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Iskandar et al.:

# 3-D Nasal Cultures: Systems Toxicological Assessment of a Candidate Modified-Risk Tobacco Product

## Supplementary Data

### Supplementary Materials and Methods

***Normalization of the adenylate kinase (AK)-based cytotoxicity assay***

For each of the five experimental repetitions, the value of the luminescence signal was normalized using the mean of the positive control (Triton X-100-treated cultures; considered as 100% cytotoxicity) and negative control (PBS-treated or untreated cultures; considered as 0% cytotoxicity):

$$Cytotoxicity\ (\%) = \frac{AK_{Tissue} - AK_{Neg\ CTRL}}{AK_{Pos\ CTRL} - AK_{Neg\ CTRL}}\ x\ 100, \text{ where}$$

$$AK_{Pos\ CTRL} = \sum_{i=1}^{nbPhase} \frac{AK_{TX-100}}{nbPhase}$$

$$AK_{Neg\ CTRL} = \sum_{i=1}^{nbPhase} \frac{\sum_{j=1}^{nbCTRL^i} \frac{AK_{i,j}}{nbCTRL^i}}{nbPhase}$$

$$AK_{Tissue} = relative\ luminescence\ unit\ of\ a\ given\ sample$$

$$nbPhase = number\ of\ experimental\ phase$$

$$Neg = negative$$

$$Pos = positive$$

$$CTRL = control$$

Triton X-100 (at 1% final concentration) was added to the basolateral media of the cultures for 24 h to maximally induce cell lysis. The averages of the normalized relative luminescence unit were reported.

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***Normalization of the cytochrome P450 activity assay***
For each of the five experimental repetition, the CYP1A1/1B1 activity levels were reported relative to the luminescence signals of the mean of triplicate positive controls (TCDD-treated tissue inserts were considered as 100% activity induction) and negative controls (considered as 0% activity):

$$Normalized\ CYP\ activity\ (\%) = \frac{CYP_{Tissue} - CYP_{Neg\ CTRL}}{CYP_{Pos\ CTRL} - CYP_{Neg\ CTRL}}\ x\ 100, \text{ where}$$

$$CYP_{Neg\ CTRL} = \sum_{i=1}^{nbPhase} \frac{\sum_{j=1}^{nbCTRL^{i}} \frac{CYP_{i,j}}{nbCTRL^{i}}}{nbPhase}$$

$$CYP_{Pos\ CTRL} = \sum_{i=1}^{nbPhase} \frac{CYP_{TCDD}}{nbPhase}$$

$$CYP_{Tissue} = relative\ luminescence\ unit\ of\ a\ given\ tissue\ culture\ sample$$
$$nbPhase = number\ of\ experimental\ phase$$

$$Neg = negative$$

$$Pos = positive$$

$$CTRL = control$$

For each of the five experimental repetitions, a positive control of CYP1A1/1B1 activity induction was measured from triplicate cultures treated with 30 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Sigma-Aldrich) added to the basolateral media and incubated for 48 h. For the negative control of CYP1A/1B1 activity, luciferin-CEE (substrate only)-treated cultures were used.

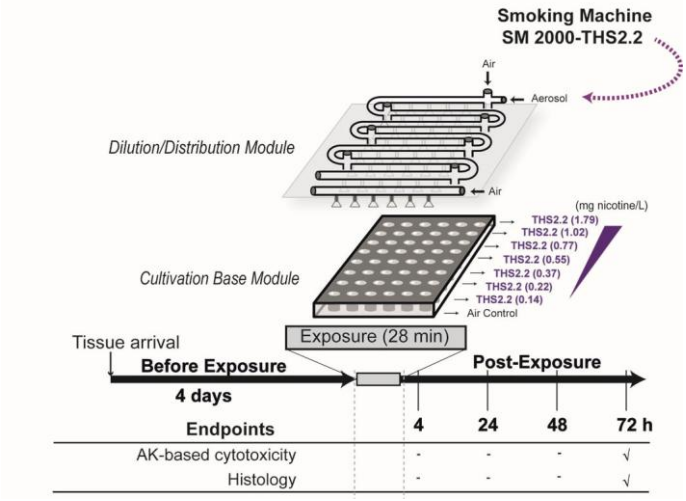
***Normalization of the FFT Power to calculate of the power of the detected signal of cilia beating***
The FFT power was normalized to the ambient noise using the following formula:

