

Regulation and role of the ER-stress transcription factor CHOP in alveolar epithelial type-II cells

Supplementary Figures S1-S12

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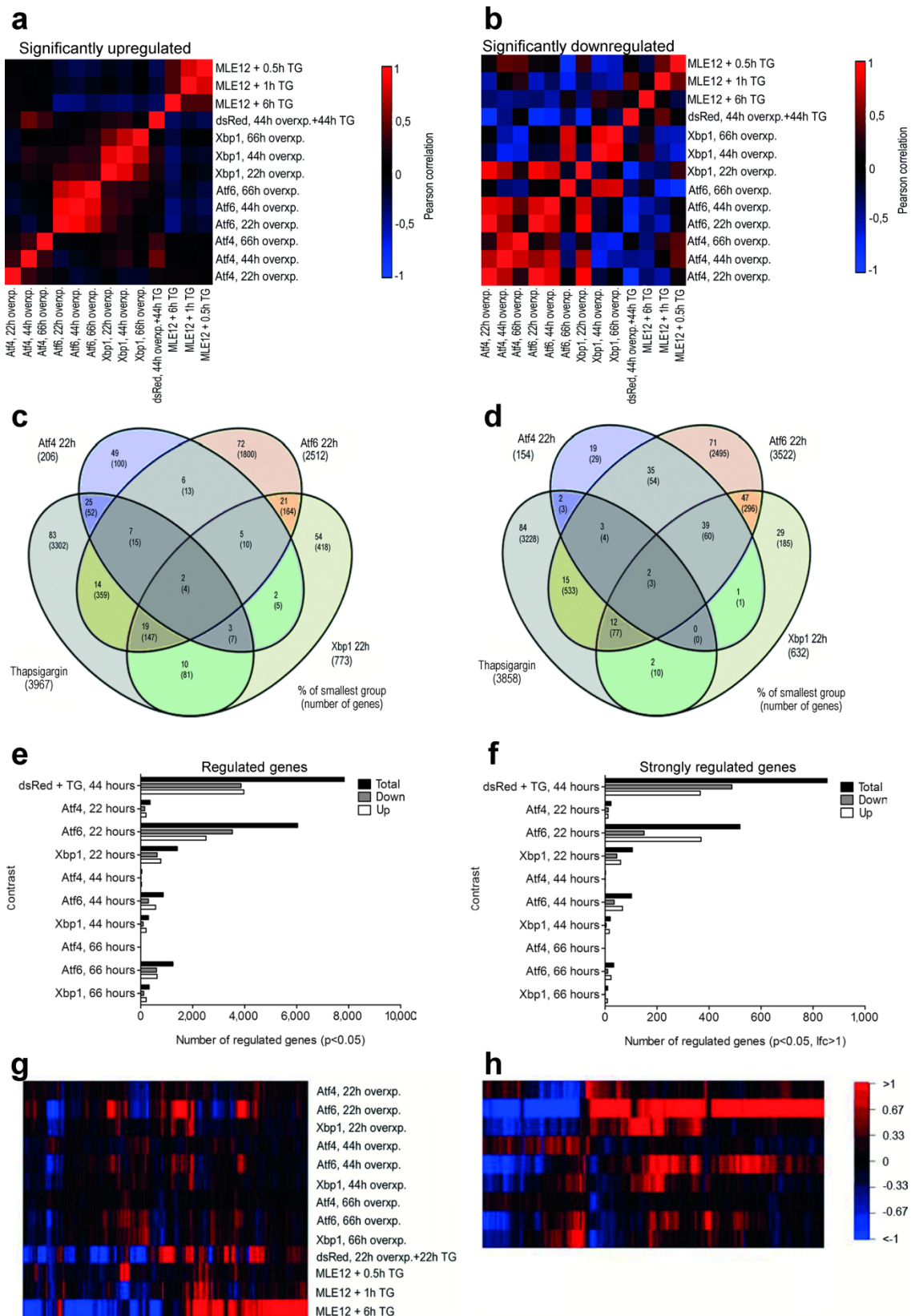


Fig. S1. Correlation of the effects elicited by overexpression of Atf4, p50Atf6 and sXbp1 and thapsigargin-effects.

(a, b) Color-coded correlation charts of UPR transcription factor effects and thapsigargin (TG)-effects: Whole genome expression analysis using RNA from MLE12 cells overexpressing Atf4, p50Atf6 or sXbp1, versus empty-vector (dsRed) and TG (1 μ M/ml)-treated cells (n=6 experiments per condition for the time periods 22h, 44h, 66h; and n=4 for the 0.5h, 1h and 6h treatments with TG, and n=12 for the respective control). Only significantly up-regulated genes are included (based on a 5% false-discovery rate). The color coding (red = Pearson correlation coefficient of 1; blue = Pearson correlation coefficient of -1) indicates the extent of correlation between the different conditions. **(b)** Same as in **(a)**, but only significantly down-regulated genes are included.

(c, d) Venn-diagrams, depicting the number of genes up-regulated **(c)** or downregulated **(d)** based on a 5% false-discovery rate and their relative distribution in subgroup and conditions.

(e, f) Extend of the transcriptomic response of MLE12 cells to single overexpression of Atf4, p50Atf6, sXbp1 or treatment with TG. Given is the number of regulated genes with $p < 0.05$ in **(e)** or strongly regulated genes ($p < 0.05$, $lfc > 1$) in **(f)**.

(g, h) Time-dependent changes in transcription regulation in response to Atf4, p50Atf6 and sXbp1 overexpression and TG effects in MLE12 cells. The left heat map **(g)** includes the effects of TG and the effects of the transgene expression (selection for both: genes with $p < 0.05$ and $lfc > 1$ in at least one contrast). On the right one **(h)**, exclusively the effects of single transgene expression are depicted [on the same lane as in **(g)**]. Red and blue indicate increased and decreased gene expression, respectively.

