ONLINE DATA SUPPLEMENT

C/EBPα Regulates Protease/Anti-protease Required for Regeneration of the Bronchiolar Epithelium

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SUPPLEMENTAL METHODS

Immunohistochemistry and X-gal Staining.

Lungs were inflation fixed at 25cmH_2O and tissue sections were immune stained with anti-mouse antigens, FoxJ1 (Sevenhills, Cincinnati, OH), Scgb1a1 (Santa Cruz Biotechnology, Santa Cruz, CA), C/EBPα (Santa Cruz), Fibronectin (Abcam, Cambridge, MA), GFP (Abcam) and negative control serum, with or without microwave-citrate antigen retrieval. Using the antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 594 fluorechromes (Invitrogen, Camarillo, CA), tissue sections were stained for double immunofluorescent and mounted with DAPI containing reagent (Invitrogen). The Zeiss Axioplan 2 microscope equipped with AxioVision software was utilized for immunofluorescent microphotographs. X-gal enzymatic reaction was carried out for LacZ staining as reported previously (E1).

Mitotic Index

Lung tissue sections fixed 3d after naphthalene injury were immunostained with Ki-67 antibody (Dako, Carpinteria, CA) and proliferation of epithelial cells in 200μm terminal bronchiole from bronchoalveolar duct junction (Mitotic Index) was calculated. Mitotic Index (%) = Ki-67 positive cell/total cell x 100.

Laser Capture Micro-dissection (LCM) and mRNA Microarray Analyses

Terminal bronchiolar epithelial cells lining approximately 200 μm from bronchoalveolar duct junction were collected from five mice for each group by LCM as described previously (E2). Briefly, the lungs were inflated with Tissue-Tek OCT in DEPC-PBS with 10% sucrose, immediately frozen in liquid nitrogen–cold 2-methylbutane. Lungs were sliced and fixed in 100% ethanol and dehydrated. Using a PixCell II System (Arcturus Engineering, Mountain View, CA) with amplitude 70 mW and laser diameter 28-32 μm, 100 to 250 regions, 4,000 to

7,500 cells were isolated from each mouse. All the captured samples from the same mouse were pooled and total RNA was extracted using the Arcturus PicoPure ™RNA Isolation Kit (Applied Biosystems, Carlsbad, CA). Approximately 20 to 60 ng total RNA were recovered from each mouse according to Agilent QC test. Total RNA samples were sent to CCHMC microarray core and RNA Integrity Number (RIN) was evaluated by RNA 6000 Pico Assay using the Agilent Bioanalyzer 2100 (Hewlett Packard) for quality control. Degradation lowers a RIN score and three samples with the highest RIN were selected from 5 samples for each group for microarray analyses. RIN scores of our LCM samples were 4.1 to 6.4 and were acceptable for analyses. The purity of our samples was evaluated by the ratio of wavelength 260/280 (260/280 nm score) using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE). The 260/280 nm score decrease by contamination and the highest score of the perfect sample is 2.0. The 260/280 nm score of our LCM samples were over 1.5 and were acceptable for microarray. There were no differences in quality and quantity of recovered RNA between the groups. The Ovation Pico WTA System (NuGEN, San Carlos, CA) was used to amplify and prepare target cDNA and the Encore Biotin Module (NuGEN) chemically fragmented and labeled the target sense cDNA. Fragmented cDNA was hybridized by the hybridization chamber for the array. Data were further analyzed using GeneSpring GX 11 (Agilent Technologies). Genotype and time dependent mRNA expression changes were identified using ANOVA with the threshold of expression P-value ≤ 0.01 and fold change ≥ 1.5. Probe sets expression filter was 30-100 percentile for at least two of three samples to remove probes with very low expression intensity.

Cluster discovery and Gene Ontology Analysis

Differentially expressed genes at 0, 3, and 72hr following naphthalene injury were subjected to gene ontology analysis and functional classification. Gene Ontology Analysis was

performed using publicly available web-based tool DAVID (database for annotation, visualization, and integrated discovery) (E3,4) and Ingenuity Pathway Analysis (IPA, Ingenuity). Ontology categories are considered to be significant when a Fisher's exact test P value ≤ 0.01 and gene hits ≥ 10.