$$\frac{FFT\ Magnitude - \ Lowest\ FFT\ Magnitude^{2}}{Lowest\ FFT\ Magnitude}$$

$$FFT = \text{Fast Fourier Transformation}$$

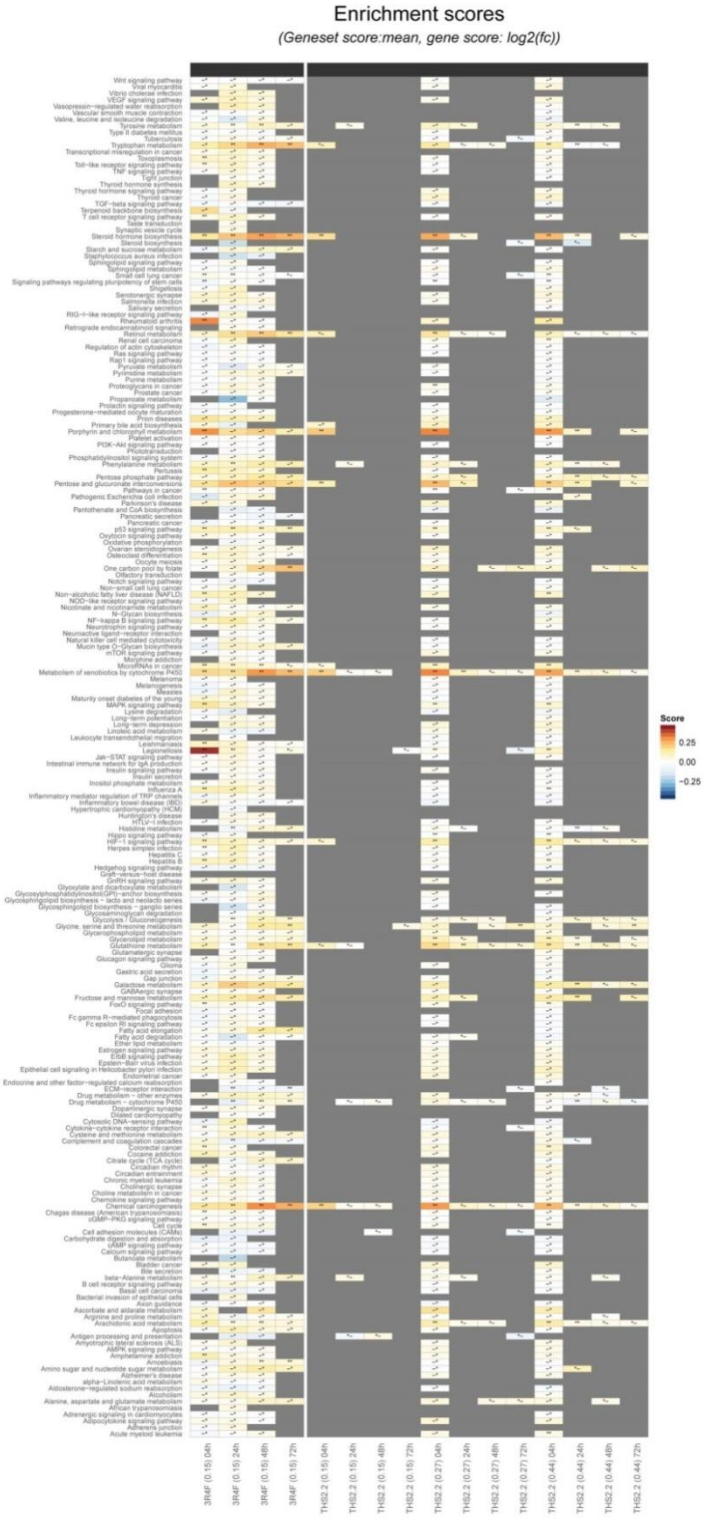
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Supplementary Fig. 1




**Supplementary Fig.1: Exposure run and endpoint schematic for the dose range assessment of THS2.2 aerosol.**
Tissue cultures were exposed to THS2.2 aerosols at the apical side using Vitrocell® 24/48 exposure systemconnected to the smoking machine (SM) 2000-THS2.2. The Vitrocell® 24/48 exposure systemhas a Dilution/Distribution Module where dilutions of THS2.2 aerosol were applied to reach the target nicotine concentrations in the THS2.2 aerosol. The numbers in parentheses indicate the concentration of nicotine in the THS2.2 aerosol (mg/L). For each group, cytotoxicity and histological analyses were measured at 72 h post-exposure.

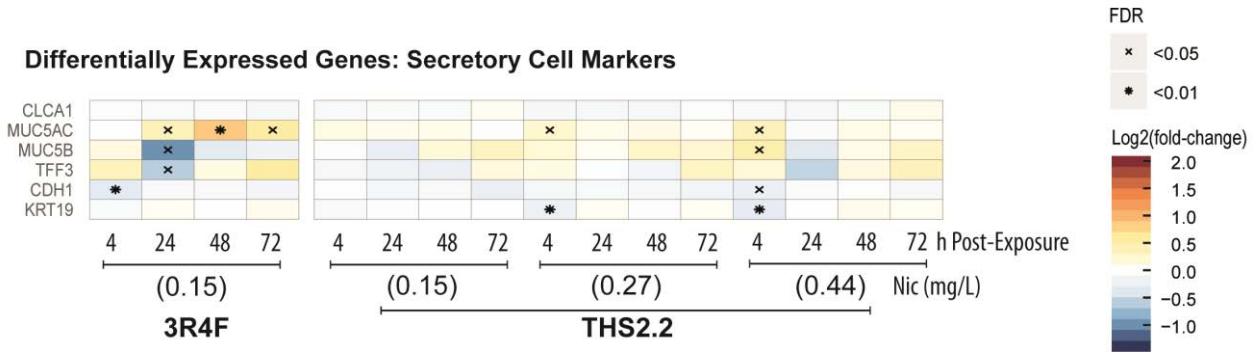
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**Supplementary Fig.2: Gene-set analysis showing various biological pathways enriched in the datasets.**
A heatmap showing all KEGG pathways that were significantly enriched in the dataset.The color gradient represents the score enrichment: the mean of the gene score ($log_2$(fold-change)). Two categories of significance are shown: first and second characters indicate Q1 and Q2, respectively (see Materials and Methods).