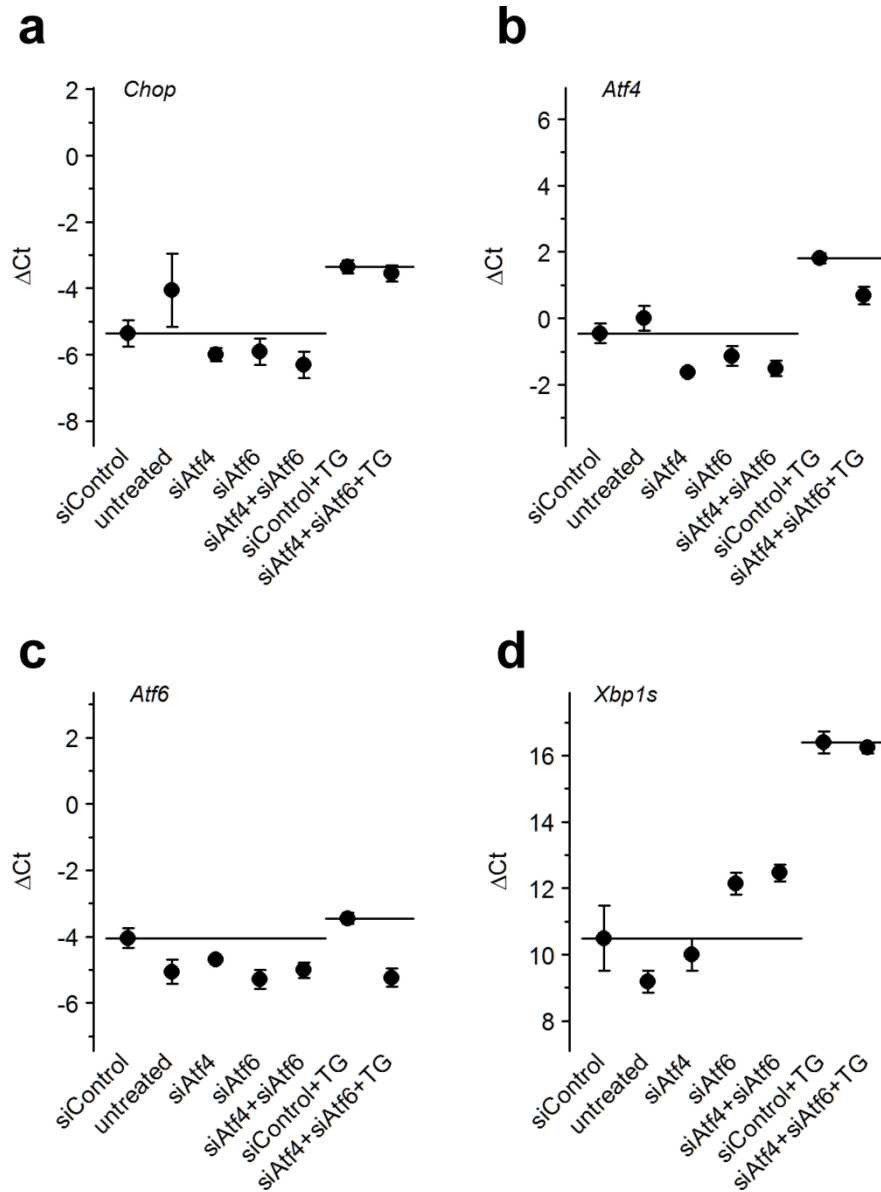


Fig. S2. Effects of Atf4 and/or Atf6 knockdown on thapsigargin (TG)-induced and non-induced *Chop*-expression in MLE12 cells.

(a-d) Quantitative RT-PCR analysis for *Chop* **(a)**, *Atf4* **(b)**, *Atf6* **(c)** and spliced *Xbp1* (*Xbp1s*) **(d)** in untreated MLE12 cells, and cells transfected with non-targeting siRNA (siControl), *Atf4* and/or *Atf6* targeting siRNA in the presence (1 μ M/ml) or absence of TG. Data are expressed as means $\pm$ SD, from n=4 independent experiments.

CHOP Promoter
>*DDIT3* 9999bp genomic DNA (5'-3')

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 AGGCCATGGTAAAAGATTACAGCCAGGCGCTCCCGAGGTC +1 → transcription initiation

CCAAT and 3'-TAACC-5'/5'-ATTGG-3': NF-Y NF-Y serves (amongst other functions) as a co-factor for ATF6 and binds to the CCAAR part of both ERSEs.

CCAATGCCGGCGTGCCACTTTCTGATTGG: ERSE2 (5' → 3') und ERSE1 (3' → 5')

GGGGCGGGGC: SP-1, 94.5%
CCCCGCCCC: SP-1, 95.9%
GCCCCGCCC: SP-1, 94.5%
GGGGCGGGGC: SP-1, 94.5%
GGGGCAGGGT: SP-1, 95.9%

ACTTCCGGG: c-Ets-1, 94.1%

ATGACTCACCCA: AP-1, 93.4%

CCTCCGCG: MZF-1, 93.0%
CCTCCCC: MZF-1, 93.0%
AGTGGGGA: MZF-1, 100.0% (Reverse)

ATTGCATCA: AARE1
GTTTCACCA: AARE2

TTGGGCGCC: v-Myb, 96.8%
GTTGGGGCC: v-Myb, 96.8%

GCTGATAGCC: GATA-2, 94.5% (also GATA-1, 93.1%)

CCTCCCAAAGTG: Lyf-1, 100%
TTTGGGAGA: Lyf-1, 98.7%

TCAAGTGA: Nkx-2.5, 100%

ACCACA: AML-1 α , 100%

CGATTCTTCTGCC: HSF2, 93.6%

GTTTGTTTGTTT: HFH-2, 95.4%

AGTGATGGGG: GATA-1, 93.1%

GCCTAACC~~AA~~AGA: C/EBP, 96.2%

GAGACCATCTGGTCC: Tal-1a, 92.4%; Tal-1b, 92.2%

TTGCGGGAA: STATx, 92.3%
TTCCCAGAA: STATx, 96.2%

NCGTG = HRE consensus sequence, where N can be A or G, has been reported in Wikipedia and in various publications)

5'-GCGTG-3' HRE consensus sequence

5'-ACGTG-3' HRE consensus sequence

Fig. S3. Sequence of the 10.0-kb 5'-flanking region of the human *CHOP* gene.

High scoring (>90%) transcription factor binding sites in the 2.7-kb 5'-flanking region of the human *CHOP* gene are depicted, in addition to the known and well conserved ERSE - (ER stress-response element) and AARE (amino-acid response element) regulatory elements. NF-Y, HRE and MZF-1 regulatory elements are also found in the very distal region of the *CHOP*

promoter. Abbreviations: NF-Y: Nuclear transcription factor Y; SP-1: transcription factor SP-1; c-Ets-1: Protein c-Ets-1 (or p54); AP-1: Activator protein 1 (or c-Jun); MZF-1: Myeloid zinc finger 1; HRE: Hypoxia response element; v-Myb: Transcriptional activator v-Myb; GATA: GATA family of transcription factors (GATA1 and-2); Lyf-1: Lymphoid transcription factor Lyf-1 or DNA-binding protein Ikaros; Nkx-2.5: homeobox protein Nkx-2.5; AML-1 α : Acute myeloid leukemia 1 protein or Runt-related transcription factor 1; HSF2: Heat shock factor 2; HNF2: Hepatocyte nuclear factor 3 forkhead homolog 2; CEBP: CCAAT/enhancer-binding protein; Tal-1a/1b: T-cell acute lymphocytic leukemia protein 1a/b; STATx: Signal transducer and activator of transcription.

