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

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Major histocompatibility complex (MHC) class I and II molecules play critical roles in the activation and regulation of adaptive immunity through antigen presentation to CD8+ and CD4+ T cells, respectively. Strict regulation of MHC expression is critical for proper immune responses. CIITA (MHC class II transactivator), an NLR (nucleotide-binding domain, leucine-rich-repeat containing) protein, is a master regulator of MHC class II (MHC-II) gene transcription. Although it has been known that CIITA activity is regulated at the transcriptional and protein levels, the mechanism to determine CIITA protein level has not been elucidated. Here, we show that FBXO11 is a bona fide E3 ligase of CIITA and regulates CIITA protein level through ubiquitination-mediated degradation. A nonbiased proteomic approach for CIITA-binding protein identified FBXO11, a member of the Skp1–Cullin-1–F-box E3 ligase complex, as a binding partner of CIITA but not MHC class I transactivator, NLRC5. The cycloheximide chase assay showed that the half-life of CIITA is mainly regulated by FBXO11 via the ubiquitin–proteasome system. The expression of FBXO11 led to the reduced MHC-II at the promoter activity level, transcriptional level, and surface expression level through downregulation of CIITA. Moreover, human and mouse *FBXO11*–deficient cells display increased levels of MHC-II and related genes. In normal and cancer tissues, *FBXO11* expression level is negatively correlated with MHC-II. Interestingly, the expression of *FBXO11*, along with *CIITA*, is associated with prognosis of cancer patients. Therefore, FBXO11 is a critical regulator to determine the level of MHC-II, and its expression may serve as a biomarker for cancer.

FBXO11 | MHC-II | CIITA | ubiquitination | NLRC5

Significance

MHC class II (MHC-II) molecules are essential for both humoral and cellular immunity as they are required for CD4+ T cell activation through antigen presentation. The expression of MHC-II requires the activity of a master regulator, CIITA. Although posttranslational regulation is critical for CIITA activity, the regulation of CIITA protein has been poorly understood. Here, we report that an E3 ligase, FBXO11, ubiquitinates and degrades CIITA through the ubiquitin–proteasome system, thereby suppressing MHC-II expression. FBXO11 expression levels were negatively correlated with MHC-II expression in both normal and cancer cells. Our findings elucidate molecular mechanism that regulates CIITA at the protein level and determine MHC-II expression level, which may provide a therapeutic target for inflammatory diseases and cancer.

Major histocompatibility complex class II (MHC-II) molecules are essential for antigen presentation to and activation of CD4+ T cells, thus play critical role in both humoral and cellular immunity. Unlike MHC-I which is expressed in any cell types, MHC-II molecules are expressed in limited cell types where they perform antigen presentation (HLA-DP, -DQ, -DR in human or H2-A, -E in mice) or regulate peptide loading (HLA-DM, -DO in human or H2-M, -O in mice). The expression of MHC-II is tightly regulated and its constitutive expression is restricted in classical antigen-presenting cells such as dendritic cells, macrophages, and B cells, as well as thymic epithelial cells (1). Most nonimmune cells do not express MHC-II, but induced expression is widely observed in various cell types, predominantly upon stimulation with interferon (IFN)-γ (2). MHC-II expression can be observed in solid tumors which do not normally express MHC-II (3). Although the exact role of aberrant MHC-II in such cancers is not clear, it has been reported that therapeutic efficacy of checkpoint blockade immunotherapy is positively correlated with MHC-II expression levels in those cancers (4, 5).

