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Supplemental information

Aspergillus fumigatus hijacks human p11 to redirect fungal-containing phagosomes to non-degradative pathway

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Supplementary Figures

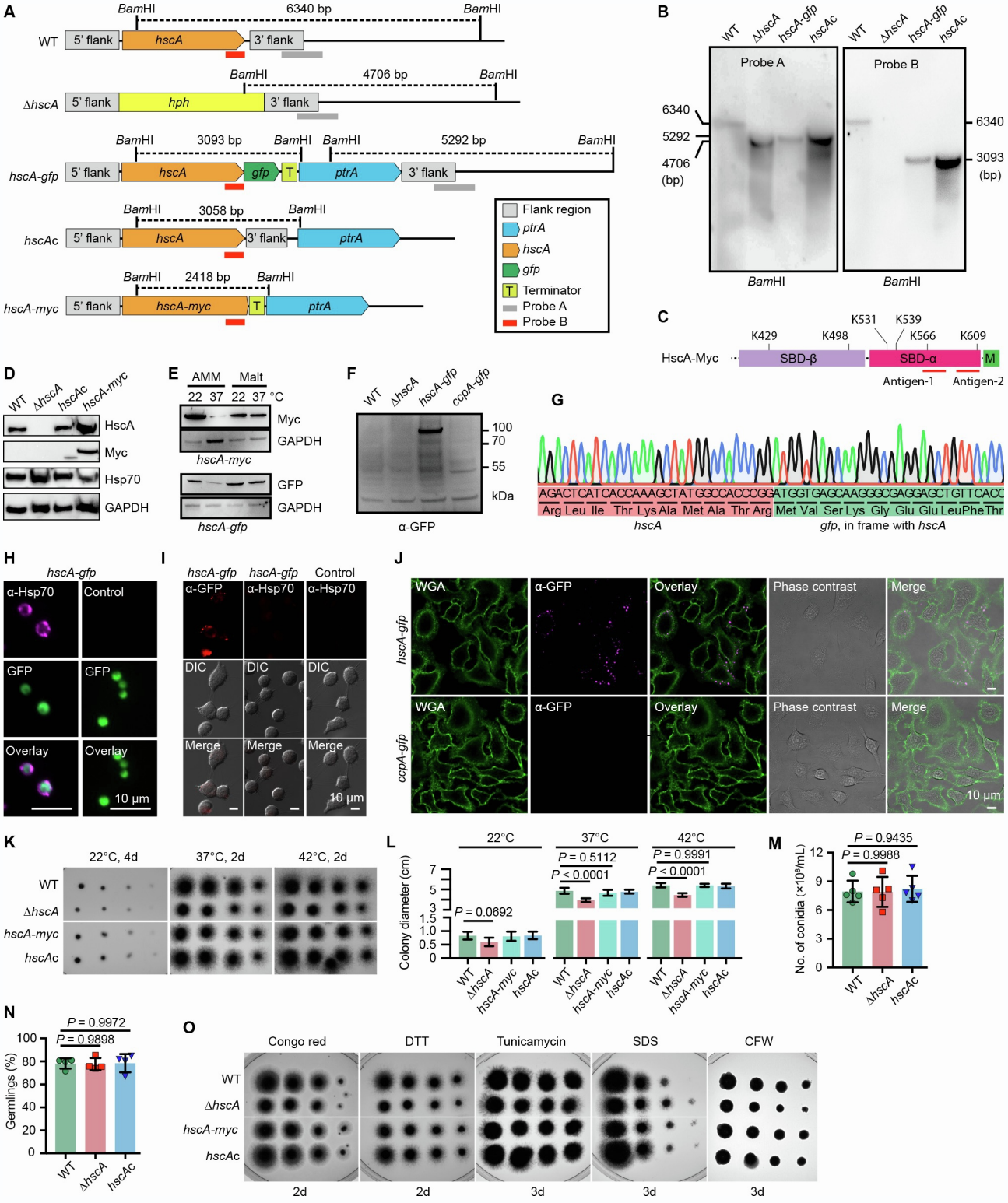

Figure S1. Verification and phenotypic analysis of *A. fumigatus hscA* mutant strains. Related to Figure 1.

(A) Chromosomal organization of the *hscA* locus in different *A. fumigatus* strains. Size of generated DNA fragments by *Bam*HI restriction and binding sites of hybridization probe A and B are indicated. *ptrA*, pyrithiamine resistance gene.

(B) Southern blot analysis of chromosomal DNA cut by *Bam*HI to confirm the generated recombinant *A. fumigatus* strains. A DNA band obtained with probes A and B with the size of 6,340 base pairs (bp) is characteristic of the WT strain, a band obtained with probe A with the size of 4,706 bp of Δ*hscA* strain and 5,292 bp of *hscA-gfp* strain. A band obtained with probe B with the size of 3,093 bp is indicative of both strain *hscA-gfp* and *hscA*c.