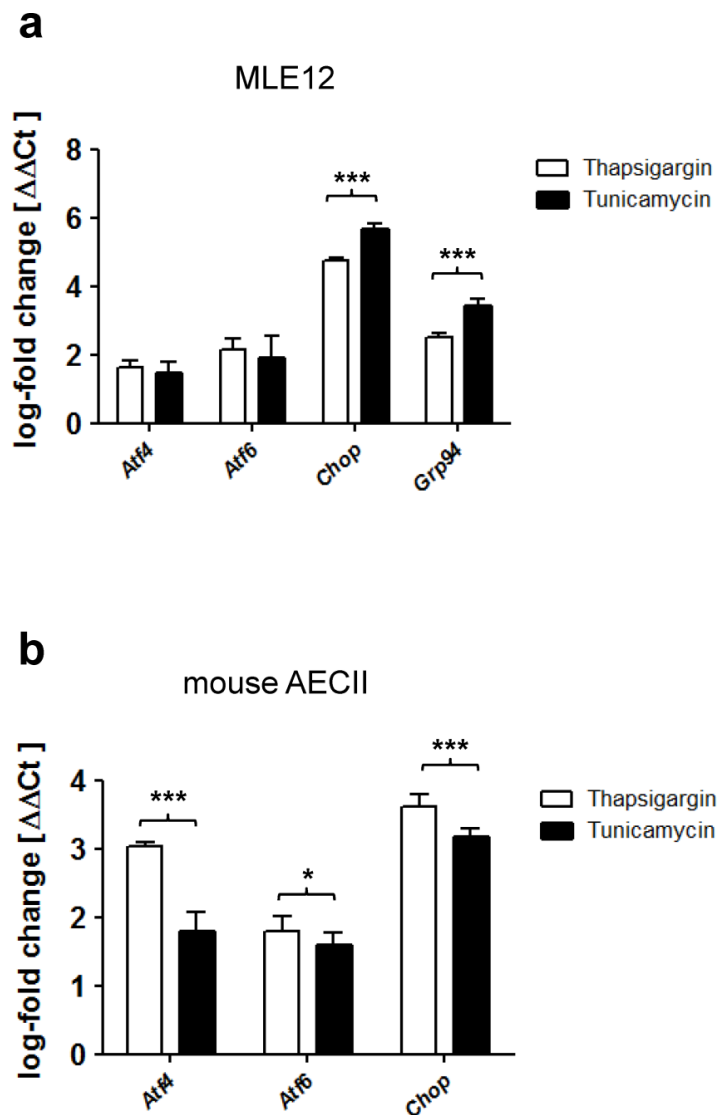


Fig. S4. Comparison of the effects of thapsigargin- and tunicamycin-treatment on ER-stress induction in MLE12 and primary mouse alveolar epithelial type-II cells (AECII).

(a, b) MLE12 cells (a) or primary mouse AECII (b) were treated for 22h with vehicle (0.025% DMSO), thapsigargin (1 μ M/ml) or tunicamycin (1 μ g/ml), followed by quantitative RT-PCR analysis for *Atf4*, *Atf6* and *Chop* (and *Grp94* in MLE12). *B2m* served as reference gene. From real-time PCR data, dCt values were calculated as $dCt = Ct[\text{reference}] - Ct[\text{target gene}]$ using the Ct values of *B2m*. The $\log_2$ fold-changes are given by the ddCt values, where $ddCt = dCt[\text{treatment}] - dCt[\text{empty-vector-transfection}]$. All Ct values were measured in triplicate. Data are expressed as means $\pm$ SD, from n=2 independent experiments.

