Supplementary Information

FBXO11 constitutes a major negative regulator of MHC class II through ubiquitin-dependent proteasomal degradation of CIITA

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SI Materials and Methods

Cell lines. HEK293T and HeLa cells were from RIKEN Bio BRC, Japan. RAW264.7 cells were from ATCC. Cells were maintained at 37 °C in a humidified incubator with 5% CO2. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively). FLAG-tagged human CIITA-, NLRC5- and NOD2-expressing stable cell line was generated with HeLa cells by transfecting with pQCXIP-FLAG-CIITA, pQCXIP-FLAG-NLRC5, pQCXIP-FLAG-NOD2 vector, followed by puromycin (2 μg/mL) selection. Tetracycline-inducible (Tet-ON) FLAG-tagged human FBXO11 stable cell line was generated with HeLa cells by transfecting with pTRIPZ FLAG-FBXO11 vector, followed by puromycin (2 μg/mL) selection.

Plasmids. The expression vector of pcGN containing HA-tag (pcGN-HA), p3×FLAG-CMV7.1, and pQCXIP plasmids are kind gifts of Dr. Hatakeyama (Hokkaido University). pRL Renilla null control reporter vector is purchased from Promega. *FBXO11* expression vectors were created by amplifying the FBXO11 (NM_001374325.1) coding sequence using cDNA transcribed from RNA of HeLa cells. The PCR amplified DNA fragments were cloned into the pcGN vector using SalI and NotI. pTRIPZ FLAG-FBXO11 was created by inserting PCR amplified FLAG-FBXO11 site into pTRIPZ (M)-HT-Cbx6, a gift from Xiaojun Ren (Addgene plasmid # 82514 ; http://n2t.net/addgene:82514 ; RRID:Addgene_82514) using XhoI and MluI. CIITA, NLRC5 and NOD2 expression vectors were created by amplifying each coding sequence using cDNA transcribed from RNA of HeLa cells and cloned into p3×FLAG vector using XbaI and SalI for CIITA and NLRC5, or NotI and SalI for NOD2, respectively. The mutant of FBXO11 or CIITA was generated by standard site-directed mutagenesis method by PCR using designed primer sets (SI Table2). The luciferase reporter plasmids for HLA promoters, pGL3-HLA-DRA, pGL3-HLA-DPA and pGL3-HLA-A are kind gifts from Dr. van den Elsen (Leiden University, Netherlands). The sequences of all of the cloned constructs were confirmed by Sanger sequencing. The sequence information of the primers for cloning is shown in Supplementary Table 2.

Retrovirus expression system. CIITA, NLRC5 and NOD2 retrovirus vectors were created by amplifying each coding sequence using cDNA created from RNA of HeLa cells and cloned into pQCXIP-FLAG vector using EcoRI and XhoI for CIITA, or NotI and XhoI for NLRC5 and NOD2 respectively. The retrovirus vectors (1 μg) were transfected with the pCL10A1 vector (Novus Biologicals) into 1×10^6 of HEK293T cells to generate recombinant retroviruses. HeLa cells (1×10^5) were infected with the recombinant retroviruses in the presence of polybrene (1ug/ml) and selected in a medium containing puromycin (5 μg/ml).

Reporter assay. For luciferase assays, 1×10^4 of HEK293T were seeded into 24 well plates. To

analyze the effect of FBXO11 on MHC promoter activity, 50 ng reporter construct of the HLA promoter and 10ng of pRL Renilla null control reporter vector were co-transfected with the FBXO11 expression vector, and either CIITA or NLRC5. Alternatively, endogenous CIITA or NLRC5 was induced with IFN-γ (100 U/m) for 24 h. After 36 h incubation, cells were harvested, and cell lysates were analyzed using a dual Luciferase Assay System (Promega).

Quantitative reverse-transcription PCR. For the evaluation of mRNA expression, RNA was reversely transcribed into cDNA using ReverTra Ace™ qPCR RT Master Mix reagent (Toyobo), and quantitative reverse-transcription PCR was performed using THUNDERBIRD™ SYBR™ qPCR Mix reagent (Toyobo, Japan) with the specific primer sets targeting human CIITA, HLA-DRA, mouse H2-Aa, and H2-Kb genes. As an internal control, the human and mouse GAPDH gene was targeted, amplified, and used for normalization. Relative gene expression levels were calculated, and the results are shown as fold induction over untreated control. The sequence information of the primers for RT-qPCR is shown in Supplementary Table 3.

