04 | 24 |
| | Embryonic Development | 1.34E-02 – 3.78E-04 | 80 |
| CS* | | | |
| Category | Name | <i>P</i> -value range | # Molecules |
| Disease and Disorders | | | |
| | Cancer | 1.58E-02 – 1.63E-14 | 957 |
| | Endocrine System Disorders | 1.36E-02 – 1.63E-14 | 822 |
| | Organismal Injury and Abnormalities | 1.61E-02 – 1.63E-14 | 965 |
| | Gastrointestinal Disease | 1.18E-02 – 2.41E-09 | 842 |
| | Reproductive System Disease | 1.47E-02 – 1.09E-06 | 574 |
| Molecular and Cellular Function | | | |
| | Cellular Development | 1.18E-02 – 1.08E-05 | 41 |
| | Cellular Growth and Proliferation | 1.18E-02 – 1.08E-05 | 45 |
| | Cell Morphology | 9.98E-03 – 7.74E-05 | 25 |
| | Cellular Assembly and Organization | 1.54E-02 – 1.70E-04 | 87 |
| | Cellular Function and Maintenance | 1.54E-02 – 1.70E-04 | 69 |
| Physiological System Development and Function | | | |
| | Embryonic Development | 1.61E-02 – 1.08E-05 | 53 |
| | Hematological System Development and Function | 1.34E-02 – 1.08E-05 | 75 |
| | Hematopoiesis | 1.34E-02 – 1.08E-05 | 66 |
| | Humoral Immune Response | 1.24E-02 – 1.08E-05 | 52 |
| | Lymphoid Tissue Structure and Development | 1.18E-02 – 1.08E-05 | 52 |

102

103 *CS = cigarette smoke.

104

105 **Table S4. Canonical pathway analysis of upregulated genes in IQOS and CS groups**
 106 **compared to the air group.**