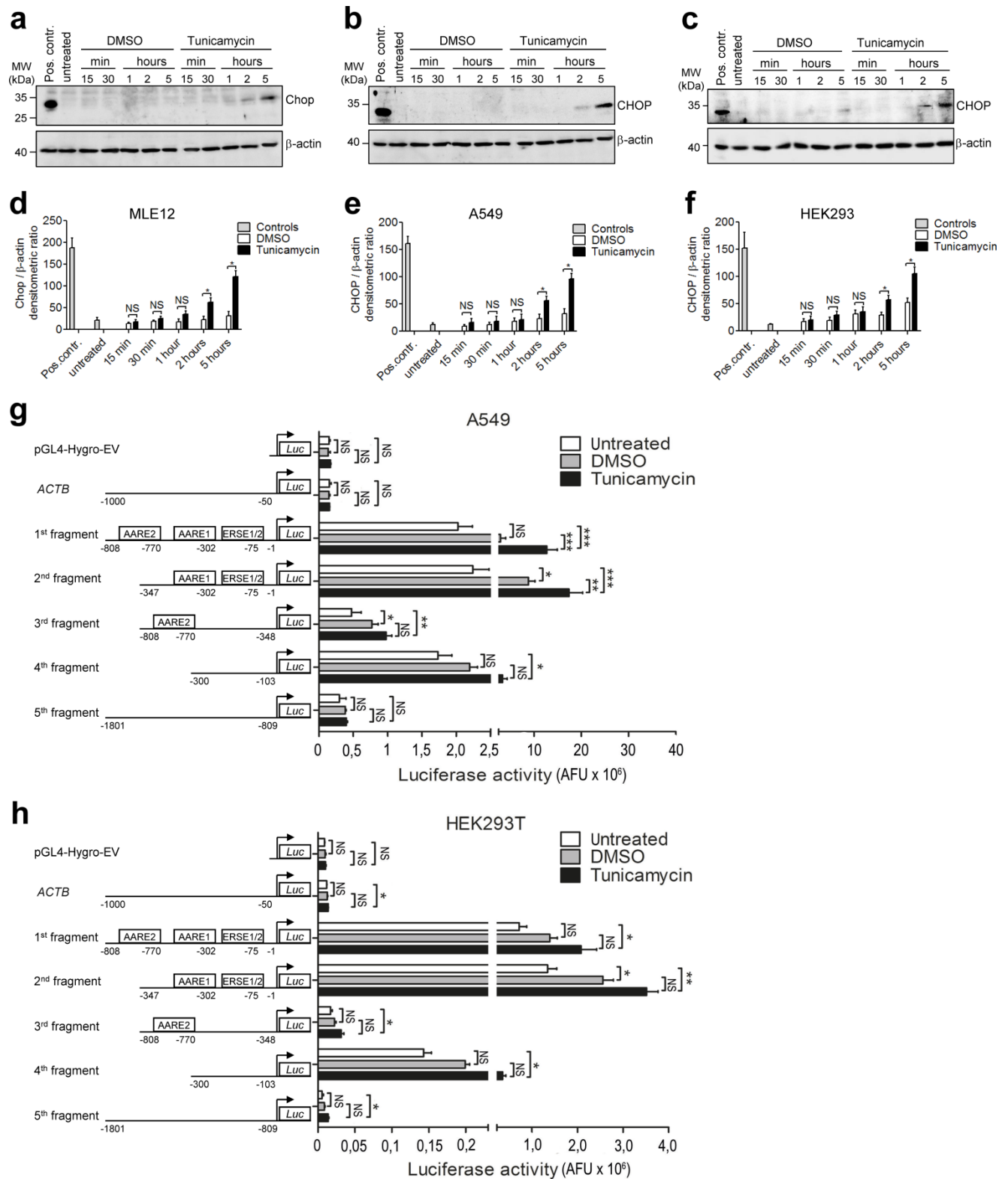


Fig. S5. Analysis of the human *CHOP* promoter in different epithelial cell lines.

(a-f) Time-dependent induction of Chop/CHOP in different epithelial cell lines MLE12 (a), A549 (b) and HEK293T (c) in response to 2 µg/ml of tunicamycin. β-actin served as loading

control. Each immunoblot was performed three times. The protein levels for induced Chop/CHOP in MLE12 (**d**), A549 (**e**) and HEK293 cells (**f**) were densitometrically quantified by using ImageJ software. For normalization, β -actin expression was used as a control. Data are expressed as means $\pm$ S.D., from n=3 independent experiments, with analysis by unpaired Student's t-test. *P < 0.05, NS = nonsignificant. Abbreviations: Pos. contr. = positive control, 24h-treatment of MLE12, A549 or HEK293T cells with 1 μ g/ml of tunicamycin.

(**g**, **h**) *Luciferase* reporter gene-assays of cloned promoter constructs containing five different fragments of the human *CHOP* promoter or one *ACTB* promoter fragment in A549 (**g**) and HEK293T cells (**h**). All fragments including pGL4-Hygro empty-vector (EV) were transiently transfected together with a vector containing a β -*Galactosidase* reporter gene into A549 (**g**) or HEK293T cells (**h**), followed by tunicamycin (2 μ g/ml) or vehicle (0.02% DMSO)-treatment 24h after transfection. After 2h, *Luciferase*- and β -*Galactosidase* assay were performed as described in the Methods. Results are presented as normalized *Luciferase* activity. Means $\pm$ S.D. are shown, from n=3 independent experiments, with analysis by Bonferroni's Multiple Comparison test. *P < 0.05, **P < 0.01, ***P<0.001, NS = nonsignificant.

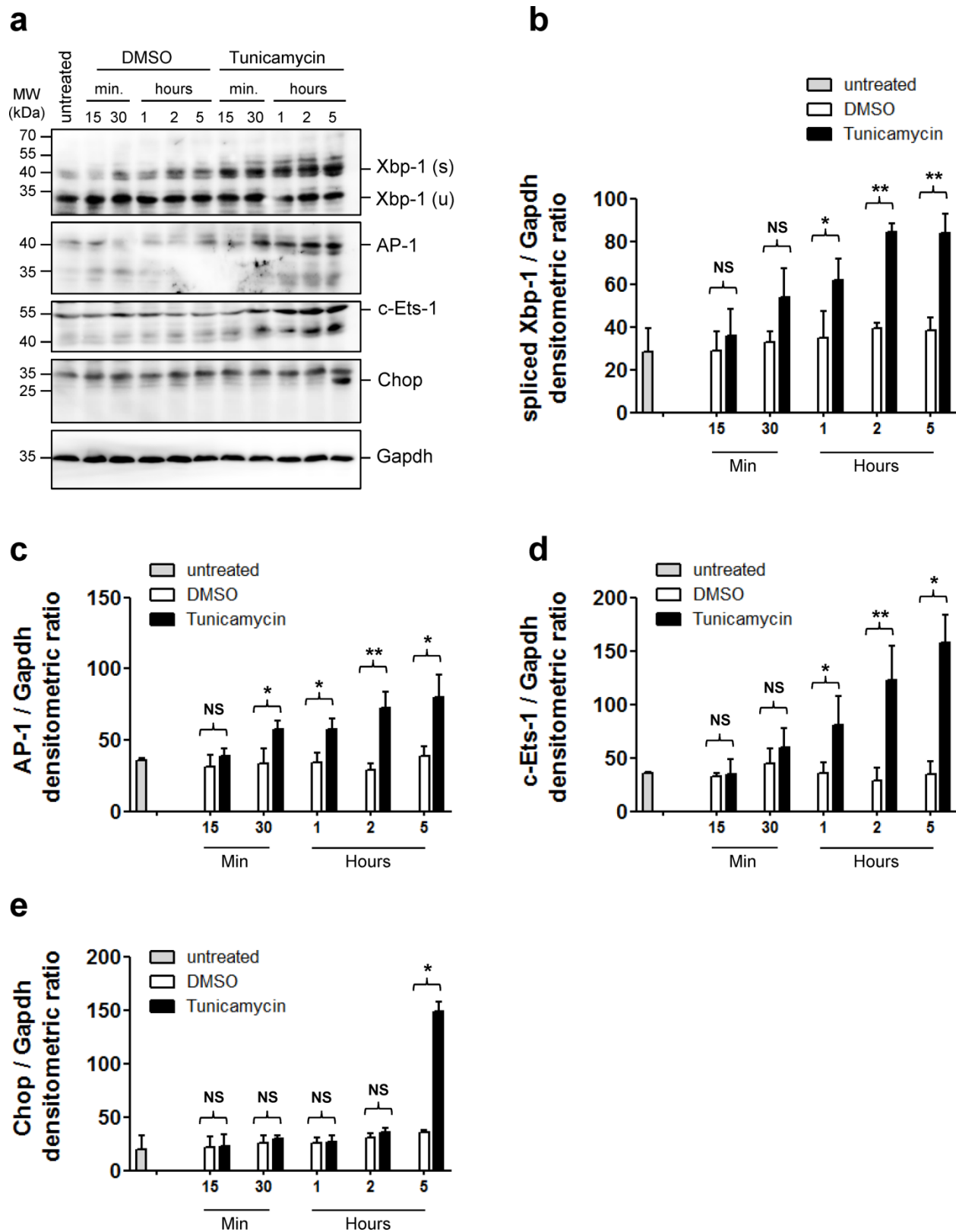


Fig. S6. AP-1 and c-Ets-1 are upregulated under ER-stress conditions in primary mouse alveolar epithelial type-II cells (AECII).