Real-time (RT) PCR

Expression patterns of mRNAs encoding *Cebpa* anti-proteases and proteases were studied by RT-PCR. Total RNAs were isolated from terminal bronchiolar epithelial cells collected by LCM, left lung lobe homogenates and isolated Scgb1a1 cells using RNeasy plus mini-kit (QIAGEN, Valencia, CA) and RNA was reverse transcribed to cDNA by using Superscript VILO (Invitrogen, Carlsbad, CA). Quantitative RT-PCRs were analyzed using TaqMan® gene expression assays (Applied Biosystems, Foster City, CA) with probes selected from the list of Applied Biosystems (Table-E-1).

Protease Activity

Caseinolytic activity was measured as a general protease activity on supernatants of homogenized lung after perfusion with 0.9% NaCl. Samples were incubated with buffer, BPTI (100 mg/ml), or EDTA (100mM) at 37^{o}C for 1 hr, followed by incubation with BODIPY TR-X labeled casein (Invitrogen, Camarillo, CA) in 96 well plate for 16 hr at 37^{o}C. BODIPY TR-X signal cleaved by protease were measured by fluorescent microplate reader (BioTek, Winooski VT) using excitation and emission filters of 575/15 nm and 645/15 nm. One unit was defined by the reaction of 10 ng trypsin analyzed as a standard.

Naphthalene and BPTI Administration

To induce bronchiolar epithelial cell injury, *Cebp*$\alpha^{\Delta/\Delta}$ and control mice were anesthetized in a 2.5% isoflurane/O_2 chamber for weighing and injection. Naphthalene (Sigma, St Louis, MO)

was dissolved in corn oil at a concentration of 30mg/ml and administered to 9wk old mice (275mg/kg) via intraperitoneal injection (E5).

To study the role of C/EBPα in protease/anti-protease balance in injured lung, the serine protease inhibitor BPTI (Sigma, St. Louis, MO, 1mg in 0.1ml) or saline was injected daily (from 2d after naphthalene injury until study day) into the trachea by placing it in the oropharynx under anesthesia with isoflurane vapors (E6).

Scgb1a1$^+$ Cell Isolation

Scgb1a1-positive (Scgb1a1$^+$) bronchial and bronchiolar epithelial cells were isolated from control and *Cebpα*$^{\Delta/\Delta}$ mice lungs as reported previously (E7) with the following modification. To further purify the isolated cells, CD45, DC16/32, CD31, and CD90 reactive cells were excluded by negative selection using magnetic bead sorting (Miltenyi Biotec, Auburn, CA). Similar to the previous study (E7), the purity of the Scgb1a1$^+$ cells isolated from control and *Cebpα*$^{\Delta/\Delta}$ mice was 73 to 83% as assessed by double-immunofluorescence staining of cytospin slides with anti-Scgb1a1 and anti-pro-SP-C antibody. Approximately 2x10^5 Scgb1a1$^+$ cells were recovered from each mouse. C/EBPα protein in isolated Scgb1a1$^+$ cells was assessed by Western blot.

REFERENCES

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FIGURE LEGENDS

Figure E1. Expression of *Cebpa* mRNA was analyzed by microarray (A) and RT-PCR (B), 0, 3, and 72hr after naphthalene on terminal bronchiolar epithelial cells isolated by LCM. *Cebpa* mRNA was low in *Cebpα*$^{\Delta/\Delta}$ mice, while *Cebpa* mRNA increased 3hr after naphthalene injection in control mice. (C): Marked recovery of C/EBPα protein in terminal bronchiolar epithelial cells after naphthalene injury was detected in control mice by immunohistochemistry). Bronchoalveolar duct junctions are included on the right end. (D): Transformation of FoxJ1^{+} cells after naphthalene injury was evaluated using FoxJ1-eGFP mice (The Jackson Laboratory, Bar Harbor, ME). The ciliated cells transformed to squamous after injury (eGFP) and those cells were less detectable by immunohistochemistry of FoxJ1. Decreased number of FoxJ1^{+} cells in *Cebpα*$^{\Delta/\Delta}$ mice after injury reflect delayed recovery from squamous to cuboidal cells. Scale bar 20 μm n=3/group