Denature and non-denature immunoprecipitation. For non-denaturing immunoprecipitation, 1.0×10^7 HEK293T cells were harvested 48 h after polyethyleneimine (PEI) transfection, lysed in 0.2% NP40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.2% NP40 and protease inhibitor cocktail [Roche]) and sonicated. For denaturing immunoprecipitation, cells were lysed in denaturing lysis buffer (50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% SDS and protease inhibitor cocktail [Roche]), sonicated, and boiled at 95 °C for 10 min. After denaturation, cell lysates were diluted 10-fold in the lysis buffer to reduce SDS concentration to 0.1% or less. Cell extracts were incubated with anti-FLAG M2 affinity gel (Sigma) for 2 h at 4 °C. Immunoprecipitants were washed with the lysis buffer three times and resuspended in SDS sample buffer, boiled for 5 min at 95 °C.

Cycloheximide chase assay. HEK293T (2×10^7 cells) or RAW264.7 (2×10^7 cells) were seeded in 10-cm dishes for 24 h and transfected with 4 μg of p3xFLAG-CIITA and 4 μg of either pcGN HA-empty, FBXO11 or ΔF mutant. 6h after transfection, cells were split into 6-well dish and cultured for an additional 18 h. Cells were then treated with cycloheximide (CHX) for 0, 0.5, 1, 1.5, and 2 h, and samples were collected and lysed in Laemmli sample buffer for Western blotting.

Immunoblot analysis. Immunoblot analysis was performed by separating protein extracts from cells lysed by sonication in Laemmli sample buffer by 10% SDS-PAGE, transferred to PVDF membranes, blocked with 10% nonfat milk, and incubated sequentially with primary antibodies and horseradish peroxidase-conjugating secondary antibodies against mouse or rabbit immunoglobulin G (1:3000 dilution in TBST containing 2% skim milk). The primary and secondary antibodies were diluted at the following rates: anti-FLAG antibody (Sigma), 1:1000; anti-HA antibody (BioLegend), 1:1000; anti-Myc

tag antibody (CST), 1:1000; anti-STAT1 antibody (CST), 1:1000; anti-pSTAT1 antibody (CST), 1:1000; anti-IRF1 antibody (CST), 1:1000; anti-FBXO11 antibody (Bethyl), 1:100; anti-CIITA (SCBT), 1:500; anti-ubiquitin P4D1 (SCBT), 1:1000; anti-ubiquitin P4D1 (SCBT), 1:1000; anti-beta actin (Proteintech), 1:5000; and secondary antibodies with horseradish peroxidase conjugate (GE Healthcare), 1:3000. Images were developed with ECL reagent (Takara) and scanned with ImageQuant™ LAS 4000 (GE Healthcare).

Flow cytometry. To analyze surface expression of MHC-I and MHC-II, 4×10^5 wild-type or *Fbxo11*-deficient RAW264.7 cells were analyzed with or without IFN-γ stimulation. To examine functional recovery of *Fbxo11*-deficient RAW264.7 cells, the expression vectors for HA-tagged FBXO11 or ΔF mutant expression vectors were additionally transfected. 48 h after the transfection, cells were used for analysis. Cells were washed with PBS and collected in FACS buffer (2% FBS/PBS) and stained with PE-conjugated isotype control (BioLegend), FITC-conjugated isotype control (BioScience), PE-conjugated anti-mouse I-A/I-E antibody (BioScience) or FITC-conjugated anti-mouse H-2Kb/H-2Db antibodies (BioLegend). After incubation on ice for 30 min, cells were washed twice with FACS buffer and used for flow cytometric analysis using FACS Canto (BD). Data analysis was performed using FlowJo software (BD).

Generation of the *Fbxo11*-deficient cells using the CRISPR/Cas9. The sgRNA sequences for mouse *Fbxo11* (NC_000083.7 REGION: 88298287. 88373473) were designed using the IDT gRNA design tool (https://sg.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE). The selected two sgRNA sequences (GCAAAGCGTGCAAGAGTGTC, targeted to Exon3; TTGTAGAGCTGCTTGCGTGT, targeted to Exon 4) was cloned into pLentiCRISPRv2 (1). To produce pseudo-lentiviruses, 4×10^5 HEK 293T cells were plated on 6-well plates and pLentiCRISPRv2 (0.67 μg, with or without sgRNA), psPAX2 (0.67 μg) and pMD2.G (0.67 μg) were transfected on the next day using PEI. Forty eight h after the transfection, the supernatant suspension was collected and used for the transduction with addition of 5 ug/ml of polybrene to RAW264.7 cells, which had been spread at a concentration of 8×10^5 in 6-wells on the previous day. Twenty four h after the transduction, culture medium was replaced with medium containing 10 μg/ml puromycin and cultured for 3 additional days. Puromycin-resistant cells were cloned by limiting dilution and single cell clones were selected. The genotype for the homozygous deletion of parts of exons 3 and 4 of the FBXO11 gene was confirmed by PCR amplification of the genomic DNA using the region-specific primers (F-primer, CAGGTCCTGGTGCACAAAATAG; R-primer, ACACACCGTCCGTCTTTCAA). The absence of the transcripts and protein expression of the Fbxo11 gene was confirmed by RT-PCR of mRNA, and Western blotting respectively. pLenti-CRISPRv2 empty vector pseudo-lentivirus was used as vector control cells, which were generated by the same method.