MHC-II expression is regulated at the transcriptional level, and a master transactivator, CIITA, is responsible for both cell type–specific and inducible expression (6, 7). CIITA is a protein composed of 1,130 amino acids and contains an acidic activation domain (AAD) important for transcriptional activity at the N terminus; P/S/T regions rich in proline, serine, and threonine; nucleotide-binding domain (also known as NACHT); and C-terminal leucine-rich repeats (LRRs) (8). Three promoters generate three isoforms, namely isoforms I, II, and III of CIITA with different lengths of N terminus (9–11). Although CIITA does not possess a DNA-binding domain, CIITA associates with multiple transcription factors and chromatin-remodeling coactivators, including Regulatory Factorbinding to the X-box (RFX) protein complex, NF-Y complex, p300/CBP, and P300/CBP-associated factor (PCAF), to generate active protein/DNA complex called MHC enhanceosome on the proximal promoter of MHC-II (12). The activity of CIITA is regulated by various posttranslational modifications such as ubiquitination, phosphorylation, and acetylation. Phosphorylation is important for transcriptional activity, nuclear localization, multimerization, and protein–protein interactions, while acetylation is

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The authors declare no competing interest.

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important for nuclear importation of CIITA (13–16). Monoubiquitination of CIITA increases transcriptional activity, whereas polyubiquitination plays a role in nuclear translocation and degradation of CIITA (17, 18). Proteins with an AAD generally turn over quickly, and the half-life of CIITA is likewise short, approximately 30 min (19). The transcriptional activation domain often closely overlaps with protein-destabilizing sequence motifs called degrons, and in the case of CIITA, regions within the P/S/T domain, AAD and the first 10 amino acids of extreme N terminus of isoform III are known to mediate degradation (19–21). However, the degradation mechanism of CIITA has been poorly understood, since the ubiquitinating enzyme that leads to degradation of CIITA is unknown.

The ubiquitin–proteasome system regulates the degradation of target proteins and plays an essential role in a variety of cellular processes. Ubiquitination is catalyzed by three enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E3 ligase determines substrates by associating with target proteins (22). The Skp1–Cullin-1–F-box (SCF) E3 ligase complex is the largest family of E3 ligases and consists of CUL1, SKP1, and F-box proteins (23). FBXO11 is a member of the F-box family with a length of 927 amino acids (24). The structure of FBXO11 contains a proline-rich domain and an F-box domain at the N-terminal end, which are essential for binding to the SKP1 protein in the SCF-complex (24). It has also three carbohydrate-binding proteins and sugar hydrolase domain in the middle, and a zinc finger-like domain called the ubiquitin–protein ligase E3 component N-recognin (UBR) domain at the C terminus (24, 25). Multiple proteins have been reported as substrates for the E3 ligase activity of FBXO11, including BCL6 (26), CDT2 (27, 28), p53 (24), SNAIL (29, 30), BAHD1 (31), CDC25A (32), and BLIMP-1 (33).

In this study, we identified FBXO11 as a specific CIITA-binding protein through unbiased proteomic screening. FBXO11 is a bona fide E3 ligase for CIITA and mediates its ubiquitination and proteasome-dependent degradation. We found that FBXO11-dependent mechanism represents major posttranslational regulation of CIITA, and indeed FBXO11 expression level is negatively correlated with MHC-II. Interestingly, the combination of expression levels of FBXO11 and CIITA transcripts is associated with MHC-II expression and prognosis in breast cancer patients, suggesting FBXO11 as a biomarker for cancers.

Results

FBXO11 Binds Specifically to CIITA through P/S/T Domain. To identify CIITA-specific binding proteins, we established HeLa cells that stably express FLAG-tagged CIITA. For comparison, cells stably expressing other NLR protein family, NLRC5 and NOD2, were also generated simultaneously. Whole-cell extracts were immunopurified with anti-FLAG antibody beads, and the purified CIITA, NLRC5, and NOD2 complexes were analyzed by mass spectrometry (Fig. 1*A*). Mass spectrometry analysis identified specific binding proteins for each protein. Peptide spectrum matches (PSMs) counts of FLAG-CIITA, -NLRC5, and -NOD2 were compared, and proteins that showed more than twofold PSM counts above the minimum PSM counts were included as specific binding proteins (Fig. 1*B* and *SI Appendix*, Table S1). The samples used for mass spectrometry analysis were also silver-stained to confirm the presence of proteins that are presumed to be specific binding proteins (Fig. 1*C*). The bait-derived peptides were most abundant in the respective stably expressing cells, indicating the validity of the analysis. Among the identified binding proteins, FBXO11 was identified only as a CIITA-binding protein, and likewise, CUL1, which forms an SCF-complex with F-box proteins, was found to bind specifically to CIITA (34). Peptides derived from SKP1 were most abundant, although not specific, in CIITA-expressing cells compared to NLRC5- and NOD2-expressing cells (*SI Appendix*, Table S1). In order to verify the specific binding between CIITA and FBXO11, HA-FBXO11 and FLAG-tagged CIITA or NLRC5 were overexpressed in HEK293T cells. Immunoprecipitation with anti-FLAG antibody showed that FBXO11 coprecipitated with CIITA but not with NLRC5 (Fig. 1*D*), confirming the specific association between FBXO11 and CIITA. To determine a binding domain of CIITA recognized by FBXO11, various expression vectors for domain deletion mutants of CIITA were generated. Immunoprecipitation of each FLAG-CIITA mutant with HA-FBXO11 revealed that P/S/T domain of CIITA is required for binding to FBXO11 (Fig. 1*E*).