(a-e) Primary mouse AECII were treated with 0.02% DMSO (vehicle) or 2 μ g/ml tunicamycin for the indicated time periods. Protein lysates were subjected to western blotting for indicated antibodies **(a)**. The protein levels for spliced Xbp-1 **(b)**, AP-1 **(c)**, c-Ets-1 **(d)**, and Chop **(e)** were densitometrically quantified by using ImageJ software. For normalization, Gapdh expression was used as a control. All data are expressed as means $\pm$ S.D., from n=3 independent experiments, with analysis by unpaired Student's t-test. *P < 0.05, **P < 0.01, NS = nonsignificant.

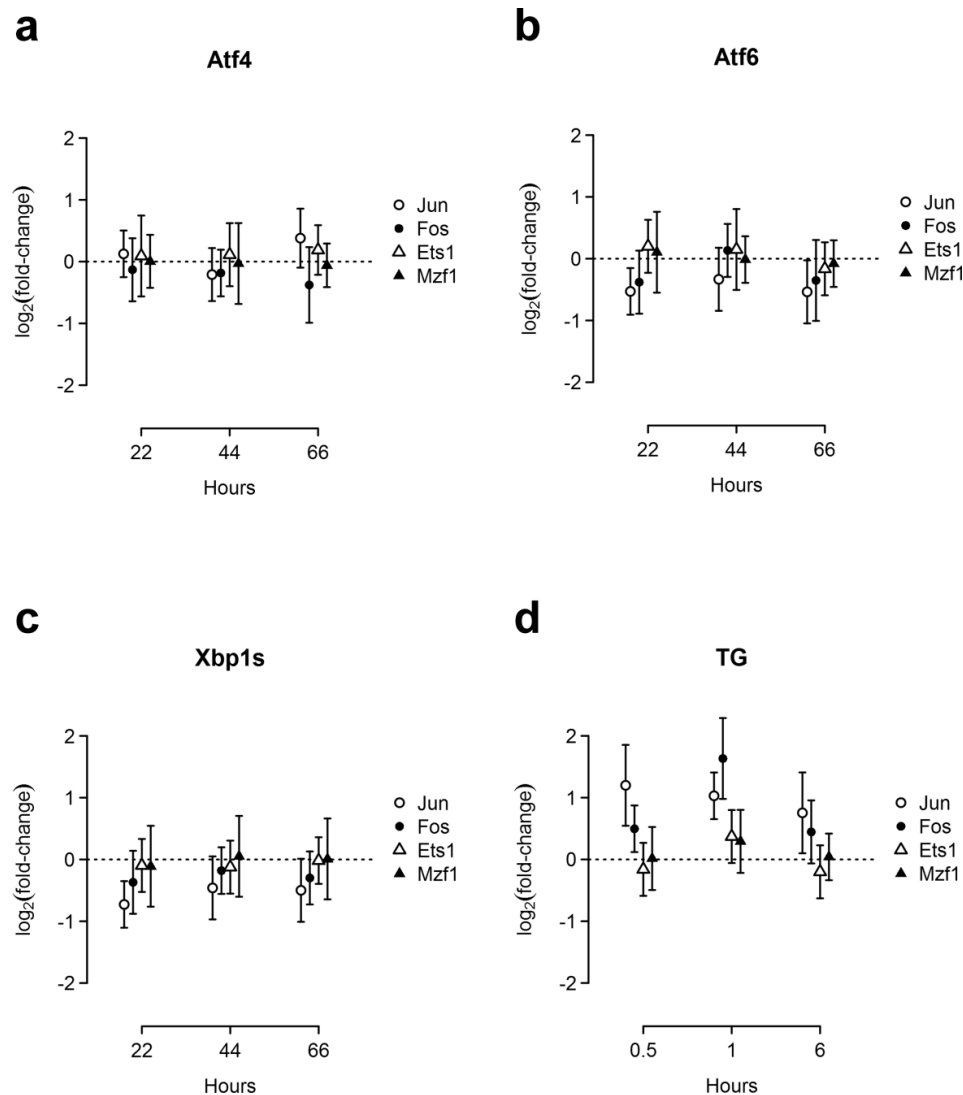


Fig. S7. Microarray analysis showing the differential expression of *Jun*, *Fos*, *Ets1* and *Mzf1* using RNA from MLE12 cells after overexpression of the UPR transcription factors Atf4 (**a**), p50Atf6 (**b**) or spliced Xbp1 (Xbp1s) (**c**), against empty-vector transfection, in comparison to thapsigargin (TG, 1 μ M/ml)-treated cells (**d**) (n=6 experiments per condition for time periods 22h, 44h, 66h; and n=4 for the 0.5h, 1h and 6h treatments with TG, and n=12 for the respective control). Error bars are 95% confidence intervals. The microarray as described in Fig. 1a and Fig. 1b (main manuscript) and Fig. S1 was used for expression analysis of indicated genes. Because c-Fos may form a heterodimer-complex with the *Jun*/AP-1 transcription factor at various gene promoters, its gene expression under conditions of ER-stress was of interest.