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Supplementary Fig. 3




**Supplementary Fig.3: Impact of exposures on cellular markers of secretory cells.**
The heatmap represents the levels of gene expression changes that were significantly impacted by exposure when compared with ording to their relevance to particular cell types. The color gradient reprethe air controls. The list of genes was compiled based on previously reported findings in: (Hupin et al., 2014), (Noruddin et al., 2007), (Brezillon et al., 1995), (LeSimple et al., 2007), (Raiford et al., 2011), (Schlage et al., 1998), and (Jiao et al., 2015). The gene names are listed on the left side of the heatmap and grouped accsents $\log_2$ of the fold-changes.

References:

Hupin, C., Gohy, S., Bouzin, C. et al. (2014).Features of mesenchymal transition in the airway epithelium from chronic rhinosinusitis.Allergy 69, 1540-1549. http://dx.doi.org/10.1111/all.12503

Noruddin, N. A., Saim, A. B., Chua, K. H. et al. (2007). Human nasal turbinates as a viable source of respiratory epithelial cells using co-culture system versus dispase-dissociation technique.Laryngoscope 117, 2139-2145.http://dx.doi.org/10.1097/MLG.0b013e3181453a1e

Brezillon, S., Dupuit, F., Hinnrasky, J. et al. (1995). Decreased expression of the CFTR protein in remodeled human nasal epithelium from non-cystic fibrosis patients. Lab Invest 72, 191-200. http://dx.doi.org/10.1371/journal.pone.0057617

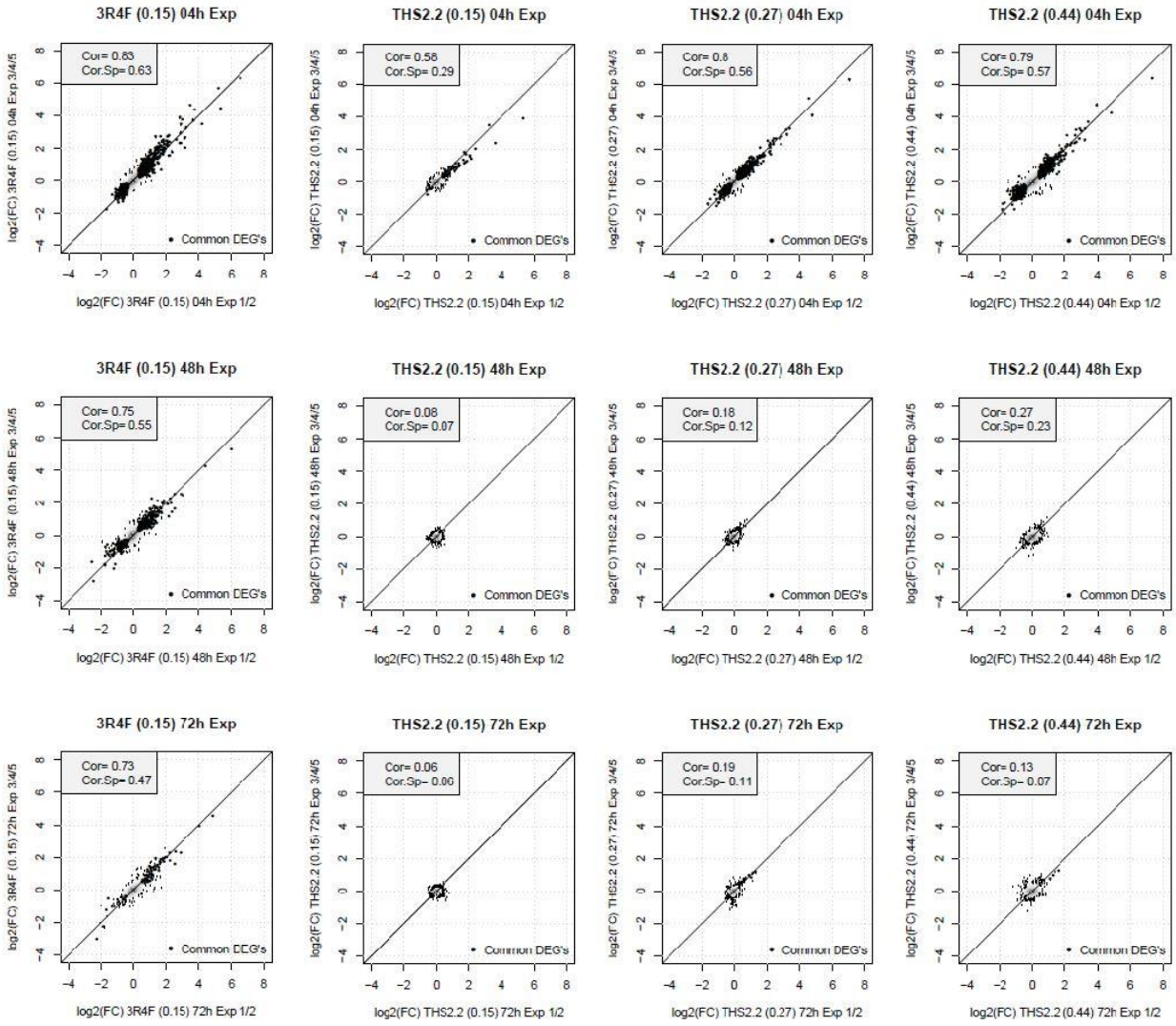
LeSimple, P., van Seuningen, I., Buisine, M. P. et al. (2007). Trefoil factor family 3 peptide promotes human airway epithelial ciliated cell differentiation. Am J Respir Cell MolBiol 36, 296-303.http://dx.doi.org/10.1165/rcmb.2006-0270OC