FBXO11 Suppresses CIITA-Dependent MHC-II Transactivation. To determine whether FBXO11 affects MHC-II transcription, we performed a dual luciferase assay. Cotransfection of FBXO11 with CIITA expression vector into HEK293T cells inhibited CIITA-mediated activation of MHC-II promoter in a dose-dependent manner for both *HLA-DRA* and *HLA-DPA* (Fig. 2*A*). However, FBXO11 did not affect MHC-I promoter activation induced by NLRC5 (Fig. 2*B*). We further examined whether the endogenous CIITA is also suppressed for their transactivation activity by FBXO11. As in the case of exogenous overexpression, FBXO11 suppressed IFN-γ-induced MHC-II promoter activity, but not MHC-I promoter activity in a dose-dependent manner (Fig. 2*C*). It has been shown that the F-box in FBXO11 is critical for its E3 ligase activity since the F-box is needed for binding to SKP1 and forming the SCF-complex (28, 35). By generating the F-box deletion mutant (ΔF) of FBXO11, we examined whether the F-box is required for the suppression of MHC-II transactivation activity. Coexpression of CIITA and either FBXO11 wild-type (WT) or ΔF mutant into HEK293T cells revealed that the ΔF mutant did not suppress the CIITA-mediated *HLA-DRA* promoter activity (Fig. 2*D*). Additionally, ΔF mutant FBXO11 did not inhibit *HLA-DRA* promoter activity induced by interferon-γ (IFN-γ) treatment, indicating that the F-box in FBXO11 is critical for inhibition of both endogenous and exogenous CIITA (*SI Appendix*, Fig. S1*A*). To determine whether FBXO11 affects CIITA protein levels, western blotting was performed using HEK293T transfected with FBXO11 together with CIITA or NLRC5 expression vectors. The expression of WT FBXO11, but not ΔF mutant, reduced CIITA protein levels, while FBXO11 did not suppress NLRC5 protein levels, indicating the specific suppression of CIITA protein by FBXO11 (Fig. 2*E*). Similarly, CIITA-dependent *HLA-DRA* expression induced by IFN-γ treatment was suppressed by overexpression of WT FBXO11 but not ΔF mutant, while FBXO11 overexpression did not display any effect on the expression of *HLA-A* or *CIITA* (Fig. 2*F* and *SI Appendix*, Fig. S1 *B* and *C*). This observation was further supported by western blotting demonstrating the reduced endogenous CIITA protein by WT FBXO11 under the treatment of IFN-γ, confirming that CIITA protein level is decreased by FBXO11 expression under more physiological conditions (Fig. 2*G*). To further confirm the negative regulation of MHC-II by FBXO11, we established a tetracycline-inducible FBXO11 system in HeLa cells, in which FBXO11 expression can be induced by the addition of doxycycline, while CIITA can still be induced by IFN-γ stimulation. Treatment with doxycycline induced the expression of *FBXO11* in a dose-dependent manner, accompanied by a reduction of IFN-γ-dependent *HLA-DRA* expression, further supporting the regulatory role of FBXO11 in MHC-II gene expression (*SI Appendix*, Fig. S1*D*). Since MHC-II functions