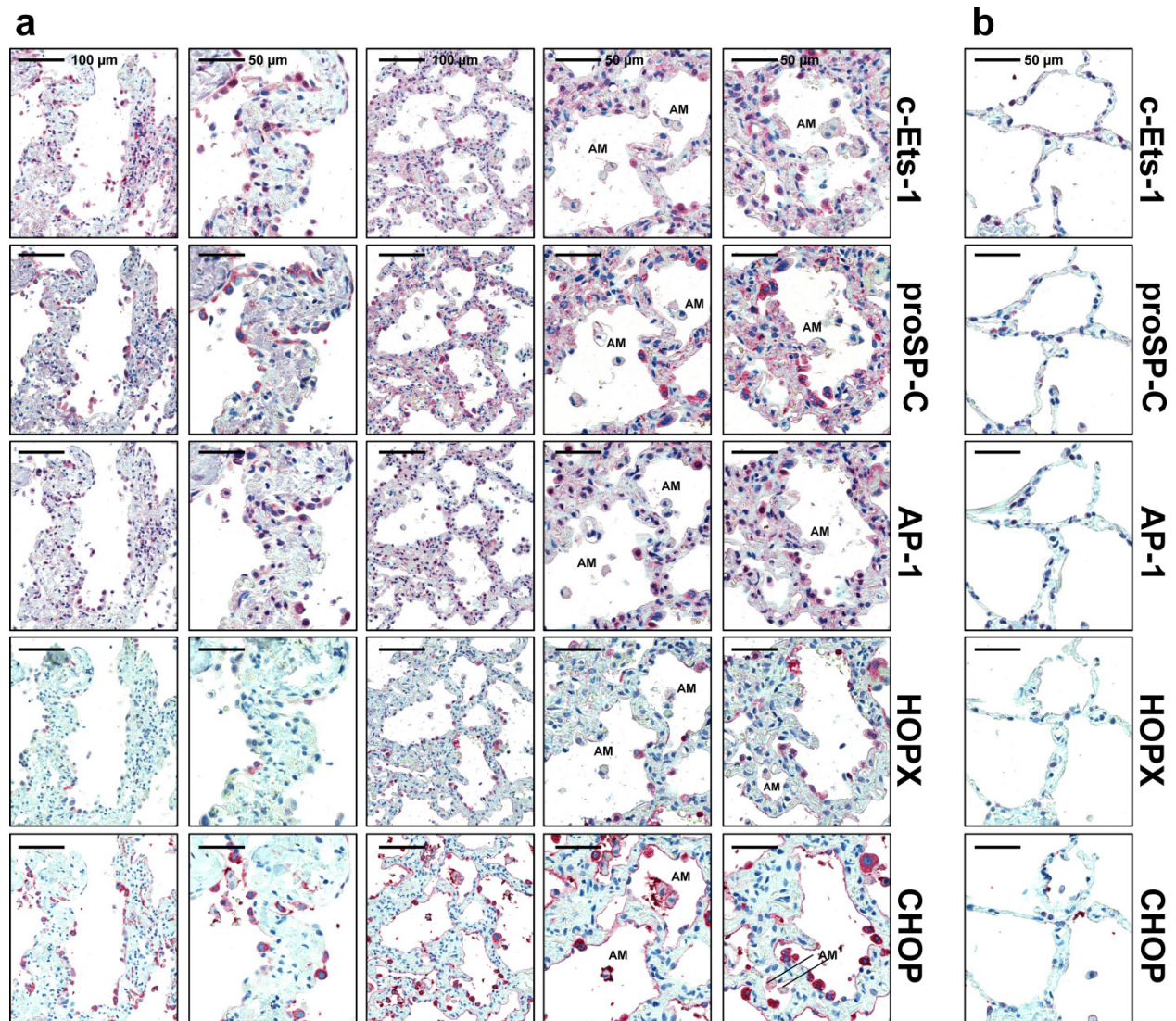


Fig. S8. AP-1, c-Ets-1 and CHOP are induced in AECII from patients with IPF.

Immunohistochemical staining of serial sections of IPF- (a) and normal donor lung tissues (b) for c-Ets-1, proSP-C (AECII-marker), AP-1, HOPX (AECI-and AECII marker) and CHOP. (a) In IPF, proSP-C expressing AECII indicated robust overexpression of c-Ets-1 and AP-1 in the nucleus and cytoplasm, and co-localized with induced CHOP expression. Alveolar macrophages (AM) of IPF-lungs indicated only faint expression of c-Ets-1 and AP-1, and no significant expression of CHOP. It should be noted, that the rabbit polyclonal anti-CHOP antibody (#5554 from CST) sometimes appeared to stain (unspecifically) mucous debris in the alveolar spaces. (b) In normal donor lungs, AP-1 and c-Ets-1 were expressed at low basal level in AEC, and occasionally found at low-to-moderate level in AM. Minimal or no immunostaining for CHOP was observed in any cells of donor lungs. Results are representative for n=6 IPF-patients and n=3 organ donors.