Figure E2. Microarray analyses of mRNAs influenced by deletion of *Cebpa* in terminal bronchiolar epithelial cells. (A): The average intensity (n=3 mice/group) of differentially expressed genes in *Cebpα*$^{\Delta/\Delta}$ and control mice 0, 3, and 72hr after naphthalene injection was demonstrated by heatmap. Samples were normalized by using mean of 0hr control as baseline. The intensity in red to green color indicates the up-and-down regulation of mRNAs. (B): Heatmap of all the significantly changed genes in each 72hr *Cebpα*$^{\Delta/\Delta}$ mouse compared to mean of 72hr control mice (n=3/group), $p \leq 0.01$, fold≥ 1.5. This figure demonstrates consistency of the microarray analyses between 3 mice in the group. (C): Total number of mRNA expressions that were increased (solid bar) or decreased (open bar). A large number of genes were induced at

72hr after naphthalene injection in control mice that were unchanged in *Cebpα*$^{\Delta/\Delta}$ mice. n=3/group (D): The top enriched bioprocesses and molecular functions of genes induced at 72hr after naphthalene injury in *Cebpα*$^{\Delta/\Delta}$ and control mice. Cell cycle (M phase) and microtubule-based process are the only enriched bioprocesses in *Cebpα*$^{\Delta/\Delta}$ mice. In control mice, the most enriched bioprocesses were "cell cycle", "microtubule-based process" and "angiogenesis". The most enriched molecular functions in control mice were "microtubule motor activity" and "peptidase inhibitor activity".

Figure E3. Role of C/EBPα during repair of the terminal bronchiolar epithelium. Intraperitoneal injection of naphthalene ablated most Clara cells from the terminal bronchiolar epithelium transformed ciliated cells to squamous, and naphthalene resistant Clara cells remained. The normal bronchiolar epithelium responds to injury with increased expression of genes encoding anti-serine proteases that suppresses protease activity. In control mice, naphthalene injury was associated with increased ECM deposition and increased ciliated cell differentiation leading to recovery. Deletion of C/EBPα (*Cebpα*$^{\Delta/\Delta}$ mice) altered these responses to injury; expression of genes regulating anti-protease activity was not increased and caused protease/anti-protease imbalance. In *Cebpα*$^{\Delta/\Delta}$ mice, increased protease activity blocked fibronectin deposition, blocked differentiation to ciliated cells and altered the cellular composition of the terminal bronchiolar epithelium. C/EBPα plays a critical role in recovery of the ciliated cells after injury by regulating protease/anti-protease balance.