Sample preparation for mass spectrometry analysis. HeLa cells (2.0×10^7) that stably expressed FLAG-tagged CIITA, NLRC5 or NOD2 were lysed in the lysis buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 10% glycerol, 0.2% NP-40, 10 mM iodoacetamide (Sigma-Aldrich), 10 mM N-ethylmaleimide (Sigma-Aldrich), 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Roche, Branchburg, NJ), 10 μM MG132 (Merck, Darmstadt, DE), and PhosStop phosphatase inhibitors (Sigma-Aldrich). The cell lysates were sonicated and centrifuged at 20,000×g for 10 min at 4 °C, and the resulting supernatants were incubated with anti-FLAG M2 agarose (Sigma-Aldrich) for 2 h at 4 °C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and eluted twice with 50 μl of lysis buffer containing 0.25 mg/ml FLAG peptide (Sigma-Aldrich). Eluted peptides were dried by vacuum centrifugation, reduced (55 mM dithiothreitol, 5 min at 95 °C) (Thermo Fisher Scientific), alkylated by incubating in 10 mM iodoacetamide (Thermo Fisher Scientific), and digested with trypsin (50 ng/μl, overnight at 37 °C) (Promega) using 0.01% RapiGest SF (Waters Corporation). After tryptic digestion, the samples were acidified with TFA and desalted by solid-phase extraction using GL-Tip GC and GL-Tip SDB (GL Sciences, Tokyo, Japan).

Mass spectrometry analysis. Desalted tryptic digests were analyzed by nanoflow ultra-HPLC (EASY-nLC 1000; Thermo Fisher Scientific) on-line coupled to an Orbitrap Elite instrument (Thermo Fisher Scientific). The mobile phases were 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). Peptides were directly loaded onto a C18 Reprosil analytical column (3 μm in particle size, 75 μm in i.d., and 12 cm in length; Nikkyo Technos, Tokyo, Japan) and separated using a 150-min two-step gradient (0-35% for 130 min, 35-100% for 5 min, and 100% for 15 min of solvent B) at a constant flow rate of 300 nano Litter/min. For ionization, a liquid junction voltage of 1.6 kV and a capillary temperature of 200 °C were used. The Orbitrap Elite instrument was operated in the data-dependent MS/MS mode using Xcalibur software (Thermo Fisher Scientific) with survey scans acquired at a resolution of 120,000 at m/z 400. The top 10 most abundant isotope patterns with a charge ranging from 2 to 4 were selected from the survey scans with an isolation window of 2.0 m/z and fragmented by collision-induced dissociation with normalized collision energies of 35. The maximum ion injection times for the survey and MS/MS scans were 60 ms, and the ion target values were set to 1e6 for the survey and MS/MS scans.

Protein identification from MS data. Proteome Discoverer software (version 2.2; Thermo Fisher Scientific) was used to generate peak lists. The MS/MS spectra were searched against a UniProt Knowledgebase (version 2017_10) using the SequestHT search engine. The precursor and fragment mass tolerances were set to 10 ppm and 0.6 Da, respectively. Methionine oxidation, protein amino-terminal acetylation, Asn/Gln deamidation, Ser/Thr/Tyr phosphorylation, diglycine modification of Lys side chains, and Cys carbamidomethyl modification were set as variable modifications for database searching. Peptide identification was filtered at a 1% false discovery rate.

RNA sequence data analysis of MDS cells. RNA seq data were obtained from accession number GSE156708. Data from MDS-Cas9 and MDSL-Cas9-FBXO11KO samples were reanalyzed using TCC-GUI with default parameters (number of iteration = 3, FDR cut-off = 0.1, elimination of potential DEGs = 0.05) using DESeq2 estimation (2, 3).