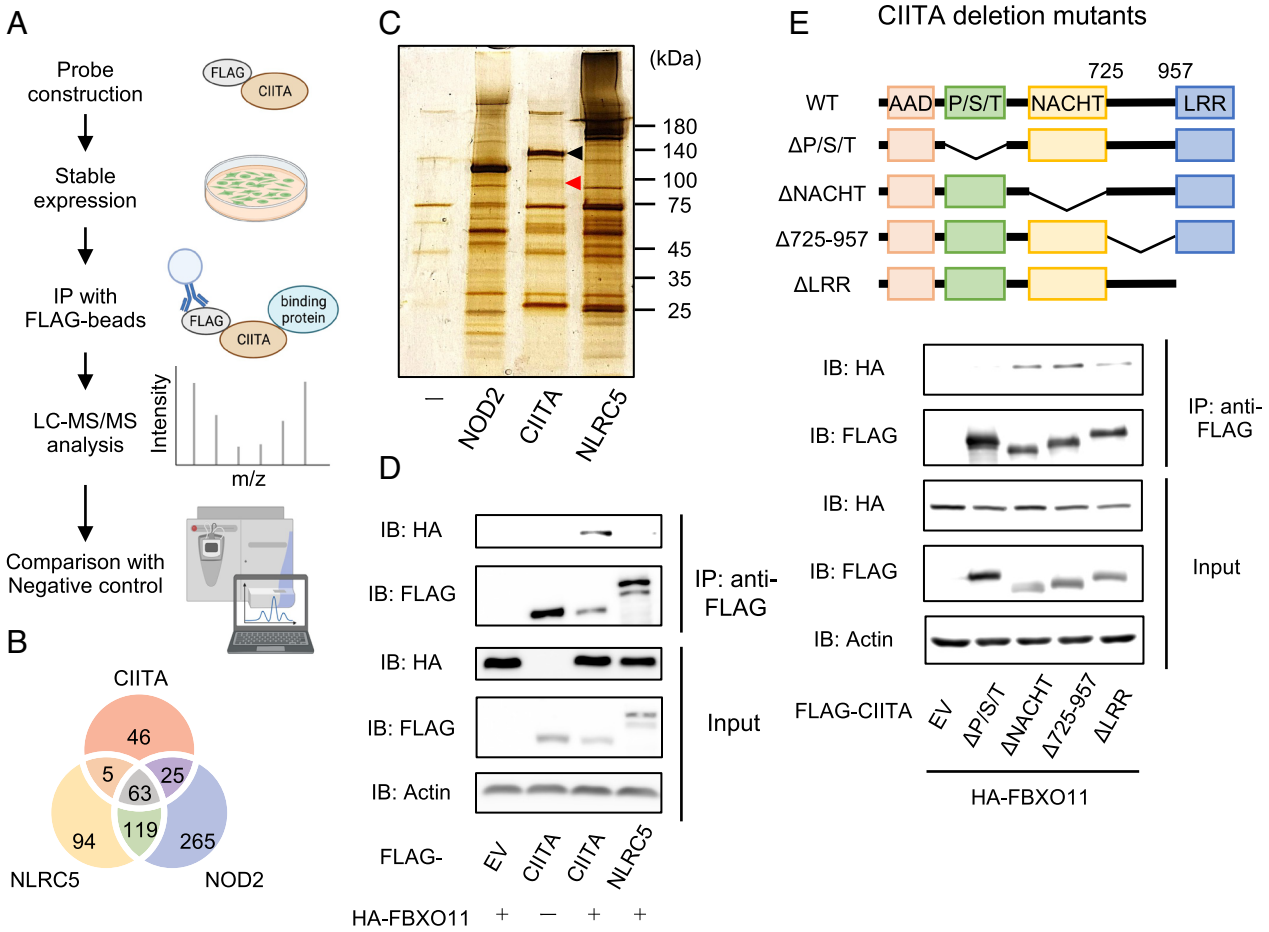

Fig. 1. Identification of FBXO11 as a CIITA-binding protein (*A*) A workflow to identify CIITA-binding proteins. (*B*) Venn diagram showing the number of proteins identified as CIITA-, NLRC5-, and NOD2-binding proteins. (*C*) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and silver staining of total cell protein extracts from WT and FLAG-NOD2, -CIITA, or -NLRC5 stably expressing HeLa cells after immunoprecipitation with FLAG antibody for the mass spectrometry analysis. A black arrow indicates FLAG-CIITA, and a red arrow indicates presumed FBXO11. (*D*) HEK293T cells were transfected with plasmids expressing FLAG-CIITA or -NLRC5, along with HA alone or HA-FBXO11 as indicated. After 48 h incubation, cell lysates were immunoprecipitated with anti-FLAG M2 beads and analyzed by western blotting with indicated antibodies. (*E*) Schematic representation of deletion mutants of CIITA and immunoblot after immunoprecipitation. HEK293T cells were transfected with plasmids expressing FLAG-CIITA WT, ΔP/S/T, ΔNACHT, Δ725-957, or ΔLRR, along with HA-FBXO11. Cells were harvested after 48 h incubation and immunoprecipitated with anti-FLAG M2 beads, followed by western blot analysis.

primarily in antigen-presenting cells, we examined whether FBXO11 could also negatively regulate MHC-II expression in immune cells. Mouse macrophage–derived RAW264.7 was used in the experiments given its relatively high transfection efficiency (36, 37). The overexpression of FBXO11 suppressed the protein level of MHC-II, but not MHC-I, as confirmed in nonimmune cells, and the inhibition was not conferred by the catalytically inactive ΔF mutant (Fig. 2*H*).

FBXO11 Deficiency Induced the Upregulation of the MHC-II, but Not MHC-I, Gene Expression. To further examine the effect of FBXO11 on MHC-II expression, we generated *Fbxo11*-deficient RAW264.7 cells using the CRISPR/Cas9 system (*SI Appendix*, Fig. S2 *A–C*) (38). The catalytically critical exons 3 and 4 were largely deleted (*SI Appendix*, Fig. S2*A*) (30, 39). The genotype of *Fbxo11*−/− cells was confirmed by PCR, and the absence of the *Fbxo11* transcript was confirmed by RT-PCR (*SI Appendix*, Fig. S2 *B* and *C*). MHC-II mRNA expression was elevated both in the absence and presence of IFN-γ in *Fbxo11*-deficient cells, while no change was observed for MHC-I expression (Fig. 3*A*). Similarly, the surface expression of MHC-II, but not MHC-I, was up-regulated in *Fbxo11*-deficient cells (Fig. 3*B* and *SI Appendix*, Fig. S2 *D–F*). In order to examine whether FBXO11 may have any impact on IFN-γ signaling, we assessed protein expression levels of STAT1 and IRF1. We found no obvious difference in the protein levels of these transcription factors between *Fbxo11*+/+ and *Fbxo11*−/− cells in the presence or absence of IFN-γ, indicating that regulatory roles of FBXO11 in CIITA/MHC-II levels are unlikely due to altered IFN-γ signaling pathway (*SI Appendix*, Fig. S2*G*). In order to rescue the phenotype of *Fbxo11*-deficient cells, the expression vectors for human FBXO11 were transfected. It is highly likely that human and mouse FBXO11 genes behave similarly because they are highly conserved, differing only by two amino acids when comparing isoform1 of human FBXO11 to isoform2 of mouse Fbxo11. Overexpression of human FBXO11 in *Fbxo11*-deficient RAW264.7 cells resulted in MHC-II surface expression at similar levels to WT, but introduction of the ΔF mutant failed to rescue the phenotype (Fig. 3*C*). To comprehensively address transcriptional alterations in *FBXO11*-deficient cells, we reanalyzed the published RNA-seq data of *FBXO11* WT and deficient MDS-L cells which were originally established from a myelodysplastic syndrome patient (40). The volcano plot for the gene expression showed that *FBXO11* deficiency was associated with the elevation of MHC-II gene expression, but did not alter the expression of its master regulator, *CIITA* (Fig. 3*D*). This indicates that the changes in MHC-II expression by *FBXO11* occur after the *CIITA* transcription, consistent with our observation that FBXO11 reduces CIITA at the protein levels.