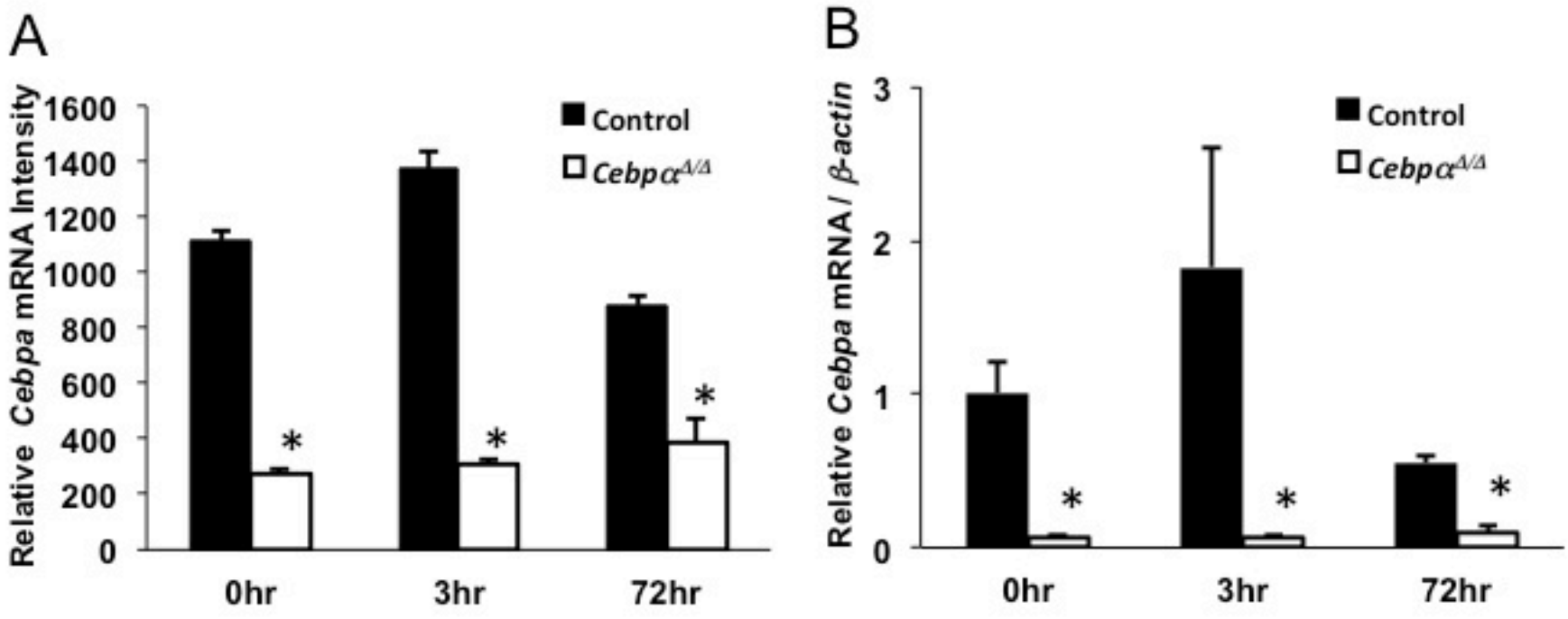
Figure E4. Expression of genes related to Notch signaling pathway was not influenced by deletion of *Cebpa*. There were no statistical differences in these genes between control and

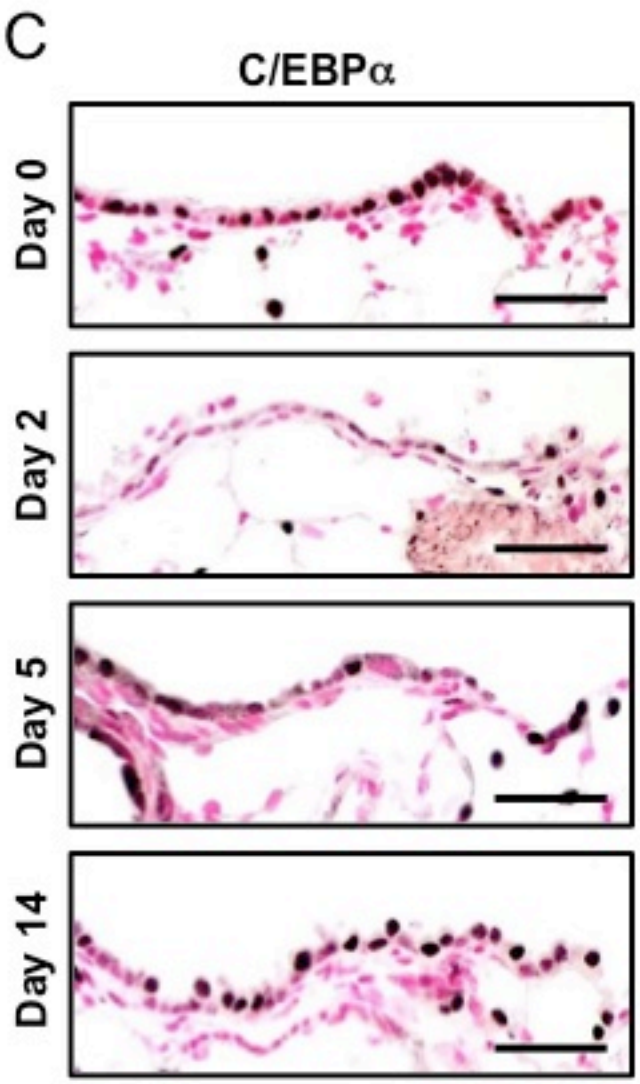
Cebpα$^{\Delta/\Delta}$ mice at 0, 3, and 72hr after naphthalene injection by microarray.