Raiford, K. L., Park, J., Lin, K.-W. et al. (2011). Mucin granule-associated proteins in human bronchial epithelial cells: the airway goblet cell "granulome". Respir Res 12, 1-10. http://dx.doi.org/10.1186/1465-9921-12-118

Schlage, W. K., Bulles, H., Friedrichs, D. et al. (1998). Cytokeratin expression patterns in the rat respiratory tract as markers of epithelial differentiation in inhalation toxicology. I. Determination of normal cytokeratin expression patterns in nose, larynx, trachea, and lung. ToxicolPathol 26, 324-343. http://dx.doi.org/10.1177/019262339802600307

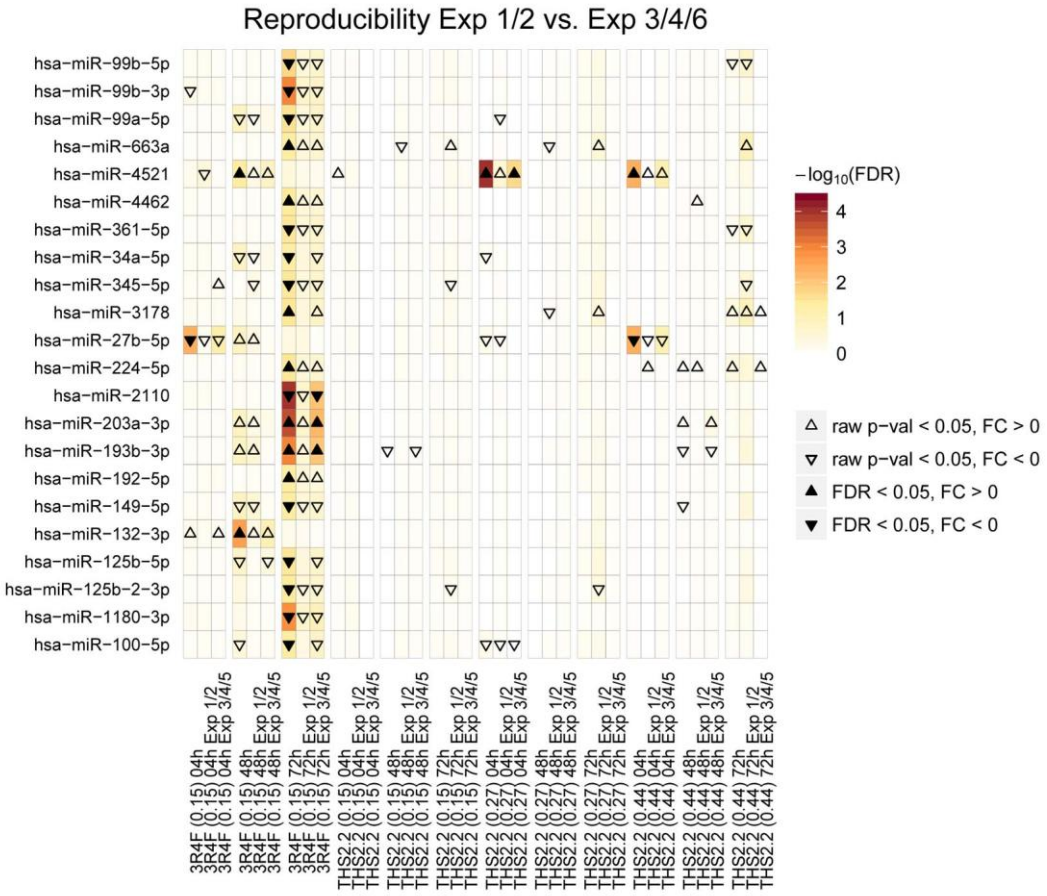
Jiao, J., Duan, S., Meng, N. et al. (2015). Role of IFN-gamma, IL-13 and IL-17 on mucociliary differentiation of nasal epithelial cells in chronic rhinosinusitis with nasal polyps.ClinExp Allergy http://dx.doi.org/10.1111/cea.12644