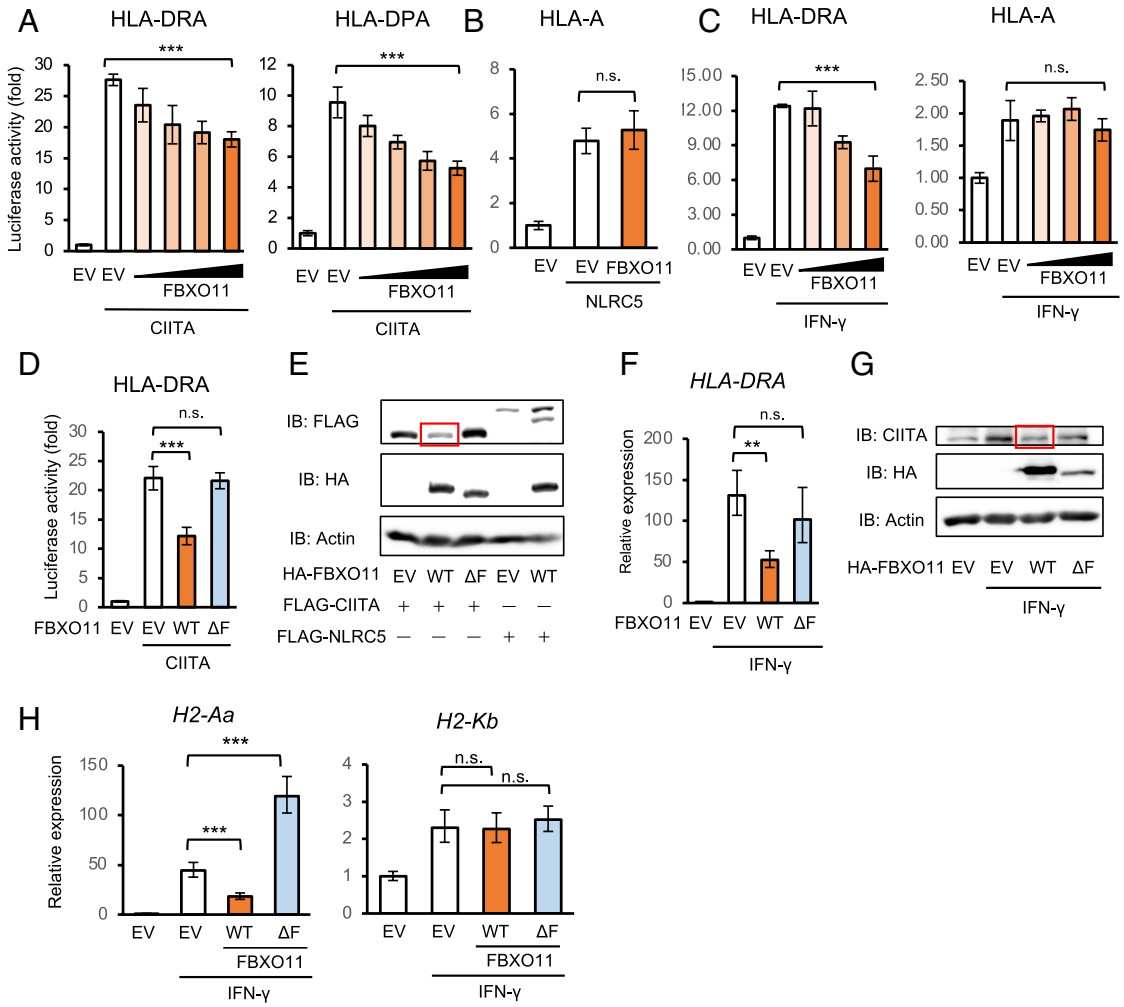

Fig. 2. FBXO11 suppresses the CIITA-dependent MHC-II promoter activity in a dose- and F-box-dependent manner. (*A*) HEK293T cells were cotransfected with plasmids expressing FLAG-CIITA, increasing amounts of HA-FBXO11, and 50 ng of either HLA-DRA or HLA-DPA luciferase reporter construct. Thirty-six hours after transfection, luciferase activity was measured. (*B*) HEK293T was cotransfected with 50 ng of FLAG-NLRC5 expression vector, 800 ng of HA-FBXO11 expression vector, and 50 ng of HLA-A luciferase construct. Thirty-six hours after transfection, luciferase activity was measured. (*C*) HeLa cells were transfected with increasing amounts of HA-FBXO11 expression vector and 100 ng of either HLA-DRA or HLA-A luciferase reporter construct. Twenty-four hours after transfection, cells were stimulated with IFN-γ (500 U/mL) for 18 h and luciferase activity was measured. (*D* and *E*) HEK293T cells were cotransfected with 50 ng of FLAG-CIITA expression vector and 800 ng of either HA-empty, FBXO11 WT, or FBXO11 ΔF mutant expression vectors. (*D*) HLA-DRA luciferase assay was performed after 36 h after transfection. (*E*) Western blotting with indicated antibodies. (*F* and *G*) HeLa cells were transfected with the indicated HA-FBXO11 expression constructs treated with IFN-γ (500 U/mL) for 18 h. (*F*) Quantitative real-time-PCR analysis of *HLA-DRA* gene expression. (*G*) Western blotting with indicated antibodies. (*H*) Quantitative real-time-PCR analysis of *H2-Aa* and *H2-Kb* gene expression in RAW264.7 cells transfected with indicated HA-FBXO11 construct stimulated with IFN-γ (100 U/mL) for 18 h.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.

A heat map for the expression of MHC and their transactivator genes showed that elevated expression levels were observed in various MHC-II genes, but not in MHC-I, *STAT1*, *NLRC5*, or *CIITA* (Fig. 3*E*). Collectively, these results indicate that FBXO11 specifically suppresses MHC-II expression without affecting the MHC-I pathway or the CIITA transcription.