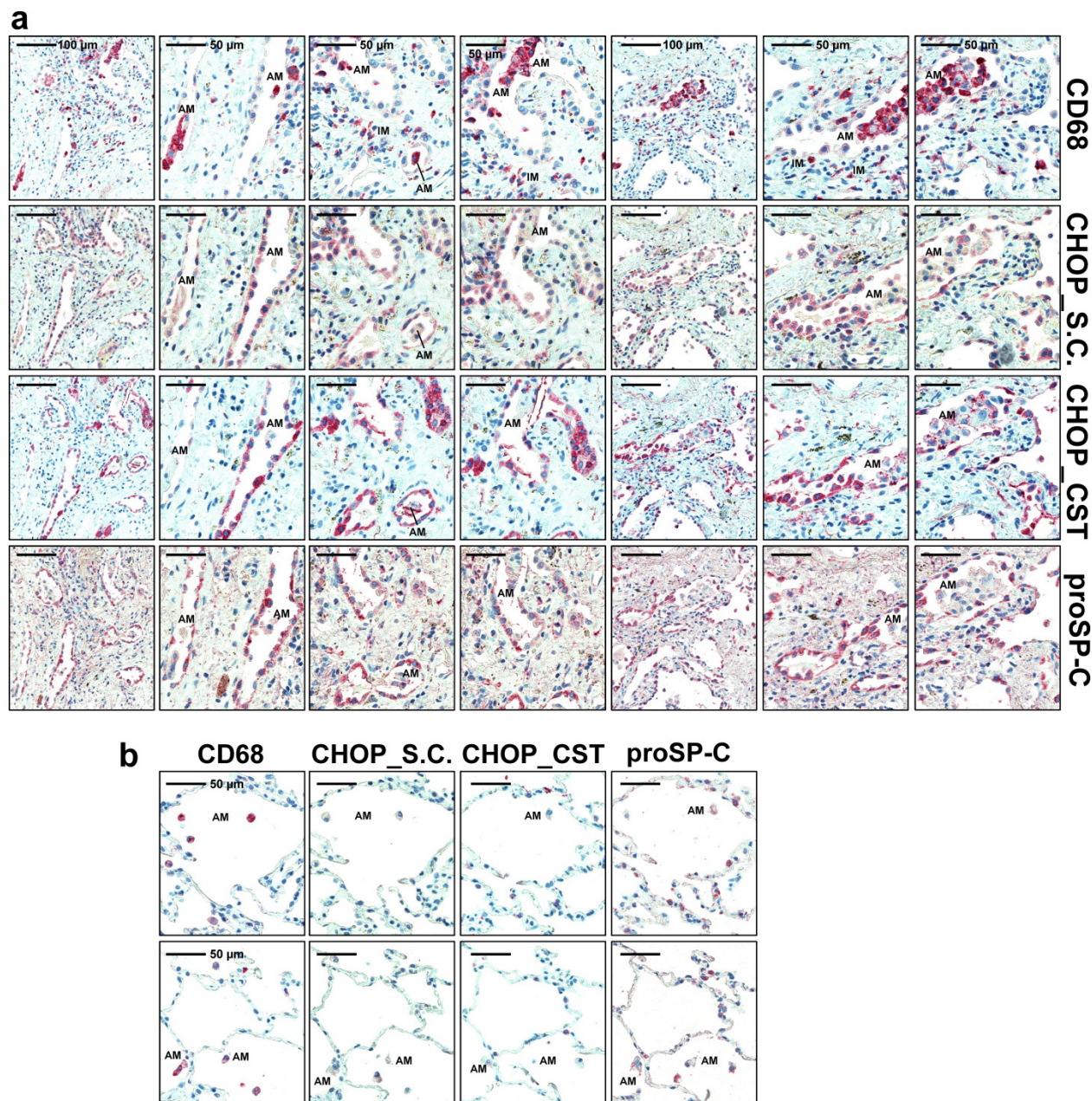


Fig. S9. CHOP is exclusively induced in AECII, but not in AM from patients with IPF.

Immunohistochemical staining of serial sections of IPF- (a) and normal donor lung tissues (b) for CD68 (macrophage marker), CHOP (mouse monoclonal anti-CHOP antibody sc-7351 from Santa Cruz, as well as rabbit polyclonal anti-CHOP antibody #5554 from CST) and proSP-C (AECII-marker). (a) In IPF, both different anti-CHOP antibodies indicated predominantly robust immunoreactivity in proSP-C expressing AECII, but not in CD68 expressing alveolar macrophages (AM). (b) No or only minimal immunostaining for CHOP was observed in any cells of normal donor lungs. Results are representative for n=6 IPF-patients and n=3 organ donors.

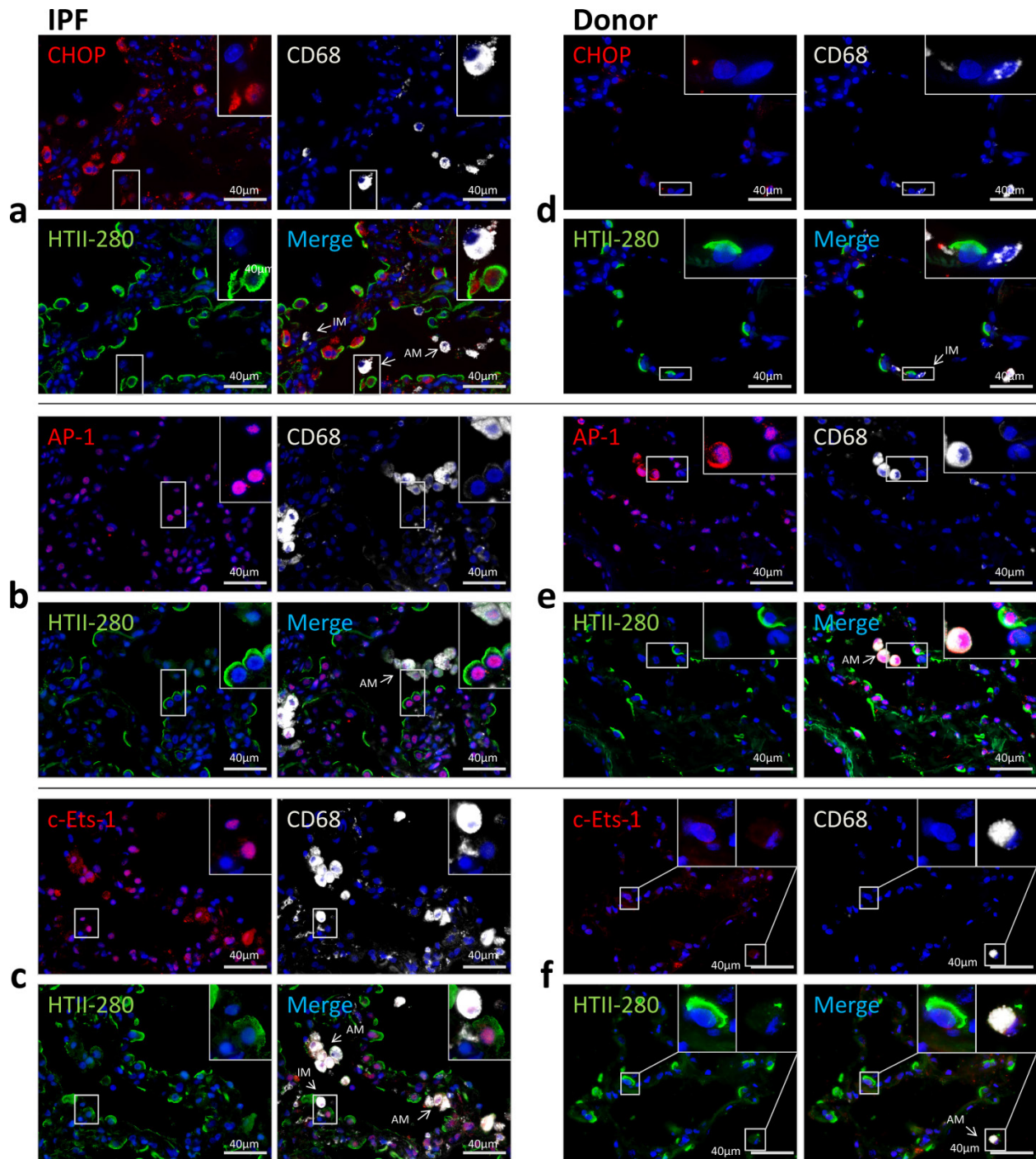


Fig. S10. CHOP, AP-1 and c-Ets-1 are exclusively induced in AECII, but not in AM from patients with IPF.