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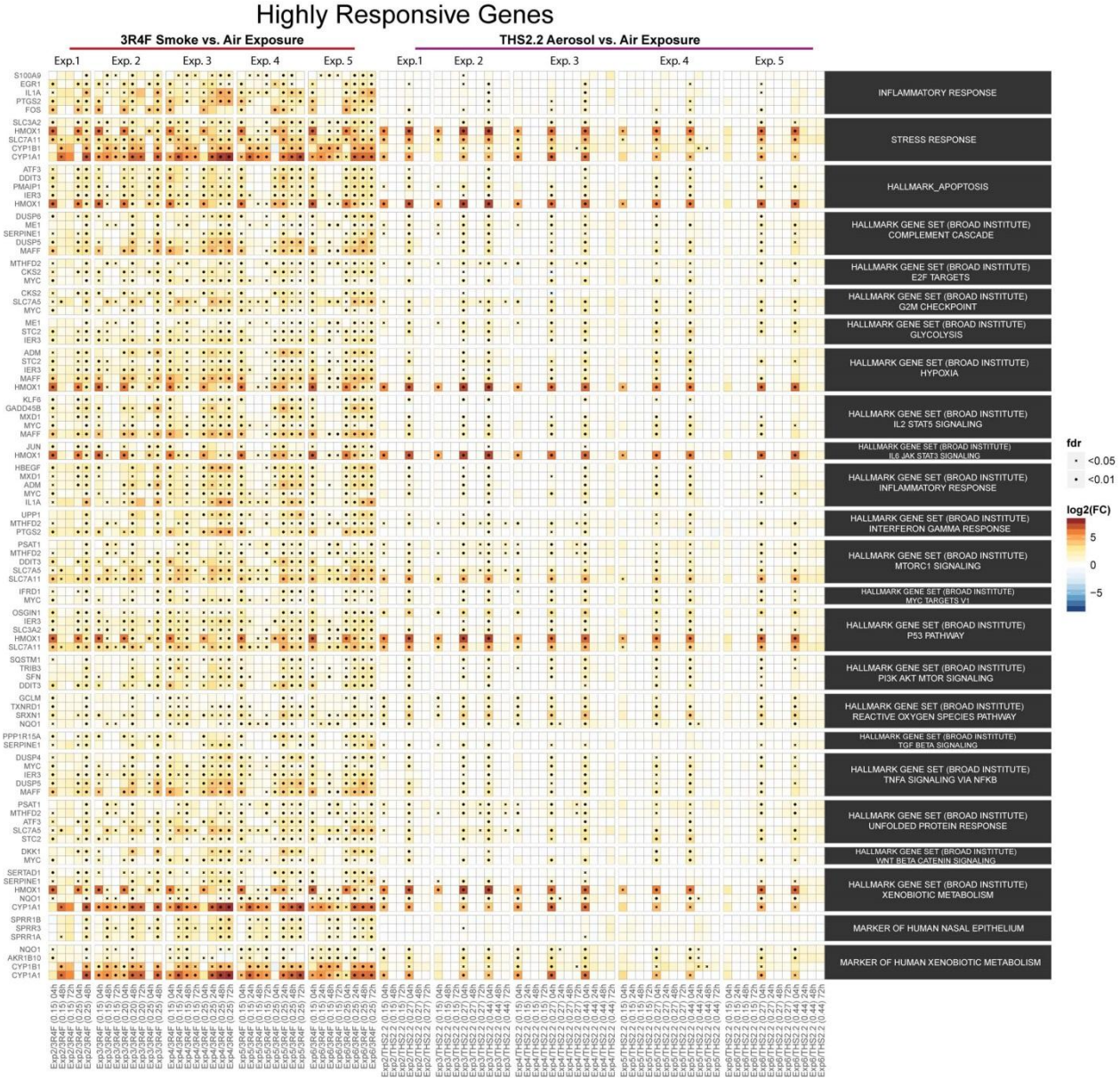

**Supplementary Fig.4: Correlation plots of gene fold-changes generated from the microarray analysis.**
Each dot represents a gene. The fold-change of a given gene from the combined experiments 1 and 2 (x-axis) was compared with the fold-change of the same gene from the combined experiment 3, 4, and 5 (y-axis). Abbreviations: Corr, correlation; Sp, Spearman correlation; DEG, differentially expressed gene.

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**Supplementary Fig.5: Comparability of miRNA fold-changes generated across the experimental repetitions**
The miRNAs are listed on the left side of the heatmap. Each exposure condition (and post-exposure time-point) comprised three columns: first, combined data from experimental repetition 1/2/3/4/5; second, combined data from experimental repetition 1/2; and combined data from experimental 3/4/5. The color gradation indicates the level of the differentially expressed miRNA (-$\log_{10}$(FDR). The $\triangle$ and$\triangledown$ signs indicate increased and decreased expression levels, respectively. Abbreviations: Exp, experiment; FC, fold-change; FDR, false discovery rate; hsa, homo sapiens.