RNA sequence data analysis and survival analysis of breast cancer patients. The RNA-seq data for the gene expression in the biopsy samples and survival data of breast invasive carcinoma (BRCA) gene expression RNA-seq table of biopsy and patient survival data was accessed through UNSC XENA (https://xenabrowser.net/datapages/). RNA-seq log2(x+1) transformed RSEM normalized count was z-score normalized to analyze whether each gene relates with patients' survival (4). For each gene, patients with z-score > 0 were defined as "high" group and those with z-score < 0 were defined as "low" group. Patients whose overall survival time were more than 3650 days were removed from survival analysis. To show synergistic effect of *FBXO11* and *CIITA* on breast invasive carcinoma patients' survival, we defined patients whose z-score of *FBXO11* < 0 and *CIITA* > 0 as "*FBXO11* low *CIITA* high" group. Similarly, we defined patients whose z-score of *FBXO11* > 0 and *CIITA* < 0 as "*FBXO11* high *CIITA* low" group. Overall survival outcomes were visualized in the Kaplan–Meier curve by the survminer package in R. P values were determined by the log-rank test.

Correlation analysis. RNA-seq log2(x+1) transformed RSEM normalized count was used to study the correlation of gene expression between *FBXO11* and *CIITA*, or MHC class II. Pearson correlation coefficient was used to assess the correlation between the expression of *FBXO11* and MHC class II genes in BRCA biopsy samples. Null hypothesis was rejected by p value of < 0.05.

Statistics. All statistical analyses were performed by a two-tailed unpaired t-test, except for the analysis for RNA-seq datasets. The p-values were indicated as *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant using data from three independent experiments. The error bars represent mean values ± SD.

Data availability. The mass spectrometric datasets were available in ProteomeXchange under the accession number PXD037963 via the jPOST repository (5).

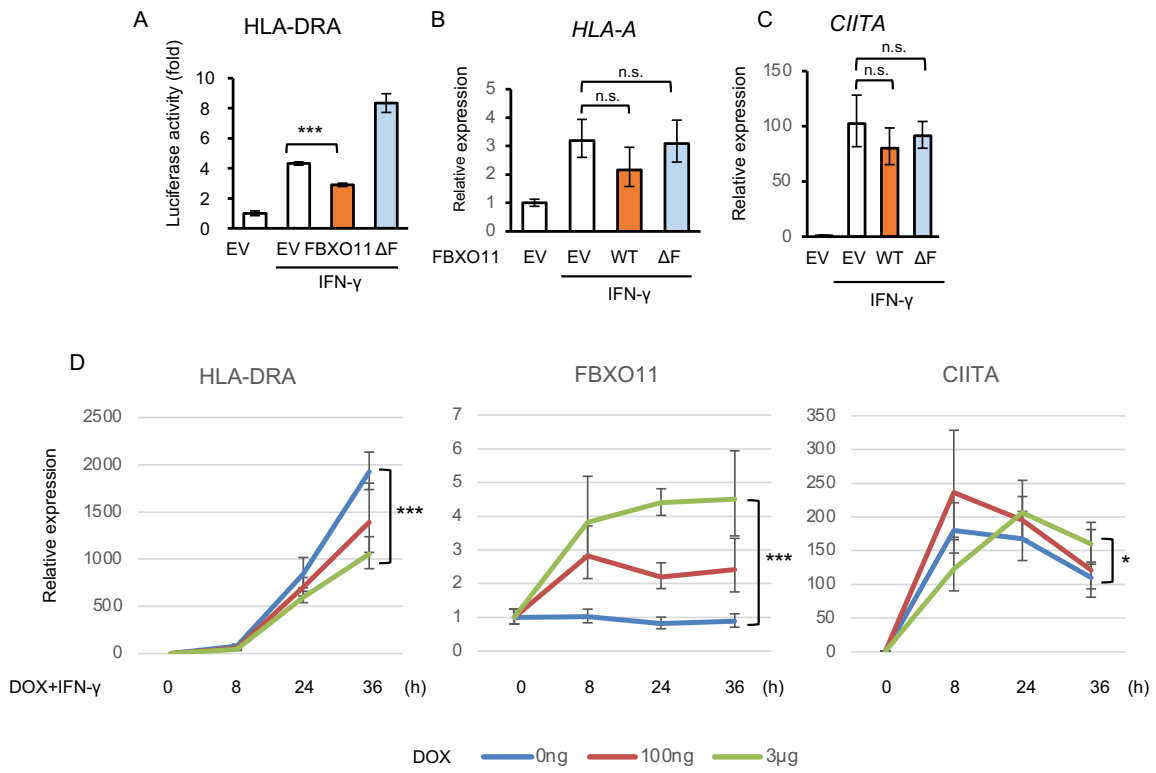


Fig. S1. FBXO11 suppresses the MHC-II transactivation ability of CIITA. (A) HeLa cells were transfected with the indicated FBXO11 construct and 100 ng of the HLA-DRA luciferase reporter construct. Twenty four hours after transfection, cells were stimulated with IFN-γ (500 U/mL) for 18 h and dual luciferase assay was performed. (B, C) Quantitative real-time PCR analysis of *HLA-A* (B) and *CIITA* (C) gene expression in HeLa cells transfected with the indicated FBXO11 expression vectors treated with IFN-γ (500 U/ml) for 18 h. (D) HeLa tet-ON FLAG-FBXO11 cells were treated simultaneously with IFN-γ (500U/ml) and doxycycline (0ng, 100ng, 3μg) then harvested at 0, 8, 24 and 36h after the treatment for quantitative real-time PCR analysis of the indicated genes.