| IQOS | | |
|---|---------------|---|
| Ingenuity Canonical Pathways | -log(P-value) | Molecules |
| Mitotic Roles of Polo-Like Kinase | 2.10 | <i>FZRI, PLK5, PPP2R2A, ANAPC5, PKMYT1, CDC23</i> |
| cAMP-mediated Signaling | 1.97 | <i>ENPP6, CAMK4, PDE4C, GABBR1, CREB5, AKAP7, CNGA1, CNGA3, OPRL1, BRAF, AKAP13, CNR2, PKIB</i> |
| Nur77 Signaling in T Lymphocytes | 1.92 | <i>CD247, CASP9, CAMK4, HLA-A, EP300</i> |
| The Visual Cycle | 1.79 | <i>RDH12, RBP3, RDH13</i> |
| Calcium-induced T Lymphocyte Apoptosis | 1.69 | <i>CD247, CAMK4, HLA-A, CD4, EP300</i> |
| Histamine Biosynthesis | 1.54 | <i>HDC</i> |
| Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells | 1.51 | <i>CD247, CASP9, HLA-A</i> |
| Role of Lipids/Lipid Rafts in the Pathogenesis of Influenza | 1.50 | <i>IFNA4, IFNAR1</i> |
| iNOS Signaling | 1.43 | <i>TLR4, CAMK4, MYD88, IRAK2</i> |
| Th2 Pathway | 1.42 | <i>CD247, SOCS3, STAT5A, NOTCH4, HLA-A, CD4, ICOS, BMPR2</i> |
| CS* | | |
| Ingenuity Canonical Pathways | -log(P-value) | Molecules |
| Neuroinflammation Signaling Pathway | 6.54 | <i>TRAF3, KLK3, PSENEN, PYCARD, HLA-A, MAPK15, PIK3R5, TLR8, IL6, MAPK13, TGFB1, PPP3R1, TLR7, IRS2, NFE2L2, BIRC3, NAIP, IFNG, PIK3C2A, TYROBP, CD200R1, CREB3, TLR2, RIPK1, PLA2G2D, CD80, TREM2, APH1A, NCF2, GLUL, CD86, IL1B, Tlr13, PTGS2, TNF, MMP9, WNT1, P2RX7</i> |
| IL-10 Signaling | 6.47 | <i>SOCS3, MAP3K14, IL4R, FCGR2A, ARG2, IL6, STAT3, MAPK13, IL33, IL1R2, NFKBID, IL1RN, CD14, IL1B, MAP2K3, TNF</i> |
| Communication between Innate and Adaptive Immune Cells | 6.47 | <i>IFNG, HLA-A, TLR8, IL6, IL33, TLR2, CCL4, CD80, IL1RN, TLR7, FCER1G, Tlr13, IL1B, CD86, TNF, HLA-E</i> |
| Altered T Cell and B Cell Signaling in Rheumatoid Arthritis | 6.13 | <i>MAP3K14, IFNG, TRAF3, HLA-A, TLR8, IL6, IL33, TLR2, CD80, IL1RN, TGFB1, TLR7, FCER1G, IL1B, Tlr13, CD86, TNF</i> |
| Graft-versus-Host Disease Signaling | 5.84 | <i>IL33, IFNG, CD80, HLA-A, IL1RN, FCER1G, IL1B, CD86, IL6, TNF, HLA-E</i> |
| Granulocyte Adhesion and Diapedesis | 5.53 | <i>CCL17, SDC4, CLDN7, ITGAL, Cxcl9, SELPLG, IL33, IL1R2, CXCL3, ITGB2, CCL4, IL1RN, Ccl2, CCL28, EZR, IL1B, CXCL17, TNFRSF1B, CXCL2, Ccl6, TNF, MMP9, MSN, MMP19</i> |
| Xenobiotic Metabolism Signaling | 5.05 | <i>CYP3A7, GSTM5, UGT1A6, PIK3R5, GCLC, KRAS, IL6, MAPK13, ALDH2, Ces1g, Gstm3, SUMO1, IRS2, NFE2L2, ALDH3A1, GSTA3, MAP3K9, MAP3K14, MGST1, CYP1A1, MAP3K6, PIK3C2A, NQO1, CYP1B1, AHRR, FTL, SMOX, IL1B, MAP3K8, MAP2K3, TNF, UGT1A7 (includes others), GSTP1</i> |
| TREM1 Signaling | 4.78 | <i>TLR2, CXCL3, TREM1, TYROBP, TLR7, TLR8, Tlr13, IL1B, CD86, STAT3, IL6, STAT5B, TNF, ITGAX</i> |
| Crosstalk between Dendritic Cells and Natural Killer Cells | 4.78 | <i>IFNG, TYROBP, HLA-A, IL6, ITGAL, CSF2RB, CD80, TREM2, TLR7, CD86, TNFRSF1B, TNF, ACTG1, HLA-E</i> |
| LXR/RXR Activation | 4.71 | <i>ABCG8, ARG2, ABCG1, IL6, IL33, IL1R2, PON1, IL1RN, SAA1, CD14, IL1B, S100A8, PTGS2, TNFRSF1B, HMGCR, TNF, MMP9, PON3</i> |

107

108 *CS = cigarette smoke.

109

110 **Table S5. Canonical pathway analysis result of downregulated genes compared to the air**
 111 **group**

| IQOS | | |
|---|-------------------------|---|
| Ingenity Canonical Pathways | $-\log(P\text{-value})$ | Molecules |
| Protein Ubiquitination Pathway | 4.74 | <i>PSMB4, ANAPC2, PSMA6, USP14, HLA-A, DNAJC1, PSMD3, HSPA5, DNAJB14, UBE3A, DNAJC21, DNAJC24, PSMC6, DNAJC8, PSMA4, PSMA3, PSMD14, PSMD1, UBA1, PSMD4, PSMA2, UBE2E1</i> |
| Melanoma Signaling | 3.86 | <i>BRAF, TP53, RAP2A, AKT2, MAP2K2, GAB1, MITF, FGFR1, RALB</i> |
| NRF2-mediated Oxidative Stress Response | 3.51 | <i>MAP2K4, RAP2A, USP14, FGFR1, RALB, DNAJC1, DNAJB14, TXNRD1, DNAJC21, MAPK14, MAP2K2, GAB1, DNAJC8, ABCC1, KEAP1, GSK3B</i> |
| Integrin Signaling | 3.44 | <i>MAP2K4, RAP2A, AKT2, ARHGEF7, FGFR1, RALB, PLCG1, MYLK, BRAF, Ppp1r12b, SHC1, PPP1R12A, GAB1, MAP2K2, CAV1, GSK3B, CTTN</i> |
| ErbB Signaling | 3.44 | <i>MAP2K4, SHC1, RAP2A, MAPK14, NRG2, MAP2K2, GAB1, FGFR1, RALB, PLCG1, GSK3B</i> |
| Thyroid Cancer Signaling | 3.35 | <i>TP53, BRAF, SHC1, RAP2A, MAP2K2, RALB, TCF3</i> |
| Cancer Drug Resistance by Drug Efflux | 3.29 | <i>BRAF, TP53, RAP2A, AKT2, MAP2K2, ABCC1, RALB</i> |
| ErbB2-ErbB3 Signaling | 3.24 | <i>SHC1, RAP2A, NRG2, MAP2K2, Nrg1, GAB1, FGFR1, RALB, GSK3B</i> |
| Myc Mediated Apoptosis Signaling | 3.2 | <i>MAP2K4, TP53, SHC1, RAP2A, AKT2, GAB1, YWHAB, FGFR1, RALB</i> |
| Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency | 3.18 | <i>TP53, SHC1, RAP2A, AKT2, RIF1, MAP2K2, GAB1, SMAD9, FGFR1, WNT7B, RALB, GSK3B</i> |