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**Supplementary Fig. 6: Highly responsive genes consistently observed across the experimental repetitions**
The list of genes are provided as an example from which a gene panel can be developed and used in future studies. From each of the experimental repetition (Exp.), for all exposure condition and post-exposure time points, highly responsive genes were chosen if they were: i) present in at least 20% of all comparisons (listed in the bottom of the heatmap); ii) has FDR < 0.05 and absolute $\log_2$(fold-change) > 1; and iii) the mean expression levels ($\log_2$(FRMA value)) in the air control and exposed groups were both > 4. A total of 49 genes are present in the heatmap (they belong to multiple processes listed on the right side of the heatmap): *ADM, AKR1B10, ATF3, CKS2, CYP1A1, CYP1B1, DDIT3, DKK1, DUSP4, DUSP5, DUSP6, EGR1, GADD4B, GCLM, HBEGF, HMOX1, IER3, IFRD1, IL1A, JUN, KLF6, MAFF, ME1, MTHFD2, MXD1, MYC, NQO, NQO1, OSGIN1, PMAIP1, PPP1R1A, PSAT1, PTGS2, S100A9, SERPINE1, SERTAD1, SFN, SLC3A2, SLC7A11, SLC7A5, SPRR1A, SPRR1B, SPRR3, SQSTM1, SRXN1, STC2, TRIB3, XNRD1, UPP1*. The multiple processes are taken from the hallmark gene sets (Broad Institute) and gene sets developed by our group.

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**Supplementary Table 1: Sample number**

| Collection Time | Group | Total sample number per endpoint | | | | | |
|---|---|---|---|---|---|---|---|
| | | AK-based cytotoxicity | CYP1A1/1B1 activity | Secretion of Mediators | Histology/immunostaining | Ciliary beating frequency | mRNA and miRNA profiles |
| **Before** | 3R4F (Air) | - | - | - | - | 9 | - |
| | 3R4F (0.15) | - | - | - | - | 9 | - |
| | 3R4F (0.25) | - | - | - | - | 9 | - |
| | THS2.2 (Air) | - | - | - | - | 9 | - |
| | THS2.2 (0.15) | - | - | - | - | 9 | - |
| | THS2.2 (0.27) | - | - | - | - | 9 | - |
| | THS2.2 (0.44) | - | - | - | - | 9 | - |
| **0h PE** | 3R4F (Air) | | | | | 9 | |
| | 3R4F (0.15) | | | | | 9 | |
| | 3R4F (0.25) | | | | | 9 | |
| | THS2.2 (Air) | | | | | 9 | |
| | THS2.2 (0.15) | | | | | 9 | |
| | THS2.2 (0.27) | | | | | 9 | |
| | THS2.2 (0.44) | | | | | 9 | |
| **4 h PE** | 3R4F (Air) | 15 | - | - | - | - | 14 |
| | 3R4F (0.15) | 15 | - | - | - | - | 15 |
| | 3R4F (0.25) | 15 | - | - | - | - | - |
| | THS2.2 (Air) | 15 | - | - | - | - | 15 |
| | THS2.2 (0.15) | 15 | - | - | - | - | 15 |
| | THS2.2 (0.27) | 15 | - | - | - | - | 15 |
| | THS2.2 (0.44) | 12 | - | - | - | - | 12 |
| **24 h PE** | 3R4F (Air) | 9 | 6 | 9 | - | 9 | 9 |
| | 3R4F (0.15) | 9 | 6 | 9 | - | 9 | 9 |
| | 3R4F (0.25) | 9 | 6 | 9 | - | 9 | - |
| | THS2.2 (Air) | 9 | 6 | 9 | - | 9 | 9 |
| | THS2.2 (0.15) | 9 | 6 | 9 | - | 9 | 9 |
| | THS2.2 (0.27) | 9 | 6 | 9 | - | 9 | 9 |
| | THS2.2 (0.44) | 9 | 3 | 9 | - | 9 | 9 |
| **48 h PE** | 3R4F (Air) | 15 | 12 | 15 | 15 | 9 | 14 |
| | 3R4F (0.15) | 15 | 12 | 15 | 14 | 9 | 15 |
| | 3R4F (0.25) | 15 | 12 | 15 | - | 9 | - |
| | THS2.2 (Air) | 15 | 12 | 15 | 15 | 9 | 14 |
| | THS2.2 (0.15) | 15 | 12 | 15 | 15 | 9 | 14 |
| | THS2.2 (0.27) | 15 | 12 | 15 | 15 | 9 | 15 |
| | THS2.2 (0.44) | 12 | 9 | 12 | 12 | 9 | 12 |
| **72 h PE** | 3R4F (Air) | 15 | 6 | 9 | 15 | - | 14 |
| | 3R4F (0.15) | 15 | 6 | 9 | 15 | - | 15 |
| | 3R4F (0.25) | 15 | 6 | 9 | - | - | - |
| | THS2.2 (Air) | 15 | 6 | 9 | 15 | - | 14 |
| | THS2.2 (0.15) | 15 | 6 | 9 | 15 | - | 15 |
| | THS2.2 (0.27) | 15 | 6 | 9 | 15 | - | 15 |
| | THS2.2 (0.44) | 12 | 3 | 9 | 12 | - | 12 |
| **72 h PE – Dose Range Assessment** | THS2.2 (Air) | 3 | - | - | 3 | - | - |
| | THS2.2 (0.14) | 3 | - | - | 2 | - | - |
| | THS2.2 (0.22) | 3 | - | - | 3 | - | - |
| | THS2.2 (0.37) | 3 | - | - | 3 | - | - |
| | THS2.2 (0.55) | 3 | - | - | 3 | - | - |
| | THS2.2 (0.77) | 3 | - | - | 3 | - | - |
| | THS2.2 (1.02) | 3 | - | - | 3 | - | - |
| | THS2.2 (1.79) | 3 | - | - | 3 | - | - |