Table-E1. TaqMan® gene expression assays for quantitative RT-PCR

Gene symbol	Assay ID
Cebpa	Mm_00514283_s1
Spink5	Mm_00511522_m1
Serpina3n	Mm_00776439_m1
Timp1	Mm_01341361_m1
Cstb	Mm_00432769_m1
Slpi	Mm_00441530_g1
Dpep2	Mm_01250602_m1
Ctsk	Mm_00484039_m1
Lgmn	Mm_01325350_m1
Mmp14	Mm_00485054_m1
Mmp2	Mm_00439498_m1

Figure E1


A
Relative Cebpa mRNA Intensity
1600
1400
1200
1000
800
600
400
200
0
Control
Cebpα^Δ/Δ
*
*
*
0hr
3hr
72hr
B
Relative Cebpa mRNA / β-actin
3
2
1
0
Control
Cebpα^Δ/Δ
*
*
*
0hr
3hr
72hr


C
C/EBPα
Day 0
Day 2
Day 5
Day 14

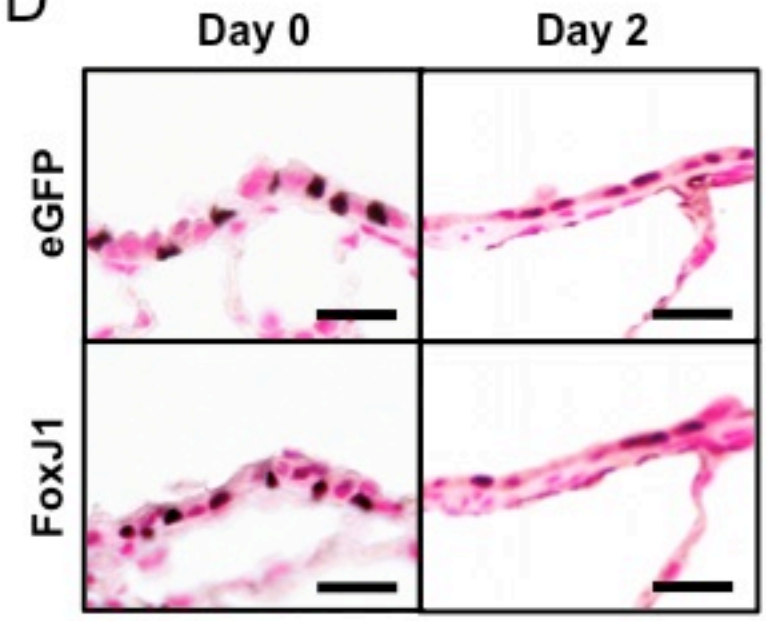

D
Day 0
Day 2
eGFP
FoxJ1

Figure E2

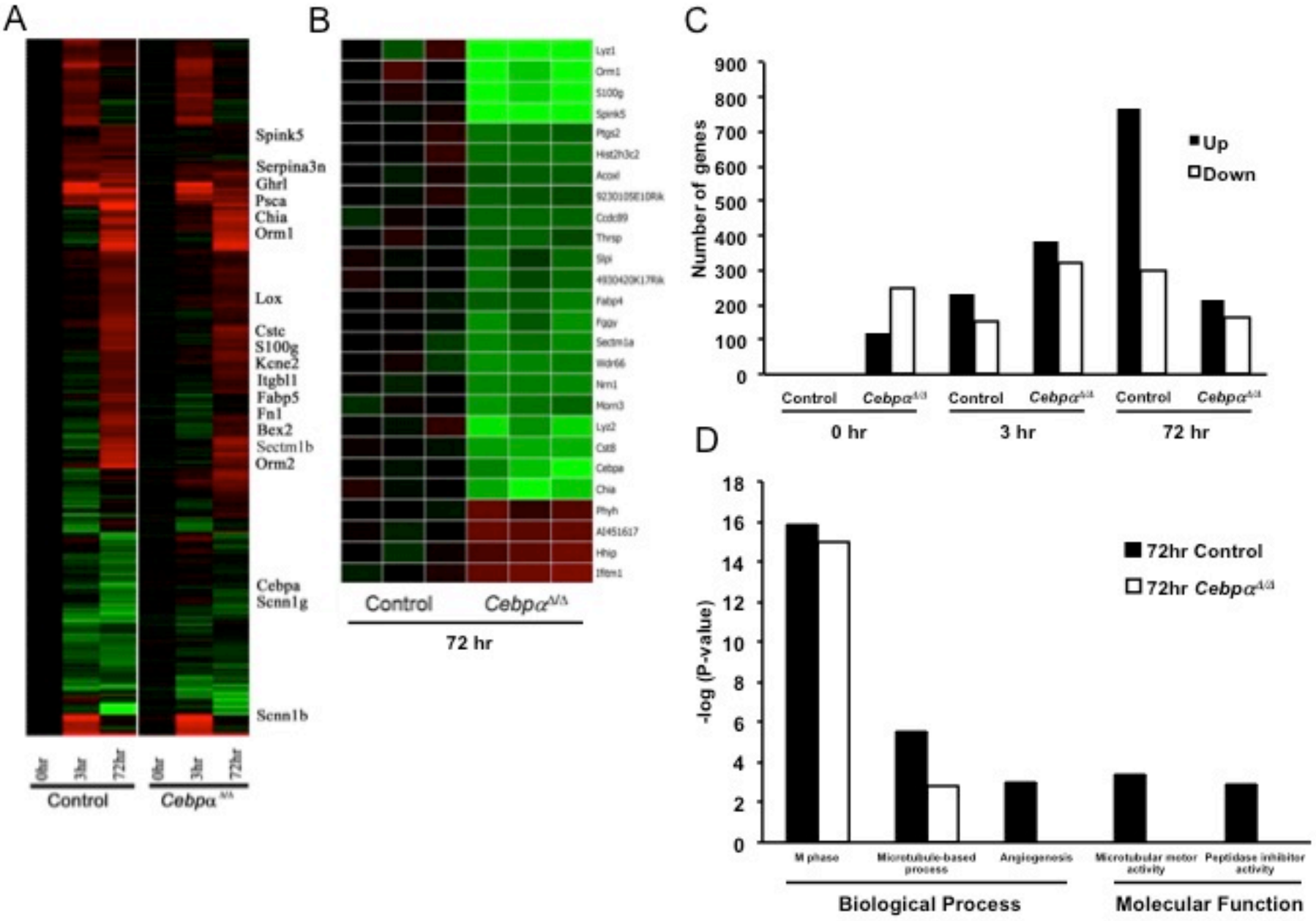

A
Spink5