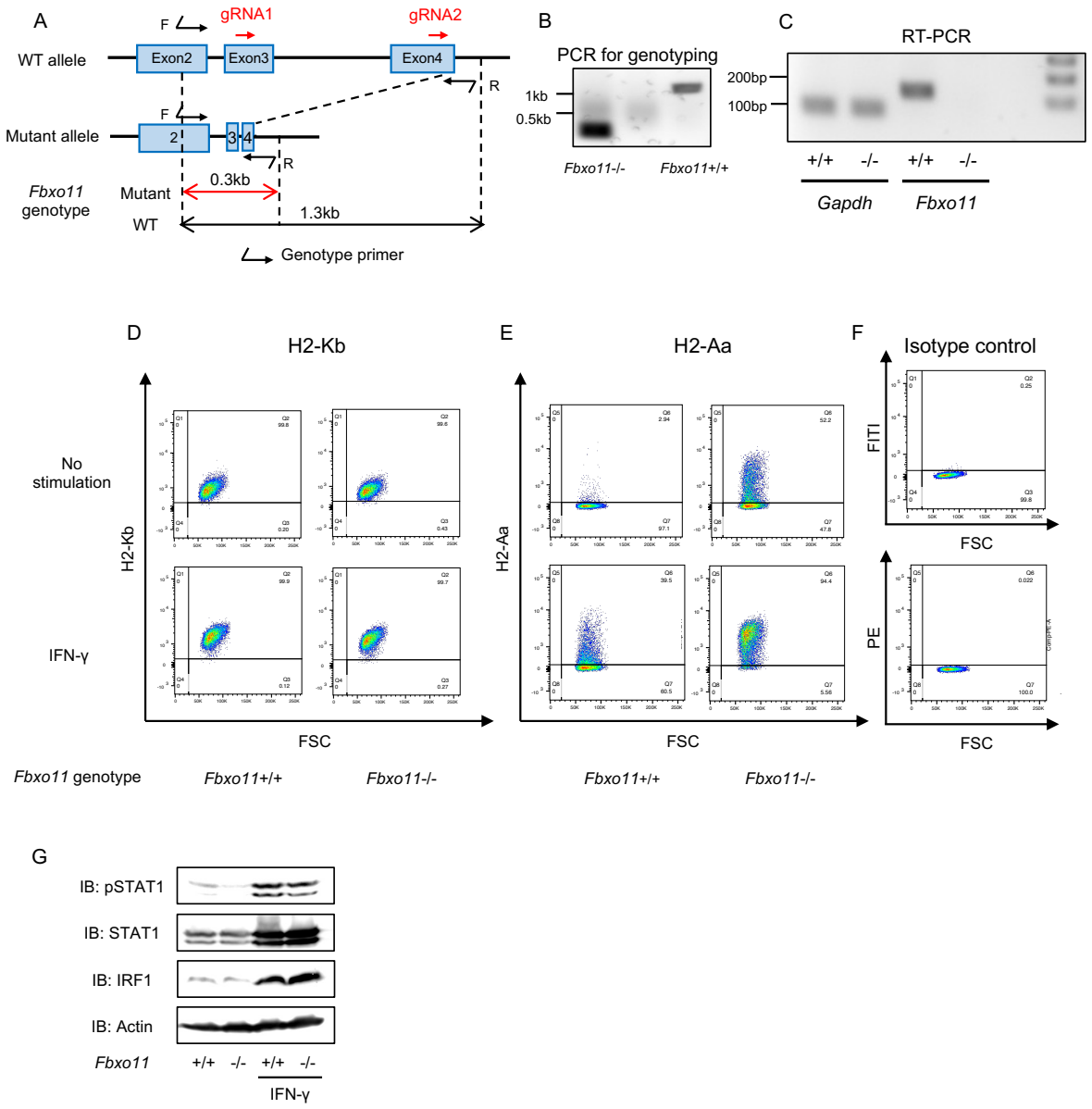


Fig. S2. Generation of *Fbxo11*-deficient RAW264.7 cells. (A) Schematic presentation for the *Fbxo11* knockout strategy. The gRNAs were designed on Exon3 and Exon4 (red arrows), deleting approximately 1.0 kb. The black arrows indicate the primers used for genotyping. Approximately 1.3 kb of DNA is amplified in wild type and 0.3kb in mutant allele. (B) Genotyping the *Fbxo11* region in *Fbxo11*+/+ and *Fbxo11*-/- RAW264.7 cells by PCR (C) The absence of *Fbxo11* transcript in *Fbxo11*-/- RAW264.7 cells was confirmed by RT-PCR. The expression of Gapdh was used as internal controls. (D-F) Surface expression of MHC-I and II in *Fbxo11*+/+ and *Fbxo11*-/- RAW264.7 cells were examined by flow cytometry. (D) Surface expression of