(C) Schematic representation of HscA-Myc substrate binding domains (SBDs) consisting of SBD-β (purple) and SBD-α (lid domain, red) [S1]. Myc tag (M) was fused to the C-terminus of SBD. Positions of antigens 1 and 2 used for polyclonal antibody generation are marked with red lines. Lysine residues with biotinylation marks are indicated [S2].
(D) Western blot of protein extracts from dormant conidia of indicated strains with antibodies against HscA, Myc-tag, Hsp70, or GAPDH. Conidia were harvested from malt agar plates after 7 days of cultivation at 22°C.
(E) Western blot of HscA in dormant conidia. Strains *hscA-myc* and *hscA-gfp* were inoculated on AMM or malt agar and incubated at 22°C or 37°C for 7 days. Protein extracts from dormant conidia were probed with anti-Myc, anti-GFP or anti-GAPDH antibodies. See also Figure 1F and 1G.
(F) Western blot for the detection of the HscA-GFP fusion protein with an anti-GFP antibody.
(G) DNA sequence of the fusion site of genes *hscA* and *gfp* present in the *hscA-gfp* strain. The DNA fragment containing the 3' region of *hscA* and the 5' region of *gfp* was PCR amplified using the primer pair oJLJ19-45 and oJLJ18-57, and then sequenced using primer oJLJ19-45.
(H) Immunofluorescence staining of Hsp70 localized on the surface of *hscA-gfp* dormant conidia with the anti-Hsp70 antibody. Conidia incubated with secondary antibody served as negative control.
(I) Immunofluorescence staining of HscA-GFP binding to A549 cells. A549 cells were incubated with protein extracts of strain *hscA-gfp* at room temperature for 1 h. Cells were then incubated with anti-GFP antibody or anti-Hsp70 antibody. A549 cells without incubation with fungal protein extracts served as negative control.
(J) Immunofluorescence staining of HscA-GFP binding to A549 cells. A549 cells were incubated with protein extracts of strains *hscA-gfp* or *ccpA-gfp* at room temperature for 1 h. Cytoplasmic membrane of A549 cells was stained with Oregon Green™ 488 conjugated wheat germ agglutinin (WGA). Protein was stained with anti-GFP antibody.
For H–J, goat anti-rabbit IgG Dylight 633 or goat anti-mouse IgG Dylight 633 were used to detect primary antibodies.
(K–O) *hscA* gene deletion caused no severe growth defects.
(K) Images of serial 10-fold dilutions of conidia of the indicated *A. fumigatus* strains inoculated onto AMM agar and incubated for 4 days at 22°C, or 2 days at 37°C or 42°C.
(L) Colony diameter of indicated strains. 10^5 conidia were inoculated at the center of AMM agar plates and incubated at the indicated temperatures for 3 days.
(M) Number of conidia of indicated strains on AMM agar plates after 3 days of incubation at 37°C. 10^5 conidia were freshly harvested and spread onto AMM agar plates. Conidia were harvested from each agar plate with 10 mL of sterile water.
(N) Number of germlings of the indicated *A. fumigatus* strains incubated in RPMI medium for 8 hours at 37°C.
(O) Images of serial 10-fold dilutions of conidia of the indicated strains that were spotted on AMM agar plates containing 30 μg/mL Congo red, 1mM DTT, 10 μg/mL tunicamycin, 0.01% (w/v) SDS, or 300 μg/mL CFW at 37°C for 2 or 3 days.
Statistics: Error bars represent the mean ± SD; *p* values are calculated by one-way ANOVA followed by Tukey's multiple comparisons test.
Scale bars, 10 μm.

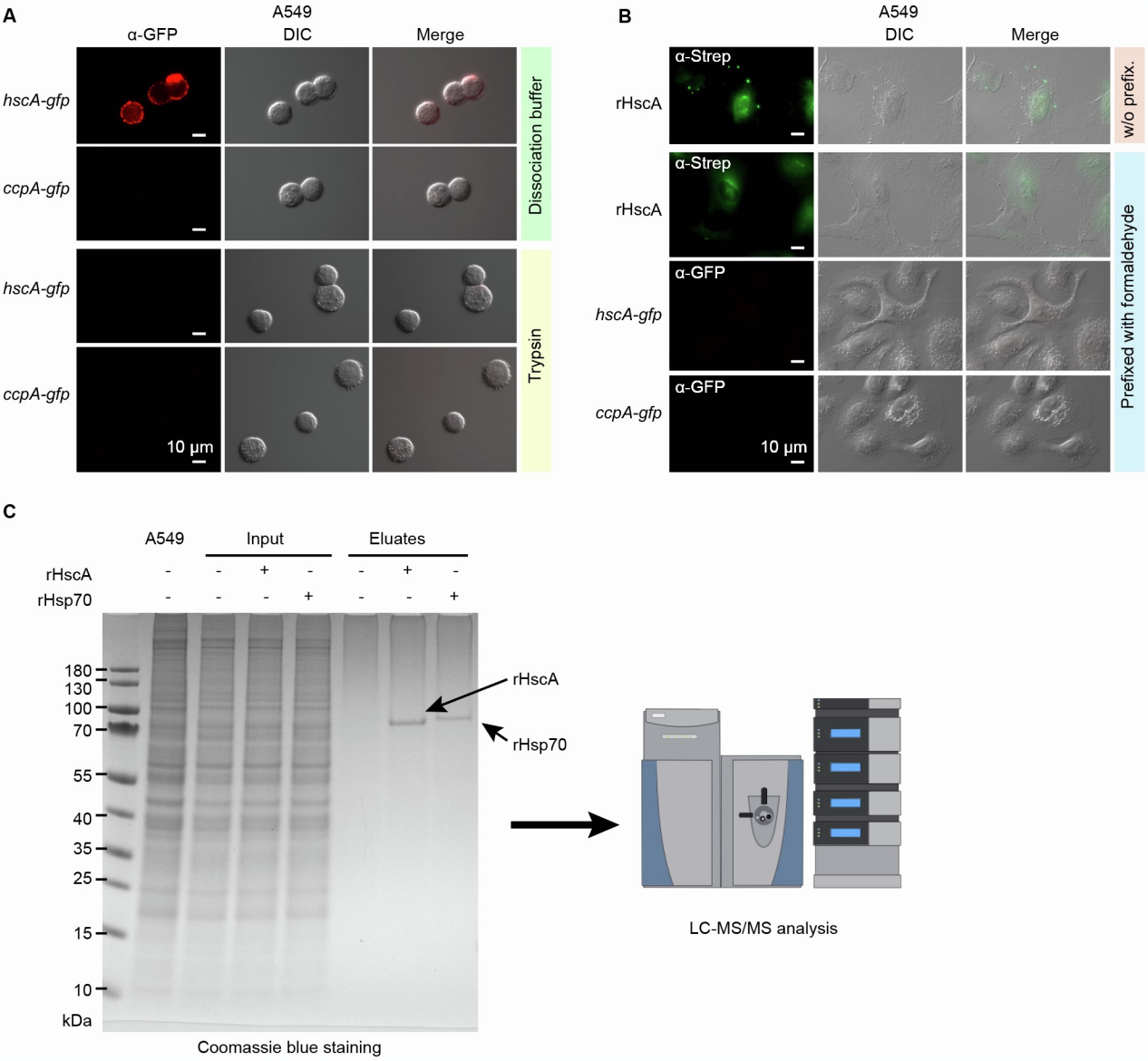

Figure S2. Identification of potential binding partners of HscA. Related to Figure 3.

(A and B) Pre-treatment of A549 cells with (A) trypsin or (B) formaldehyde abolished binding of HscA to A549 cells.

(A) Immunofluorescence staining of A549 cells incubated with protein extract of dormant conidia for 1 h at room temperature and stained with anti-GFP antibody after pre-treatment of A549 cells with trypsin. A549 cells were suspended in enzyme-free dissociation buffer or trypsin digestion buffer.