| CS* | | |
|---|-------------------------|--|
| Ingenity Canonical Pathways | $-\log(P\text{-value})$ | Molecules |
| Cellular Effects of Sildenafil (Viagra) | 5.27 | <i>CACNA1D, MPRIP, GUCY1A1, MYH14, ADCY6, PLCG1, MYLK, MYH7, ITPR1, MYH11, MYL1, CACNA1A, Ppp1r12b, ADCY9, KCNN1, CACNA1E, PDE5A, PLCB3, PLCL1, ADCY8, GUCY1B1</i> |
| B Cell Development | 4.47 | <i>CD19, CD79B, CD40, HLA-A, HLA-DOB, IGHM, DNTT, CD79A</i> |
| Apelin Liver Signaling Pathway | 3.33 | <i>COL1A2, COL1A1, COL5A3, APLNR, COL2A1, PDGFRB, COL3A1</i> |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 3.29 | <i>COL6A2, PDGFA, MYH14, COL2A1, MYH7, MYH11, MYL1, PDGFB, COL5A1, COL1A2, COL1A1, COL5A3, COL6A3, CD40, TGFB3, IGFBP3, EDNRA, COL4A4, PDGFRB, COL3A1, TIMP2, TNFRSF11B</i> |
| ATM Signaling | 2.7 | <i>MDM4, CBX1, SMC2, TOPBP1, PPP2R2B, TLK1, PPP2R5E, ZEB1, TDPI, CBX5, CBX3, RAD50, SMC1A</i> |
| Dopamine-DARPP32 Feedback in cAMP Signaling | 2.46 | <i>KCNJ12, PPP1R14C, CACNA1D, GUCY1A1, ADCY6, PLCG1, ITPR1, PRKCZ, CACNA1A, ADCY9, CACNA1E, CDK5, PPP2R2B, PLCB3, PPP2R5E, PLCL1, ADCY8, GUCY1B1</i> |
| Phospholipase C Signaling | 2.15 | <i>BLNK, MPRIP, ARHGEF12, CD79B, ADCY6, PLCG1, CD79A, ARHGEF17, ITPR1, PRKCZ, MYL1, Ppp1r12b, GNB4, ARHGEF19, ADCY9, GNG11, PLCB3, MEF2C, ARHGEF2, ARHGEF18, ADCY8, ARHGEF10</i> |
| Cardiac β -adrenergic Signaling | 2.14 | <i>GNB4, ADCY9, AKAP5, PPP1R14C, GNG11, CACNA1E, CACNA1D, PKIB, PPP2R2B, ADCY6, PDE5A, PPP2R5E, ADCY8, CACNA1A, PDE6D</i> |
| GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells | 2.06 | <i>CACNA1I, CACNA1D, ADCY6, PLCG1, ITPR1, CACNA1A, PRKCZ, ADCY9, GNG11, CACNA1E, PLCB3, ADCY8, PLCL1</i> |
| Biotin-carboxyl Carrier Protein Assembly | 2.03 | <i>ACACB, HLCS</i> |

113 *CS = cigarette smoke.