H2-Kb. (E) Surface expression of H2-Aa. (F) Isotype control. (G) Wild-type and *Fbxo11*-deficient RAW264.7 cells were treated with or without IFN-γ (100 U/ml) for 18h. Cells were lysed and followed by western blotting analysis with the indicated antibodies.

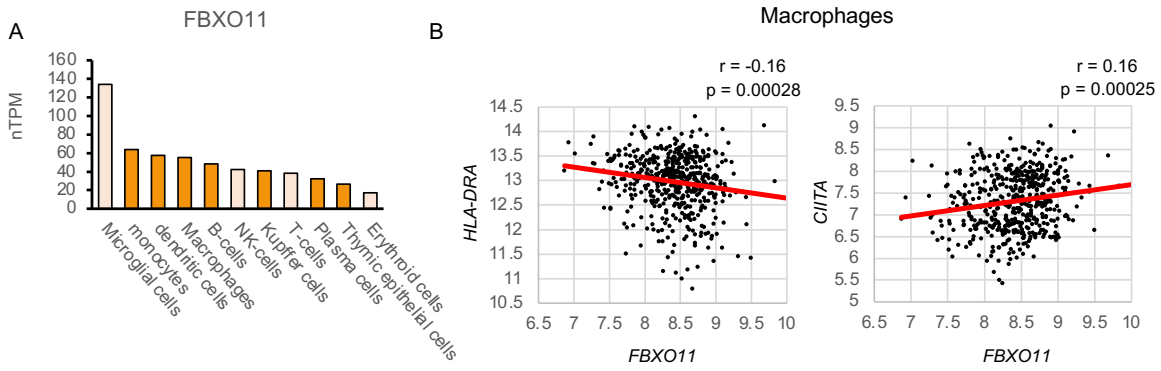
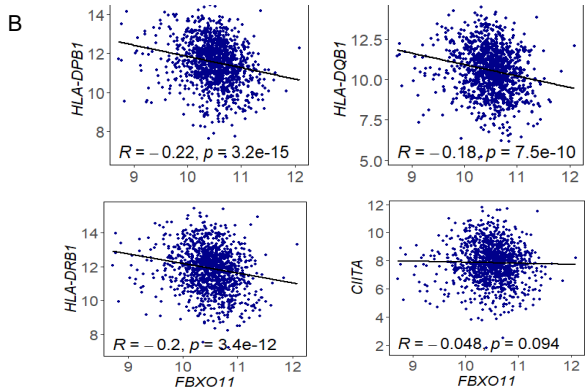


Fig. S3. The expression of FBXO11 is inversely associated with the expression of MHC-II genes, but not that of *CIITA* in immune cells. (A) Normalized TPM (nTPM) showing FBXO11 expression in immune cells. Data were downloaded from Human protein atlas RNA single cell type data (https://www.proteinatlas.org/about/download) and analyzed for immune cells. (B) Scatter plots for the expression of *FBXO11* and *HLA-DRA* or *CIITA* in human macrophages.