(B) Immunofluorescence of A549 cells pre-fixed with 4% (v/v) formaldehyde in PBS. Then, cells were incubated with rHscA or protein extracts from strains *hscA-gfp* or *ccpA-gfp* for 1h at room temperature followed by detection using indicated antibodies. All scale bars, 10 μm.

(C) SDS-PAGE of A549 protein extracts incubated with the indicated recombinant proteins. A549 cell lysates were incubated in IP buffer, with rHscA or rHsp70 for 2 h at 4°C. Samples were purified by Strep-Tactin®XT spin columns, and then analyzed by LC-MS/MS. Molecular masses of standard proteins are indicated on the left side.

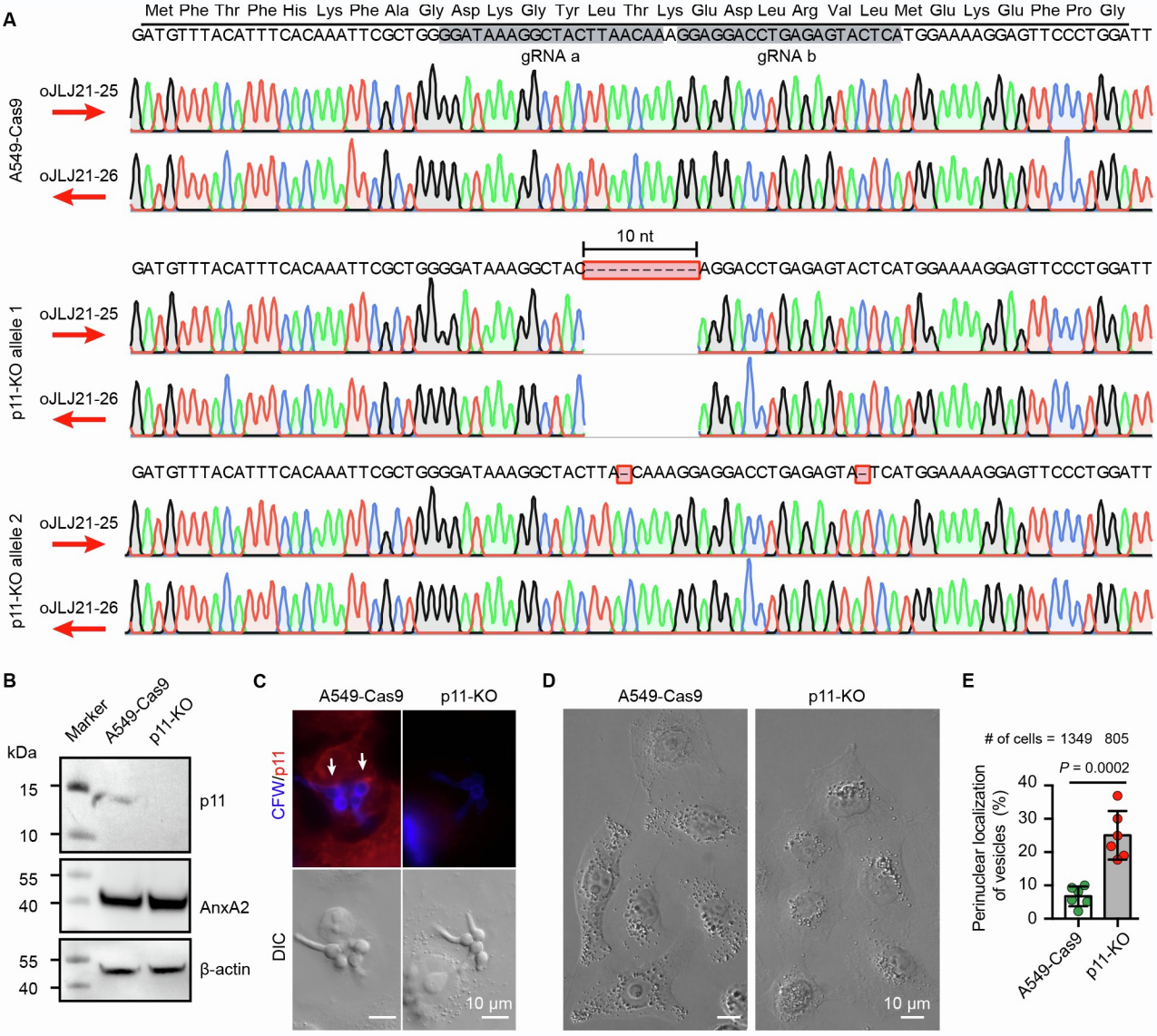

Figure S3. Knockout of the p11 gene in A549 cells. Related to Figure 3.

(A) Verification of generated p11-KO cell line by DNA sequencing. A 704 bp DNA fragment was amplified from A549-Cas9 cell line or p11-KO cell line and sequenced using primers oJLJ21-25 and oJLJ21-26. The DNA fragment obtained from p11-KO cells was further cloned into pJET1.2 for DNA sequencing. As indicated with red boxes, deletion of ten base pairs in allele 1 and two single base pairs in allele 2 causes a frame shift of the p11-coding sequence and results in a p11 knockout.

(B) Western blot of protein extracts of A549-Cas9 and p11-KO cells with antibodies against p11, AnxA2, or β-actin.

(C) Immunofluorescence staining of p11 in cell lines infected with WT conidia. Arrows indicate $p11^{+}$ phagocytic cups. See also Figure 3F.

(D) Microscopic image of perinuclear localization of vesicles in p11-KO cells.

(E) Percentage of accumulated vesicles in the perinuclear region of A549-Cas9 and p11-KO cells. Data represent the mean ± SD (n = 6 independent experiments); p = 0.0002 (unpaired, two-tailed t test). Numbers of total cells counted are indicated.

Scale bars, 10 μm.