Serpina3n
Ghrl
Psca
Chia
Orm1
Lox
Cstc
S100g
Kcne2
Itgbl1
Fabp5
Fn1
Bex2
Sectm1b
Orm2
Cebpa
Scnn1g
Scnn1b
0hr
3hr
72hr
Control
Cebpα Δ/Δ
B
Lyz1
Orm1
S100g
Spink5
Ptgs2
Hist2h3c2
Acsvl
9230105E10Rik
Ccdc89
Thrsp
Slpi
4930420K17Rik
Fabp4
Fgpr
Sectm1a
Wdr66
Nrn1
Morn3
Lyz2
Cst8
Cebpa
Chia
Phyh
AI451617
Hhip
Ifitm1
Control
Cebpα Δ/Δ
72 hr
C
Number of genes
900
800
700
600
500
400
300
200
100
0
Up
Down
Control
Cebpα Δ/Δ
Control
Cebpα Δ/Δ
Control
Cebpα Δ/Δ
0 hr
3 hr
72 hr
D
-log (P-value)
18
16
14
12
10
8
6
4
2
0
72hr Control
72hr Cebpα Δ/Δ
M phase
Microtubule-based process
Angiogenesis
Microtubular motor activity
Peptidase inhibitor activity
Biological Process
Molecular Function