Abbreviations: AK, adenylate kinase; CYP, cytochrome P450; h, hour; PE, post-exposure; THS, tobacco heating system.

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**Supplementary Table 2. List of network models considered in the study**

| Number | Abbreviated network family name | Network name |
|---|---|---|
| **1** | CFA | Apoptosis |
| **2** | CFA | Autophagy |
| **3** | CFA | Necroptosis |
| **4** | CFA | Response To DNA Damage |
| **5** | CFA | Senescence |
| **6** | CPR | Calcium |
| **7** | CPR | Cell Cycle |
| **8** | CPR | Cell Interaction |
| **9** | CPR | Clock |
| **10** | CPR | Epigenetics |
| **11** | CPR | Growth Factor |
| **12** | CPR | Hedgehog |
| **13** | CPR | Hox |
| **14** | CPR | Jak Stat |
| **15** | CPR | Mapk |
| **16** | CPR | Mtor |
| **17** | CPR | Notch |
| **18** | CPR | Nuclear Receptors |
| **19** | CPR | PGE2 |
| **20** | CPR | Wnt |
| **21** | CST | Endoplasmic Reticulum Stress |
| **22** | CST | Hypoxic Stress |
| **23** | CST | NFE2L2 Signaling |
| **24** | CST | Osmotic Stress |
| **25** | CST | Oxidative Stress |
| **26** | CST | Xenobiotic Metabolism Response |
| **27** | IPN | Epithelial Innate Immune Activation |
| **28** | IPN | Epithelial Mucus Hypersecretion |
| **29** | IPN | Tissue Damage |

Abbreviations: CFA, Cell Fate; CST, Cell Stress; CPR, Cell Proliferation; IPN, Inflammatory Process Networks; Jak Stat, janus kinase/signal transducers and activators of transcription; Mapk, mitogen-activated protein kinases; Mtor, mechanistic target of rapamycin; NFE2l2, nuclear factor, erythroid 2-like 2; PGE2, prostaglandin E2. The collection of causal biological networks used in the study(s) was the human network suite CBN v1.3 (Boué et al., 2015).

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**Supplementary Table 3. Potential target mRNA per miRNA cluster**

| Cluster a | Cluster b | Cluster c | Cluster d |
|---|---|---|---|
| *MLF2* | *CENPQ* | *SEMA7A* | *DDX5* |
| *KDELR1* | *TNRC6B* | *NEK11* | *H2AFV* |
| *SCRIB* | *RALGDS* | *SMPD2* | *EFEMP1* |
| *INPPL1* | *PBXIP1* | *KDM2A* | *SRSF6* |
| *NPDC1* | *ZNF652* | *SYMPK* | *VPS13C* |
| *SBNO2* | *ZNF789* | *GDAP2* | *MYCN* |
| *HYOU1* | | *KIF9* | *TMF1* |
| *HMGA1* | | *BAG3* | *HNMT* |
| *CCDC71L* | | *JUN* | *ANKH* |
| *MARK4* | | *PARK7* | *NET1* |
| *SLC25A3* | | *ERCC3* | *SEPW1* |
| *DLGAP4* | | *HSPA1A* | *RUVBL1* |
| *METRNL* | | *FNIP2* | *CCP110* |
| *CAP1* | | *DNAJA1* | *SH3BP5* |
| *TROVE2* | | *WWTR1* | *SUN1* |
| *NFE2L2* | | *ARHGEF4* | *ENAH* |
| *PVRL4* | | *CCP110* | *RMI1* |
| *OSTF1* | | *DUSP22* | *ATP1A1* |
| *TMBIM1* | | *ST3GAL1* | *MYLK* |
| *PROM2* | | *PLD4* | *PSIP1* |
| *EFHD2* | | *SEMA4D* | *LEF1* |
| *PDP1* | | *ARHGAP21* | *SNRK* |
| *EPRS* | | *E2F3* | *EPDR1* |
| *ESYT1* | | *MAP1B* | *EFHD1* |
| *MARCKSL1* | | *SBNO1* | *CEP128* |
| *SHMT2* | | *ZNF281* | *FOXP2* |
| *SBF1* | | *RC3H1* | *FZD6* |
| *PPP4C* | | *UBAP2* | *AZIN1* |
| *GPI* | | *PER2* | *CD164* |
| *MAN2A1* | | *YPEL5* | *SH3RF3* |
| *SSR2* | | *MARCH6* | *PECR* |
| *SDC1* | | *PREX1* | *SYNM* |
| *RFC2* | | *CUX1* | *CAPS2* |
| *TRIP6* | | *FOXN2* | *WDR35* |
| *ESPL1* | | *ARHGAP30* | *TNC* |
| *KIF1C* | | *ZNF44* | *RBM5* |
| *RAP1GAP2* | | *KIAA0753* | *CYSTM1* |
| *ARPC2* | | *KIAA0040* | *DSTN* |
| *TNPO3* | | *JUNB* | *DNAJA4* |
| *LRRC41* | | *IQCB1* | *STXBP4* |
| *NAT10* | | *GTF2E2* | *B2M* |
| *BIRC5* | | | *SMAD4* |
| *GAPDH* | | | *EYA4* |
| *YWHAE* | | | *NEDD4L* |
| *TKT* | | | *SLC7A2* |
| *TRIM28* | | | *DDX6* |