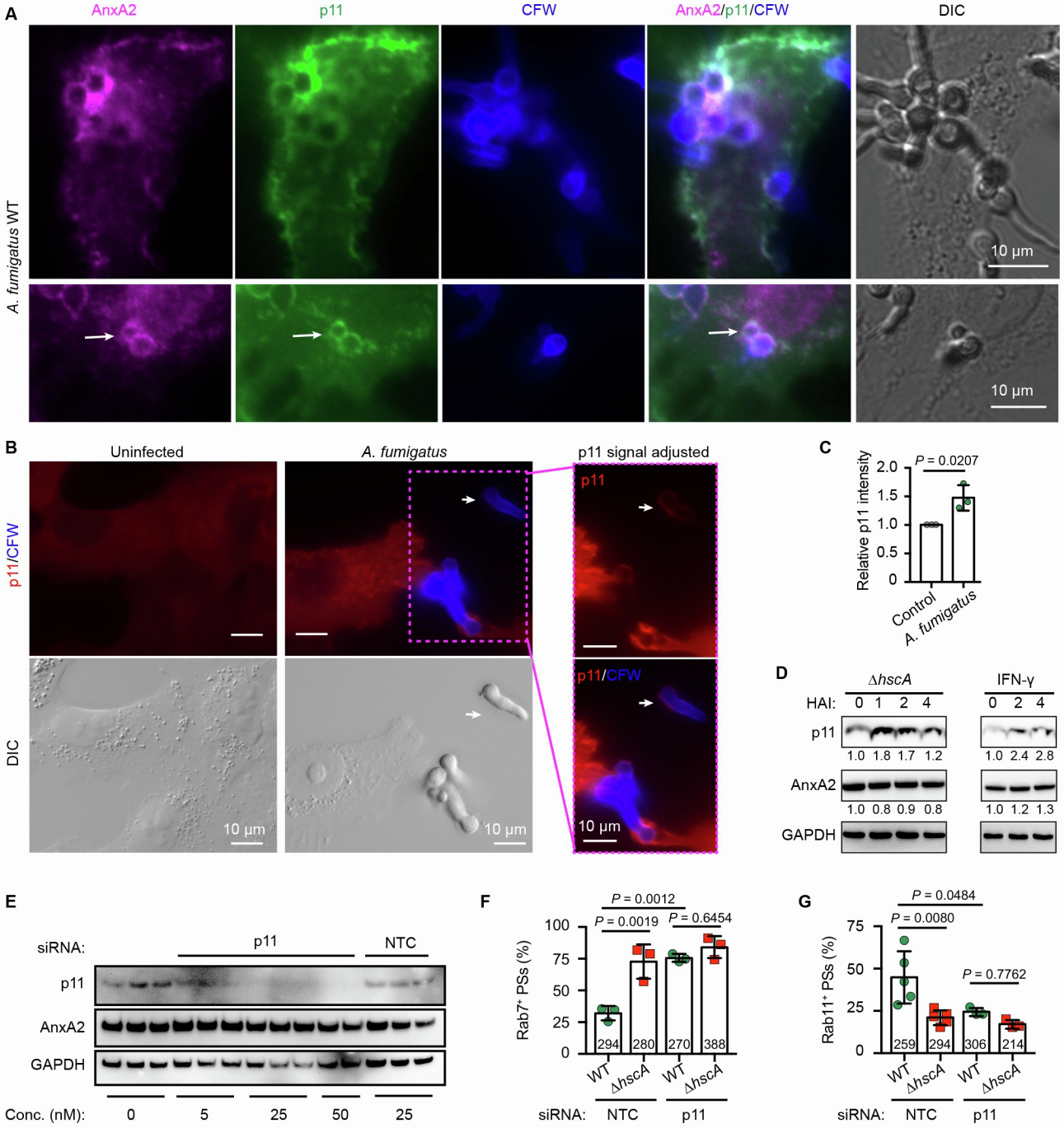

Figure S4. p11 protein level increases by *A. fumigatus* infection. Related to Figure 3 and Figure 4.

(A) Immunofluorescence staining of AnxA2 and p11 on phagocytic cups (upper row) and phagosomes (bottom row) of A549 cells infected with WT conidia for 8 h. Arrows indicate a phagosome contains a conidium. Extracellular conidia and germlings were stained with CFW. Cells were stained with anti-p11 and anti-AnxA2 antibodies. See also Figures 3F and 4A.

(B–D) p11 protein level is increased after *A. fumigatus* infection.

(B) Immunofluorescence staining of p11 in A549 cells infected without or with *A. fumigatus* for 8 hours at MOI = 10. Arrows indicate an extracellular germling with p11 staining. Extracellular *A. fumigatus* conidia and germlings were stained with CFW. Cells were stained with anti-p11 antibody. See also Figure 3K.

(C) Relative immunofluorescence intensity of p11 induced by fungal infection. Data represent the mean ± SD; p = 0.0207 (unpaired, two-tailed t test).

(D) Western blot showing induction of p11 protein expression by Δ*hscA* conidia or IFN-γ. Incubation of A549 cells with Δ*hscA* conidia (MOI = 10) or IFN-γ (50 ng/mL) for indicated time; cell lysates were analyzed by Western blot analysis and probed with anti-p11, anti-AnxA2 or anti-GAPDH antibodies. See also Figure 3L.

(E) Knockdown of p11 expression in A549 cells with p11-targeting siRNA. After incubation of cells with indicated concentrations of p11-targeting siRNA or non-targeting control siRNA (NTC, 25 nM) for 48 h, cell lysates were probed with anti-p11, anti-AnxA2 or anti-GAPDH antibodies.

(F and G) Knockdown of p11 expression in A549 cells promotes phagosomal maturation. *A. fumigatus* conidia detected in (F) $Rab7^+$ or (G) $Rab11^+$ phagosomes were counted. A549 cells treated with siRNA were incubated with conidia. Data represent the mean ± SD (*n* = 3 or 5 independent experiments); *p* values are indicated based on one-way ANOVA followed by Tukey's multiple comparisons test. Numbers of total phagosomes counted are indicated at the bottom of bar graphs. See also Figure 4I and 4O.

Scale bars, 10 μm.