A

Positive receptor	number
HER2+	95
ER+	854
PR+	740

Clinical stage	number
Stage I	189
Stage II	668
Stage III	270
Stage IV	21



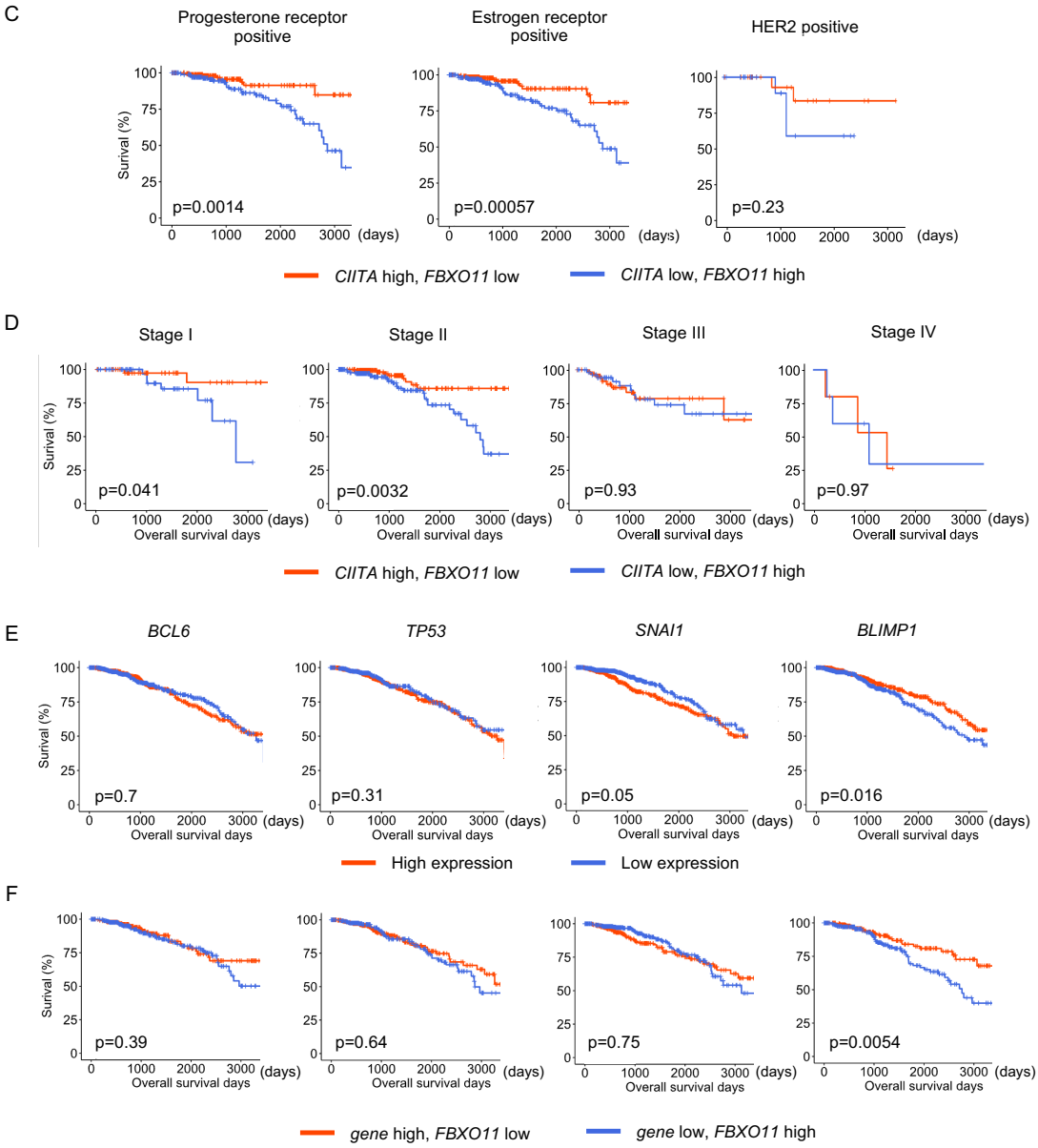


Fig. S4. The expression of FBXO11 is inversely associated with the expression of MHC-II genes, but not that of *CIITA* in breast cancer patients. (A) The number of each receptor positive and clinical

stage in the cohort of breast cancer patients used in the analysis in Figure 5 and Figure S3. (B) The expression of *FBXO11* and *CIITA*, or MHC class II in RNA-seq log2(x+1) transformed RSEM normalized count in the biopsy samples of breast invasive carcinoma (BRCA) was shown in scatter plots. The Pearson correlation coefficient (*R*) and the p value were shown. BRCA biopsy samples from 1215 patients obtained from UNSC XENA were used for data analysis. (C) Kaplan-Meier survival curves of progesterone receptor, estrogen receptor or Her2 positive breast cancer patients with low and high expression of *FBXO11* in combination with *CIITA* expression. For each gene, patients with z-score > 0 were defined as “high” group and those with z-score < 0 were defined as “low” group. (D) Kaplan-Meier survival curves of breast cancer patients at each clinical stage with low and high expression of *FBXO11* in combination with *CIITA* expression. (E) Kaplan–Meier survival curves of breast cancer patients with low and high expression of the indicated genes. (F) Kaplan-Meier survival curves of breast cancer patients with low and high expression of *FBXO11* in combination with the expression of indicated genes. For each gene, patients with z-score > 0 were defined as “high” group and those with z-score < 0 were defined as “low” group.

Supplementary Table S1. Peptide spectrum matches of CIITA specific binding protein.