| | | | |
|---|---|---|---|
| *OPTC* | | | *ARRDC3* |
| *RER1* | | | *LIFR* |
| *CNN2* | | | *SPARC* |
| *WDR41* | | | *PCNXL4* |
| *EXT1* | | | *ABI3BP* |
| *NOP9* | | | *PPAP2B* |
| *SESN2* | | | *NBEA* |
| *TTC39A* | | | *C12orf75* |
| *ATXN2L* | | | *ID4* |
| *INO80D* | | | |
| *AP2B1* | | | |
| *RARS* | | | |
| *PABPC4* | | | |
| *TIAM1* | | | |
| *BICD2* | | | |
| *MRPL10* | | | |

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**Supplementary Table 4. Enrichment analysis of the miRNA target genes using EnrichR**

| KEGG pathway | Cluster | Number of overlap genes in the pathway | Genes | EnrichR's combined score | KEGG pathway class |
|---|---|---|---|---|---|
| **Aminoacyl trna biosynthesis** | a | 2/38 | *RARS;EPRS* | 3.2081542 | Translation |
| **Arginine and proline metabolism** | a | 2/34 | *RARS;EPRS* | 3.1812275 | Amino acid metabolism |
| **Glycolysis and gluconeogenesis** | a | 2/63 | *GPI;GAPDH* | 2.687253 | Carbohydrate metabolism |
| **Cell cycle** | a | 2/104 | *YWHAE;ESPL1* | 2.1183877 | Cell growth and death |
| **Glycan structures biosynthesis 1*** | a | 2/106 | *EXT1;MAN2A1* | 2.0471369 | Glycan biosynthesis and metabolism |
| **Pancreatic cancer** | b | 1/73 | *RALGDS* | 7.7656622 | (Excluded because of disease specific) |
| **Colorectal cancer** | b | 1/84 | *RALGDS* | 7.4848943 | (Excluded because of disease specific) |
| **Axon guidance** | c | 2/126 | *SEMA7A;SEMA4D* | 1.8327301 | Development |
| **Glycosphingolipid biosynthesis globoseries** | c | 1/14 | *ST3GAL1* | 1.6650896 | Glycan biosynthesis and metabolism |
| **Circadian rhythm** | c | 1/11 | *PER2* | 1.6189169 | Environmental adaptation |
| **Glycosphingolipid biosynthesis ganglioseries** | c | 1/16 | *ST3GAL1* | 1.5454733 | Glycan biosynthesis and metabolism |
| **Keratansulfate biosynthesis** | c | 1/16 | *ST3GAL1* | 1.3647478 | Glycan biosynthesis and metabolism |
| **Basal transcription factors** | c | 1/33 | *GTF2E2* | 1.1850813 | Transcription |
| **Sphingolipid metabolism** | c | 1/36 | *SMPD2* | 1.1546578 | Lipid metabolism |
| **Adherens junction** | d | 2/75 | *SMAD4;LEF1* | 3.8679593 | Cellular community |
| **Focal adhesion** | d | 2/192 | *TNC;MYLK* | 2.6127561 | Cellular community |
| **Ether lipid metabolism** | d | 1/30 | *PPAP2B* | 1.7928805 | Lipid metabolism |
| **Histidine metabolism** | d | 1/40 | *HNMT* | 1.7270358 | Amino acid metabolism |
| **Sphingolipid metabolism** | d | 1/36 | *PPAP2B* | 1.5076568 | Lipid metabolism |
| **Glycerolipid metabolism** | d | 1/55 | *PPAP2B* | 1.3500408 | Lipid metabolism |
| **Glycerophospholipid metabolism** | d | 1/62 | *PPAP2B* | 1.2795226 | Lipid metabolism |
| **Ecm receptor interaction** | d | 1/87 | *TNC* | 1.1061538 | Signaling molecules and interaction |
| **Polyunsaturated fatty acid biosynthesis** | d | 1/13 | *PECR* | 1.0066938 | Lipid metabolism |

*** "Glycan structures-biosynthesis 1" is merged to KEGG Pathway 00510 "N-Glycan biosynthesis" (see http://www.genome.jp/kegg/docs/upd_map.html)**