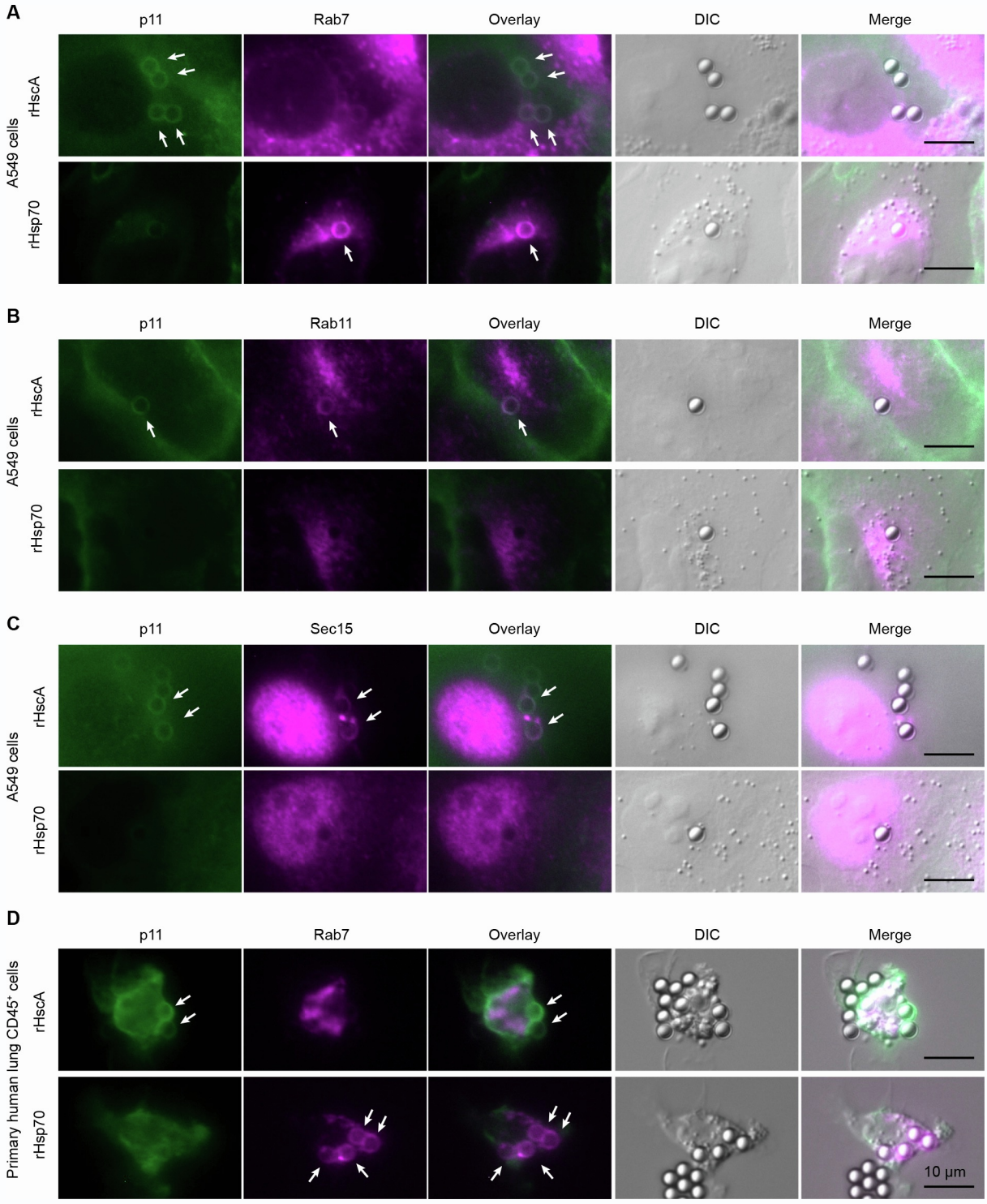

Figure S5. Differential recruitment of p11 and Rab proteins to phagosomes. Related to Figure 4 and Figure 6.
(A–C) Recruitment of phagosomal markers to latex beads with a diameter of 3 μm coated with protein rHscA or rHsp70. After incubation of A549 cells with the beads for 8 h, cells were fixed, permeabilized, and stained with the indicated antibodies: (A) anti-p11 and anti-Rab7; (B) anti-p11 and anti-Rab11; (C) anti-p11 and anti-Sec15.
(D) HscA recruits p11 to and excludes Rab7 from phagosomes containing latex beads in $CD45^+$ cells. After incubation of primary lung $CD45^+$ cells with beads for 3 h, cells were fixed, permeabilized, and stained with an anti-p11 antibody and an anti-Rab7 antibody. See also Figure 6C and 6D.
White arrows indicate positive staining of phagosomes by indicated antibodies. Scale bars, 10 μm.