Rank	Accession	Gene name	MOCK	CIITA	NLRC5	NOD2
1	P33076	CIITA		332		
2	Q96T76	MMS19		44	2	7
3	Q9NVI7	ATAD3A		33	8	7
4	Q86XK2	FBXO11		31		
5	P51531	SMARCA2		29		
6	P34931	HSPA1L	11	25	58	15
7	Q5T9A4	ATAD3B		25	4	5
8	Q13616	CUL1		17	1	
⋮	⋮	⋮	⋮	⋮	⋮	⋮
20	P63208	SKP1		7	2	4

Supplementary Table S2. DNA oligos used for cloning.

primer name	sequence (5' to 3')	use	note
FBXO11-F	ATAGTCGACATGGTTGCAGAAGAATCAG	Cloning for human FBXO11	Using SalI and NotI
FBXO11-R	AGCGGCGCCTCAGTTGTGCTGCAATG		
FBXO11-ΔFbox-F	GAAGTATTTGAATATACTCGCCCTATG	FBXO11 F-box deletion	Inverse PCR
FBXO11-ΔFbox-R	TTCAGCAGGTGCTGCTGA		
pcGN-CIITA-F	TTTCTAGAATGCGTTGCCTGGCTCCACGCCCT	Cloning for CIITA expression vector	Using XbaI and SalI
pcGN-CIITA-R	AATAATGTCGACGCGTCATCTCAGGCTGATCCGTGAAT		
pcGN-NLRC5-F	TTTCTAGAATGGACCCCGTTGGCCT	Cloning for NLRC5 expression vector	Using XbaI and SalI
pcGN-NLRC5-R	GGGGTCGACTCAAGTACCCCAAGG		
pcGN-NOD2-F	AATGCGGCCGCACCATGTGCTCGCAGGAGGCT	Cloning for NOD2 expression vector	Using NotI and SalI
pcGN-NOD2-R	AATAATGTCGACGCGTCAAAGCAAGAGTCTGGTGTC		
pQCXIP-CIITA-F	AATGAATTCACCATGCGTTGCCTGGCTCCACGCCCT	Cloning for CIITA retrovirus vector	Using EcoRI and XhoI
pQCXIP-CIITA-R	CCCCTCGAGTCATCTCAGGCTGATCCGTGAAT		
pQCXIP-NLRC5-F	AATGCGGCCGCACCATGGACCCCGTTGGCCTCC	Cloning for NLRC5 retrovirus vector	Using NotI and XhoI
pQCXIP-NLRC5-R	CCCCTCGAGTCAAGTACCCCAAGGGGCCT		
pQCXIP-NOD2-F	AATGCGGCCGCACCATGTGCTCGCAGGAGGCTT	Cloning for NOD2 retrovirus vector	Using NotI and XhoI
pQCXIP-NOD2-R	CCCCTCGAGTCAAAGCAAGAGTCTGGTGTC		
CIITA-ΔP/S/T-F	GCAGCTGGAGAGGTCTCC	CIITA P/S/T deletion	Inverse PCR
CIITA-ΔP/S/T-R	CTCAGCTGGCTTCCAGTG		
CIITA-ΔNACHT-F	AGTGGCGAAATCAAGGACAAGG	CIITA NACHT deletion	Inverse PCR
CIITA-ΔNACHT-R	TGTCTCACGCGGCC		
CIITA-Δ725-984-F	AAGAAACTGGAGTTTGCGCTGG	CIITA 725-984aa deletion	Inverse PCR
CIITA-Δ725-984-R	CAGAGCCAGCCACAGG		
CIITA-ΔLRR-F	TATCTAGAATGCGTTGCCT	CIITA LRR deletion	Using XbaI and SalI
CIITA-ΔLRR-R	ATAGAATTCTCATCTCAGGCTGAT		

Supplementary Table S3. DNA oligos used for qPCR analysis.

primer name	sequence (5' to 3')	use	note
HLA-DRA-F	GCCAACCTGGAAATCATGACA	qPCR primers for human gene analysis	SYBR greeen system
HLA-DRA-R	AGGGCTGTTCGTGAGCACA		
HLA-A-F	AAAAGGAGGGAGTTACACTCAGG		
HLA-A-R	GCTGTGAGGGACACATCAGAG		
CIITA -F	ACAGAGCACCAAGACAGAGC		
CIITA-R	CTCTGAGAGCTGGCACACTG		
mH2-Aa-F	CAACCGTGACTATTCCTTCC	qPCR primers for mouse gene analysis	
mH2-Aa-R	CCACAGTCTCTGTCAGCTC		
mH2-Kb-F	GTGATCTCTGGCTGTGAAGT		
mH2-Kb-R	GTCTCCACAAGCTCCATGTC		
mFBXO11-F	CGTGCAAGAGTGTCTGGAAA		
mFBXO11-R	ACACGCAAGCAGCTCTACAA		

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