Figure E3

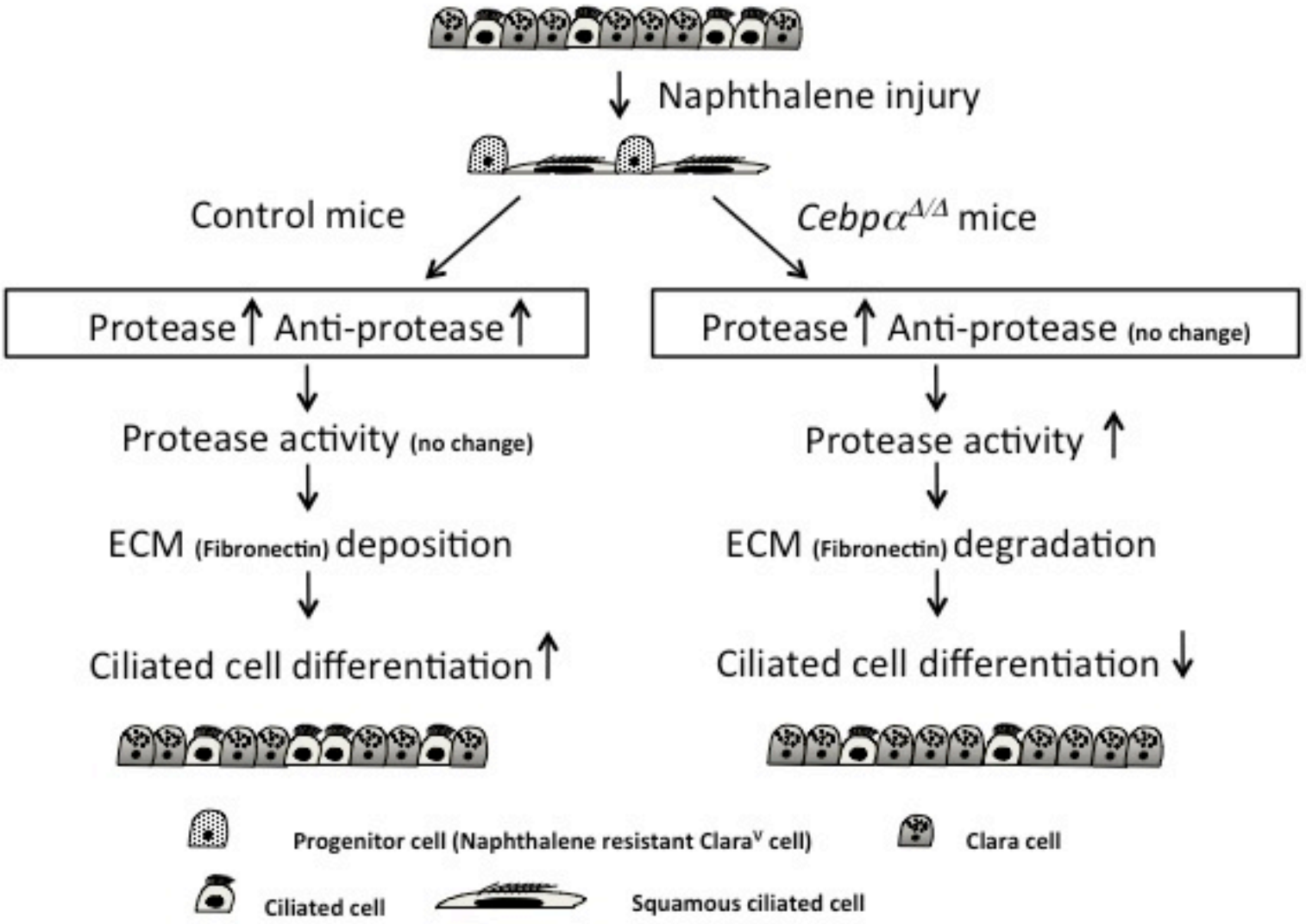
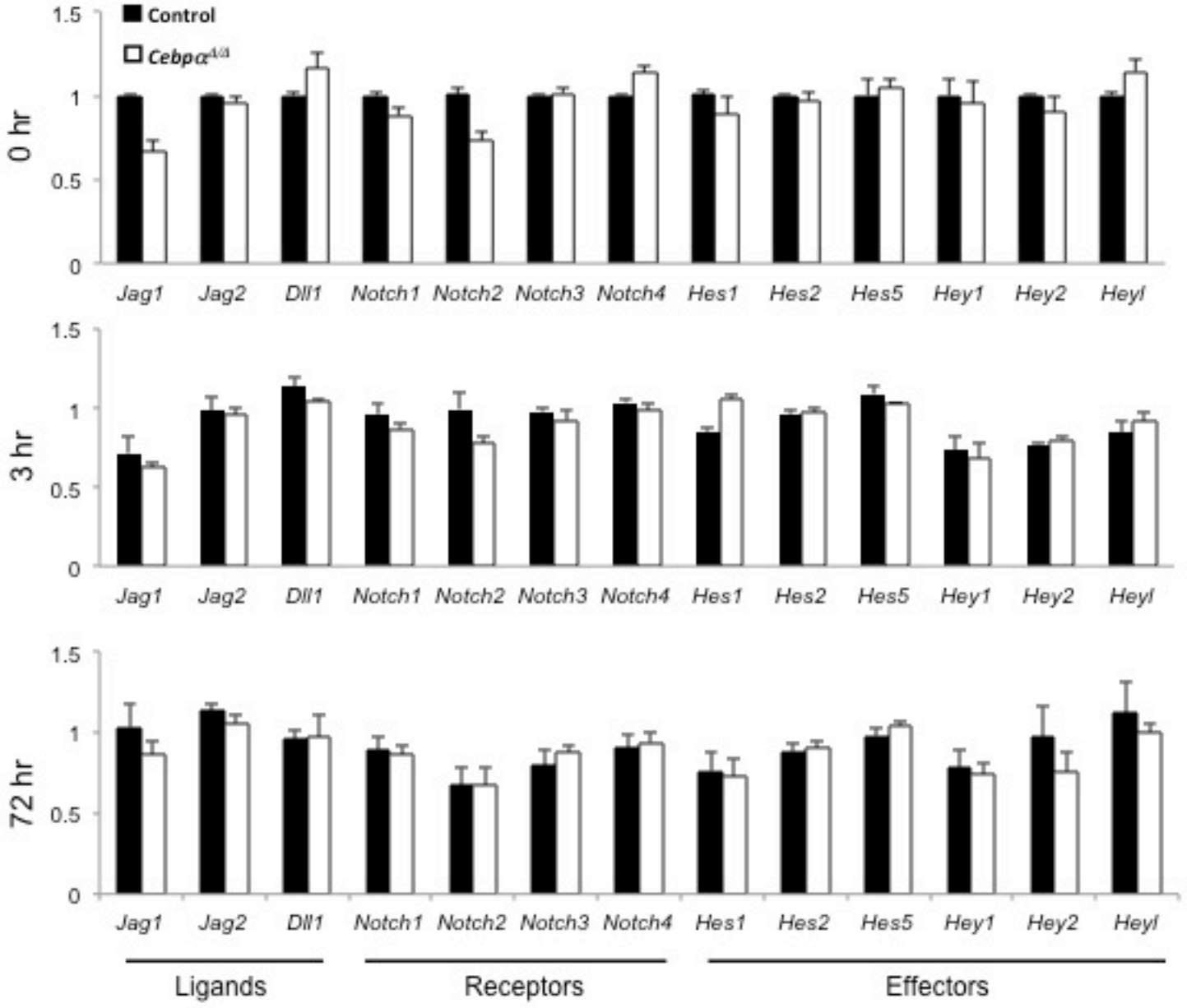

Naphthalene injury
Control mice
Cebpα^Δ/Δ mice
Protease↑ Anti-protease↑
Protease↑ Anti-protease (no change)
Protease activity (no change)
Protease activity ↑
ECM (Fibronectin) deposition
ECM (Fibronectin) degradation
Ciliated cell differentiation↑
Ciliated cell differentiation ↓
Progenitor cell (Naphthalene resistant Clara^V cell)
Clara cell
Ciliated cell
Squamous ciliated cell

Figure E4


Control
Cebpα^Δ/Δ
0 hr
3 hr
72 hr
1.5
1
0.5
0
Jag1
Jag2
Dll1
Notch1
Notch2
Notch3
Notch4
Hes1
Hes2
Hes5
Hey1
Hey2
Heyl
Ligands
Receptors
Effectors