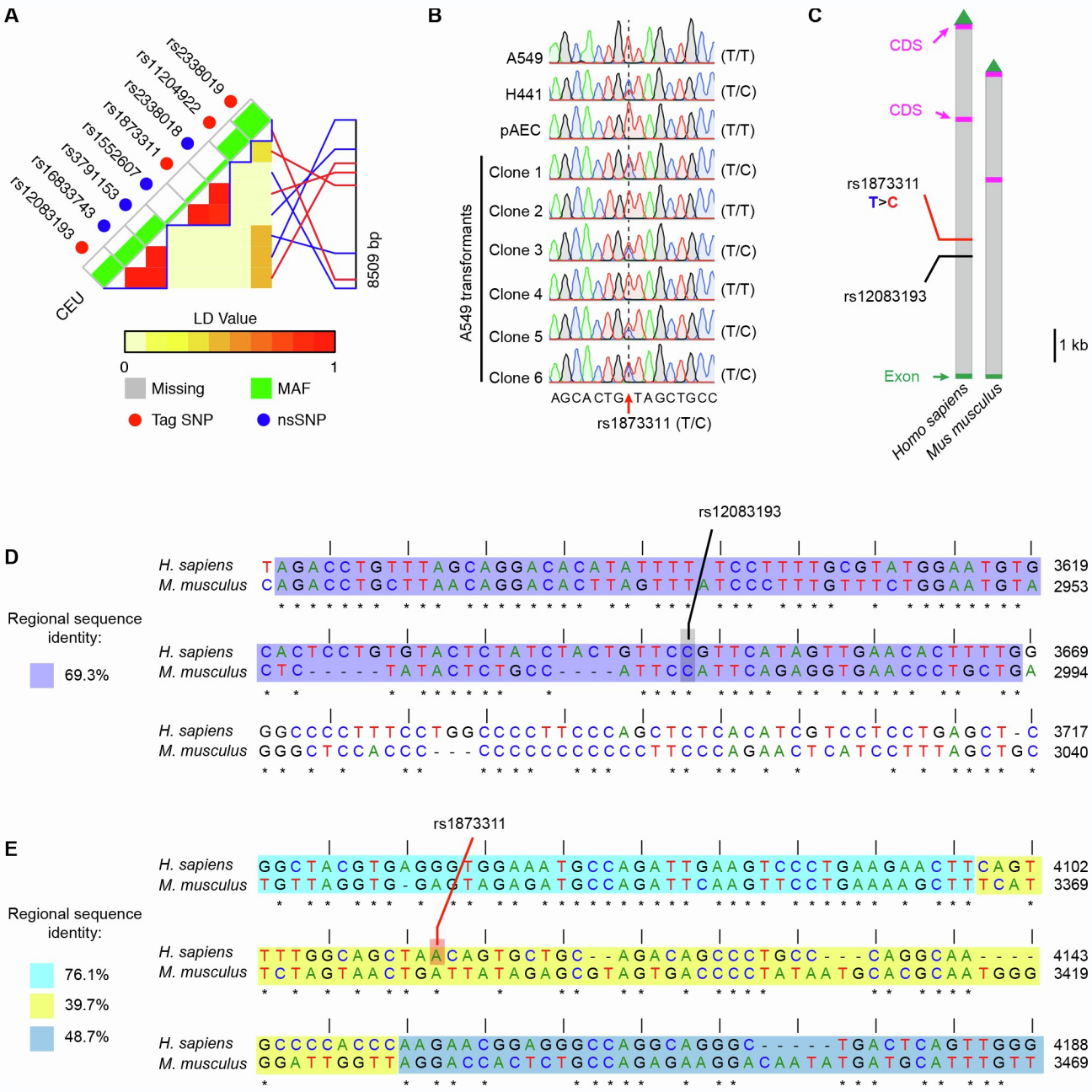

Figure S6. Sequence analysis of SNPs in *S100A10* (p11) gene. Related to Figure 6.

(A) Graphical view of the haplotype-based tagging strategy for the SNPs in the *S100A10* gene. SNPs with a minor allele frequency (MAF) above 0.05 were selected from the publicly available sequencing data from the Pilot 1 of the 1,000 Genomes Project for the CEU population (Northern Europeans from Utah). Tag SNPs are indicated with the red circles and nsSNPs are indicated with blue circles. Linkage disequilibrium (LD) values were used to define LD blocks tagged by each SNP. See also Figure 6A.

(B) DNA sequences of cell lines A549, H441, pAEC, and gene-edited A549 cells (clone 1–6) at the rs1873311 SNP locus. DNA fragments were PCR amplified using primers oJLJ21-41 and oJLJ21-42. The sequences show the T/T homozygous genotype of A549, pAEC, and clone 2 and 4 of A549-T/T, the C/T heterozygous genotype of the H441 cell line, and clones of A549-T/C.

(C–E) Alignment of human and mouse p11 gene.

(C) Signatures of human and mouse p11 gene. Introns are indicated with grey blocks, exons with green blocks, the coding sequence (CDS) of p11 is indicated with magenta blocks. Scale bar, 1 kilo base (kb).

(D and E) Alignment of p11 DNA sequences at the locus (D) rs12083193 and (E) rs1873311. Asterisks indicate aligned bases with same identity. The identity of aligned sequences labeled with the same colored box is indicated as regional sequence identity.

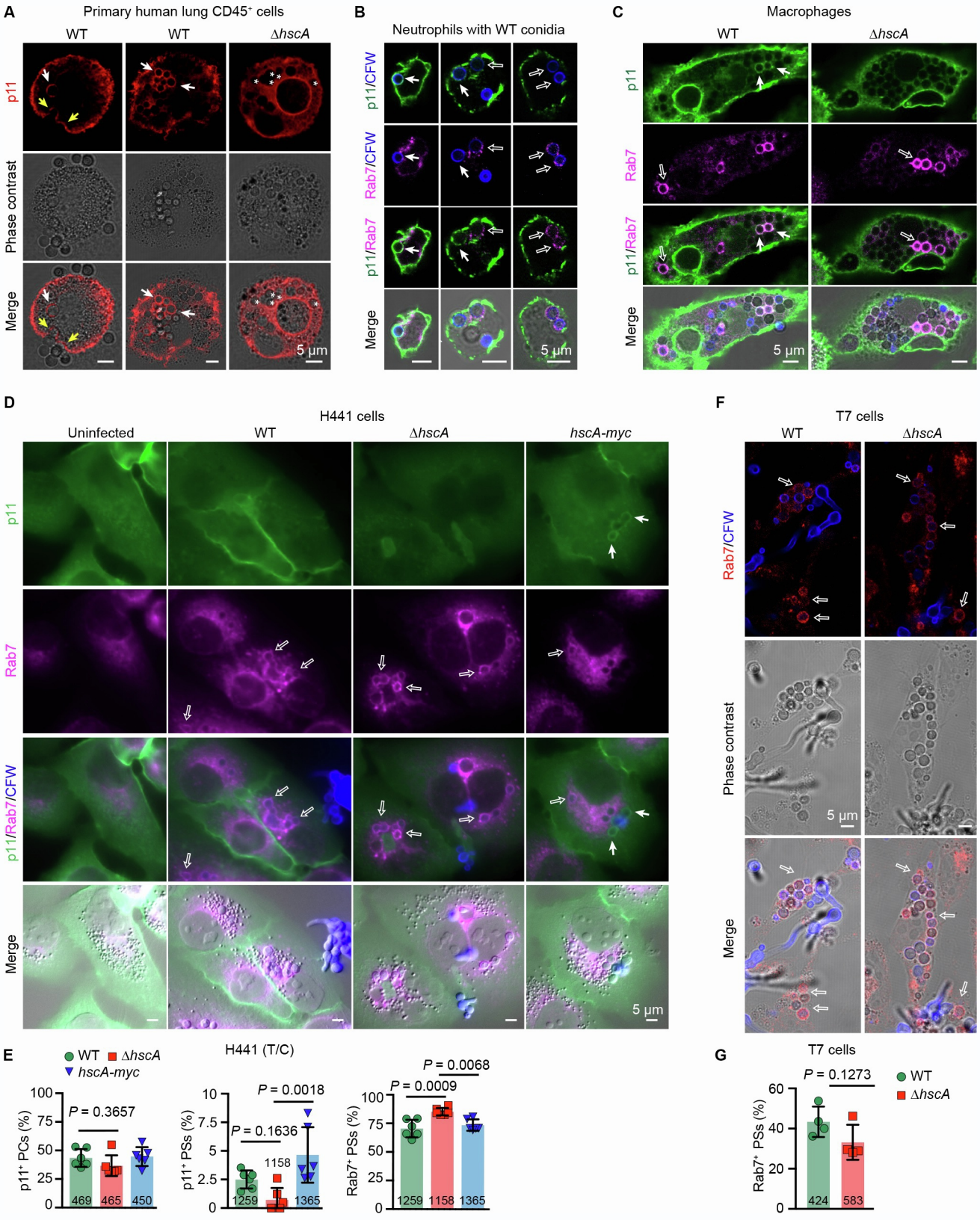

Figure S7. Immunostaining of primary cells and cell lines infected with *A. fumigatus*. Related to Figure 6.