(a-f) Immunofluorescence staining for CHOP, AP-1, c-Ets-1, CD68 (macrophage marker) and HTII-280 (AECII marker) of lung tissue sections from patients with IPF (a, CHOP; b, AP-1 and c, c-Ets-1) and organ donors (d, CHOP; e, AP-1 and f, c-Ets-1). Nuclei were counterstained with DAPI (blue color). In IPF, HTII-280 expressing AECII (green color) indicated nuclear overexpression of CHOP (a), AP-1 (b) and c-Ets-1 (c) (red color). In contrast, CD68 positive

interstitial [IM] and alveolar macrophages [AM] (indicated in white color) of IPF lungs indicated no significant expression of CHOP **(a)**, AP-1 **(b)** or c-Ets-1 **(c)**. In normal donor lungs, CHOP **(d)** and c-Ets-1 **(f)** were absent in HTII-280 expressing AECII and CD68-positive macrophages. Interestingly, nuclear expression of AP-1 was occasionally observed in HTII-280 expressing AECII and CD68-positive macrophages of donor lungs **(e)**, presumably due to its multifunctional role and involvement in various biological pathways, including (non-pathogenic) homeostasis-related signalling pathways. Results are representative for n=3 IPF-patients and n=3 organ donors.

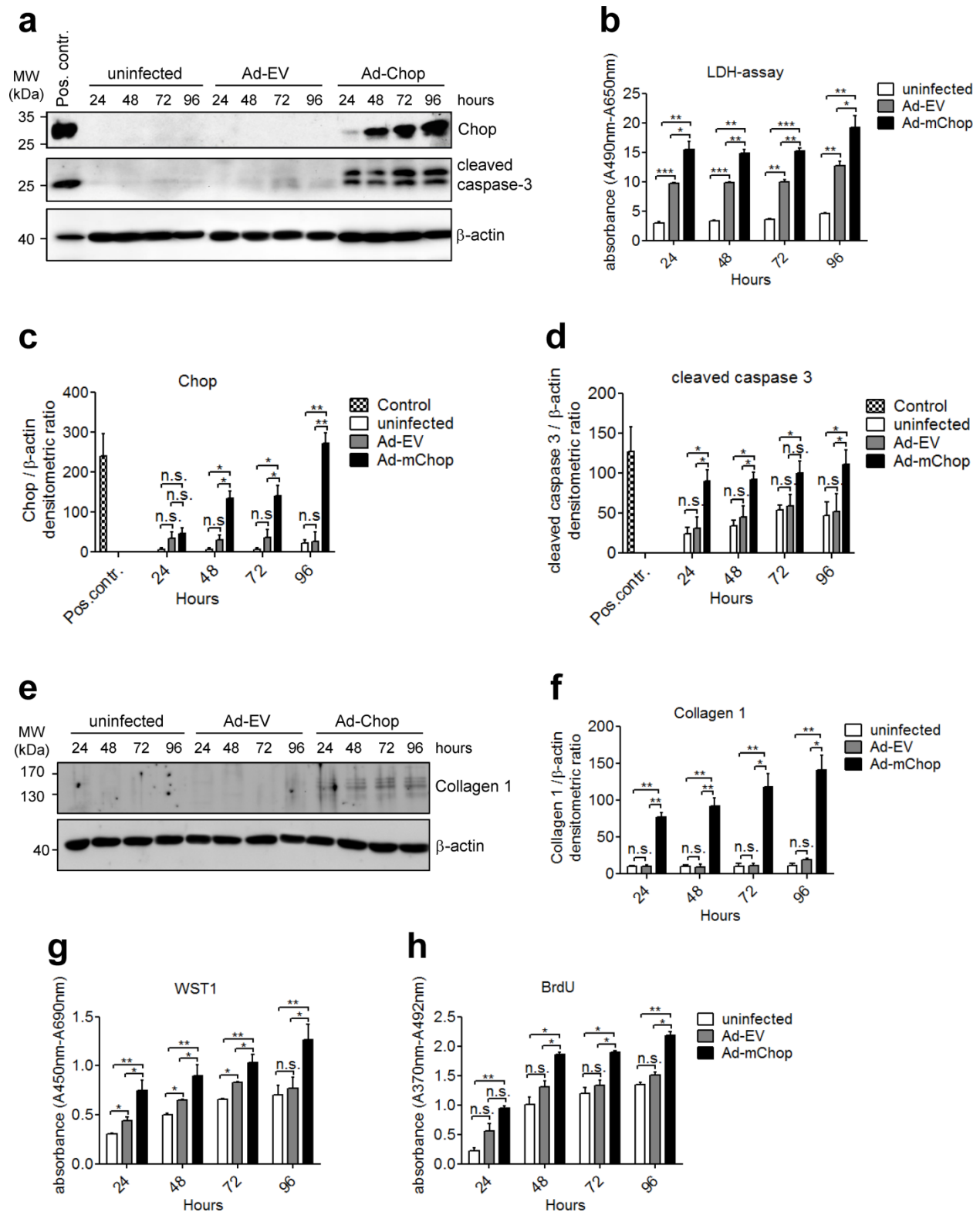


Fig. S12. Effect of Chop-overexpression on apoptosis of AECII and fibroblast proliferation *in vitro*. (a) Primary murine AECIIs were infected with adenoviral vectors Ad-EV (empty-vector-control), Ad-Chop or left uninfected, followed by quantitative immunoblot analysis for

Chop and cleaved caspase-3. β -actin served as loading control. **(b)** LDH assay quantifying AECII cell death in response to Chop-overexpression. **(c, d)** Densitometric quantification for Chop-expression **(c)** and caspase-3-activation **(d)**. Pos. contr. = positive control, AECII cells treated either with tunicamycin (1 μ g/ml for 24h) or staurosporine (1 μ M/ml for 8h), for Chop- or cleaved caspase-3-blot, respectively. **(e)** Conditioned medium from AECII cells infected with Ad-EV or Ad-Chop was applied to Mlg lung fibroblasts, followed by assessment of their collagen-synthesis by quantitative immunoblotting using anti-collagen-1 antibody and β -actin as loading control. **(f)** Densitometric quantification for collagen-1-expression. **(g, h)** Proliferation of Mlg lung fibroblasts by WST-1 assay **(g)** or BrdU incorporation **(h)** in response to culture with supernatants of AECII infected with Ad-EV or Ad-Chop. In **(b-d)** and **(f-h)**, means $\pm$ S.D. are shown, from n=3 experiments, with analysis by unpaired Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. = nonsignificant.