(A–C) p11 is detected on phagosomes containing conidia in primary cells isolated from lung and blood.

(A) CD45⁺ cells isolated from human lung tissues were infected with WT or Δ*hscA* conidia for 3 h. Cells were stained with anti-p11 antibody. "*" symbols indicate Δ*hscA* within host cell without p11 staining. See also Figure 6C and 6D.

(B and C) Neutrophils (B) and macrophages (C) were isolated from blood and were incubated with CFW-labeled conidia for 2 h. Cells were stained with an anti-p11 and anti-Rab7 antibody.

(D–G) HscA plays no significant role in preventing Rab7 recruitment to phagosomes in human H441 and mouse T7 cells.

(D) H441 cells were infected with WT, Δ*hscA,* or *hscA-myc* conidia for 8 h. Extracellular conidia and germlings were stained with CFW. Cells were stained with anti-p11 and anti-Rab7 antibodies.

(E) Percentage of $p11^+$ PCs, $p11^+$ PSs, and $Rab7^+$ PSs containing conidia in H441 cells. Data are mean ± SD (n = 6 independent experiments); p values are calculated based on one-way ANOVA followed by Tukey's multiple comparisons test. Numbers of total phagocytic cups or phagosomes counted are indicated at the bottom of bar graphs.

(F) T7 cells were infected with WT or Δ*hscA* conidia for 8 h and stained with anti-Rab7 antibody. Extracellular fungal conidia were stained with CFW.

(G) Percentage of $Rab7^+$ PSs containing WT or Δ*hscA* conidia in T7 cells. Data are mean ± SD (n = 4 independent experiments); p = 0.1273 (unpaired, two-tailed t test). Numbers of total phagosomes counted are indicated at the bottom of bar graphs.

White arrows indicate $p11^+$ phagosomes and yellow arrows indicate $p11^+$ phagocytic cups containing conidia. Open arrows indicate $Rab7^+$ phagosomes. Scale bars, 5 μm.

Table S2. Frequency of *p11* (*S100A10*) genotypes among cases of IPA and controls, and association test results. Related to Figure 6.

SNP rs# (alleles)	Genome	Patients	Genotype, n (%)			*P* value
			A/A	A/a	a/a	
rs1873311 (T>C)	R	IPA	94 (84.7)	17 (15.3)	0 (0.0)	0.96
		Controls	314 (84.4)	56 (15.1)	2 (0.5)	
	D	IPA	98 (88.3)	11 (9.9)	2 (1.8)	0.026
		Controls	304 (81.7)	67 (18.0)	1 (0.3)	
rs12083193 (G>C)	R	IPA	55 (49.6)	47 (42.3)	9 (8.1)	0.97
		Controls	179 (48.1)	162 (43.6)	31 (8.3)	
	D	IPA	54 (48.7)	41 (36.9)	16 (14.4)	0.24
		Controls	172 (46.2)	164 (44.1)	36 (9.7)	
rs11204922 (T>C)	R	IPA	38 (34.2)	57 (51.4)	16 (14.4)	0.96
		Controls	122 (32.3)	196 (53.5)	54 (14.2)	
	D	IPA	42 (37.8)	54 (48.7)	15 (13.5)	0.71
		Controls	125 (33.6)	192 (51.6)	55 (14.8)	
rs2338019 (T>C)	R	IPA	33 (29.7)	53 (47.8)	25 (22.5)	0.74
		Controls	124 (33.3)	173 (46.5)	75 (20.2)	
	D	IPA	30 (27.0)	54 (48.7)	27 (24.3)	0.32
		Controls	126 (33.9)	173 (46.5)	73 (19.6)	

SNP, single nucleotide polymorphism; IPA, invasive pulmonary aspergillosis; R, recipient; D, donor. The major and minor alleles are represented by the first and second nucleotides, respectively. A and a indicate distinct alleles of the same gene. *P* value is for Fisher's exact *t* test.

Table S3. Baseline characteristic of transplant recipients enrolled in the study. Related to Figure 6.

Variables	IPA (n=111)	No IPA (n=372)	P value
Age at transplantation, no (%)			
≤20 years	16 (14.4)	81 (21.8)	0.150
21 – 40 years	30 (27.0)	108 (29.0)	
>40 years	65 (58.6)	183 (49.2)	
Gender, no (%)			
Female	48 (43.2)	158 (42.5)	0.870
Male	63 (56.8)	214 (57.5)	
Underlying disease, no. (%)			
Acute leukemia	61 (55.0)	197 (53.0)	0.223
Chronic lymphoproliferative diseases	16 (14.4)	67 (18.0)	
Myelodysplastic/myeloproliferative diseases	17 (15.3)	34 (9.1)	
Chronic myeloproliferative diseases	8 (7.2)	21 (5.6)	
Aplastic anemia	6 (5.4)	29 (7.8)	
Other	3 (2.7)	24 (6.5)	
Transplantation type, no. (%)			
Matched, related	36 (32.4)	175 (47.0)	0.009
Matched, unrelated	40 (36.0)	91 (24.5)	
Mismatched, related	0 (0.0)	8 (2.2)	
Mismatched, unrelated	35 (31.5)	98 (26.3)	
Graft source, no. (%)			
Peripheral blood	91 (82.0)	306 (82.3)	0.645
Bone-marrow	19 (17.1)	57 (15.3)	
Cord blood	1 (0.9)	9 (2.4)	
Disease stage, no. (%)			
First complete remission	59 (53.2)	204 (54.8)	0.940
Second or subsequent remission, or relapse	19 (17.1)	63 (17.0)	
Active disease	33 (29.7)	105 (28.2)	
Conditioning regimen, no (%)			
RIC	79 (71.2)	250 (67.2)	0.452
Myeloablative	32 (28.8)	122 (32.8)	
CMV serostatus of donor and recipient, no. (%)			
D-/R+ or D+/R+	94 (84.7)	331 (89.0)	0.214
D-/R- or D+/R-	17 (15.3)	41 (11.0)	
Duration of neutropenia, mean days (range)†	13.2 (8 – 39)	14.0 (5 – 35)	0.504
Acute GVHD, no. (%)			
No GVHD or grades I – II	77 (69.4)	325 (87.4)	<0.001
Grades III – IV	34 (30.6)	47 (12.6)	
Antifungal prophylaxis, no. (%)‡			
Fluconazole	55 (49.6)	149 (40.1)	0.036
Posaconazole	31 (27.9)	120 (32.3)	
Other	9 (8.1)	15 (4.0)	
None or unknown	16 (14.4)	88 (23.7)	

Chronic lymphoproliferative diseases included cases of chronic lymphocytic leukemia, multiple myeloma, and B- and T-cell lymphomas. Chronic myeloproliferative diseases included cases of chronic myelogenous leukemia and primary myelofibrosis. Other diseases included cases of idiopathic medullar aplasia, lymphohistiocytosis, hemoglobinopathies and paroxysmal nocturnal hemoglobinuria. RIC, reduced intensity conditioning; CMV, cytomegalovirus; D, donor; R, recipient; GVHD, graft-versus-host-disease. †Neutropenia was defined as $\leq 0.5 \times 10^9$ cells/L. ‡Other antifungals used in prophylaxis included voriconazole, liposomal amphotericin B, itraconazole and caspofungin. P values were calculated by Fisher's exact probability t-test or Student's t-test for continuous variables.

Table S4. Oligonucleotides used in this study. Related to KEY RESOURCES TABLE.

Usage	Primer name	Sequence
A. fumigatus Δ*hscA* strain generation	HYG-F	CCGGCTCGGTAACAGAACTA
	HYG-R	TTGGAGCATATCGTTCAGAGC
	HscA-P1	CCTTCGAGTAGTACACATCT
	HscA-P2	TAGTTCTGTTACCGAGCCGGGTGAGAGCACCTGAAAGGAG
	HscA-P3	GCTCTGAACGATATGCTCCAACTTGAGCATTACCGTTCCCT
	HscA-P4	CCAAGGACACTCTCAAATCC
A. fumigatus hscA-gfp strain generation	PtrA-F	ATGGTGAGCAAGGGCGAGGA
	PtrA-R	CATGTGGATTACGAGCTAAC
	HscA-P5	CTTTCTCTGGCACGCTCGAG
	HscA-P6	TCCTCGCCCTTGCTCACCATCCGGGTGGCCATAGCTTTGG
	HscA-P7	GTTAGCTCGTAATCCACATGACCATATGCTCCTCTGCTAT
	HscA-P8	AGGCGCTTATGACTAACACT
hscA-gfp strain verification	oJLJ18-57	ATCTGCAGCCCGGGCGGCCGC
	oJLJ19-45	CTCTCCACCACTGAAATCGA
Probe A synthesis	HscA-P8	AGGCGCTTATGACTAACACT
	oJLJ19-18	ATGCAGCGGATACAACGACT
Probe B synthesis	oJLJ19-45	CTCTCCACCACTGAAATCGA
	oJLJ19-46	CCGGGTGGCCATAGCTTTGG
Probe C synthesis	oJLJ19-33	CGCCAAGCTCGACAAGTCCT
	oJLJ19-42	AGAAAGACGGCCCTTGTCGT
Plasmid, pLJ-HscA-Comp	HscA-Com-F	TTCGAGCTCGGTACCATCCTCCCTGGCAGGATTCT
	HscA-Com-R	CAGCCGGGCGGCCGCTTTCTTTAAATTGCATAGAT
Plasmid, pLJ-HscA-Myc	HscA-Com-F	TTCGAGCTCGGTACCATCCTCCCTGGCAGGATTCT
	HscA-Myc-R	AAGATCCTCCTCGGAGATAAGCTTCTGCTCCCGGGTGGCCATAGCTTTGG
Plasmid, pnEATST-AfHscA	AfhscANdeIf:	AACTGCATATGTCGGACGAAGTCTACGAAGGC
	AfhscABamHIr:	TTGACGGATCCTTACCGGGTGGCCATAGCTTTGGT
Plasmid, pnEATST-AfHsp70	Afhsp70NdeIf	AACTGCATATGGCTCCCGCTGTCGGTATTGAC
	Afhsp70BamHIr	TTGACGGATCCTTAGTCAAGCTCCTCAGGGCGCTC
Plasmid, pLJ-064	oJLJ21-34	AAACTGAAGTTCTTCAGGGAGGATCCTCGTCCTTTCCACA
	oJLJ21-35	TCCCTGAAGAACTTCAGTTTGTTTTAGAGCTAGAAATAGCAA
	oJLJ22-08	AGTGATAACACTGCGGCCAA
	oJLJ22-09	ACCGTAAGTTATGTAACGGG
rs1873311 T to C template ssODN	oJLJ21-43	GGGTGGGGCTTGCCTGGGCAGGGCTGTCTGCAGCACTGCTAGCTGCCAAAACT GAAGTTCTTCAGGGACTTCAAT
PCR, p11 KO verification	oJLJ21-25	CAGTTAGGAGGAATAAGTTT
	oJLJ21-26	GTTGGGGCTGTGTCTTCATT
PCR, rs1873311 sequencing	oJLJ21-41	GCCCTCCTGTCAAAATGTGTC
	oJLJ21-42	TCTCCTCAGCAACCAGTACC
qPCR, S100A10 (human)	F	GGCTACTTAACAAAGGAGGACC [S3]
	R	GAGGCCCGCAATTAGGGAAA [S3]
qPCR, S100A10 (mouse)	F	TGGAAACCATGATGCTTACGTT [S3]
	R	GAAGCCCACTTTGCCATCTC [S3]
qPCR, 18S rRNA	F	CGGCGACGACCCATTCGAAC [S3]
	R	GAATCGAACCCTGATTCCCCGTC [S3]

Supplemental References

[S1] Gumiero, A., Conz, C., Gesé, G.V., Zhang, Y., Weyer, F.A., Lapouge, K., Kappes, J., von Plehwe, U., Schermann, G., Fitzke, E., et al. (2016). Interaction of the cotranslational Hsp70 Ssb with ribosomal proteins and rRNA depends on its lid domain. Nat. Commun. *7*, 13563. https://doi.org/10.1038/ncomms13563.

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