FBXO11 Is the E3 Ligase Responsible for Ubiquitination and Degradation of CIITA. Since we found that FBXO11 suppressed the protein level of CIITA, we next attempted to determine whether the mechanism is due to ubiquitination of CIITA. HEK293T cells overexpressing FLAG-CIITA, HA-FBXO11, and His-Ubiquitin were treated with the proteasome inhibitor MG132, and ubiquitination was assessed by immunoprecipitation with anti-FLAG antibody under denaturing condition, followed by western blotting with anti-His antibody. Ubiquitination of CIITA was enhanced by cotransfection with FBXO11, but the ΔF mutant did not alter the ubiquitination status of CIITA (Fig. 4*A*). In order to examine under more physiological conditions, we performed a similar ubiquitination assay for endogenous ubiquitin. Again, the expression of FBXO11 increased ubiquitination of CIITA, but the ΔF mutant did not as much as WT FBXO11 (Fig. 4*B*). Notably, ubiquitinated CIITA was abolished in the absence of the proteasome inhibitor due to probable proteasomal degradation (Fig. 4*B*). Given these results, we concluded that FBXO11 is the putative E3 ligase responsible for the ubiquitination of CIITA. Next, to determine whether ubiquitination by FBXO11 leads to CIITA degradation, we performed a cycloheximide chase assay. As previously reported, the half-life of CIITA was very short (19), whereas overexpression of FBXO11 resulted in an even shorter half-life (Fig. 4*C*). Again, the ΔF mutant failed to shorten the half-life of CIITA. To further validate these results, we investigated whether the half-life of CIITA was affected in *Fbxo11*-deficient RAW264.7 cells. The half-life of CIITA was greatly prolonged and CIITA was hardly degraded in *Fbxo11*-deficient cells (Fig. 4*D*). Overexpression of FBXO11 in *Fbxo11*-deficient cells restored the

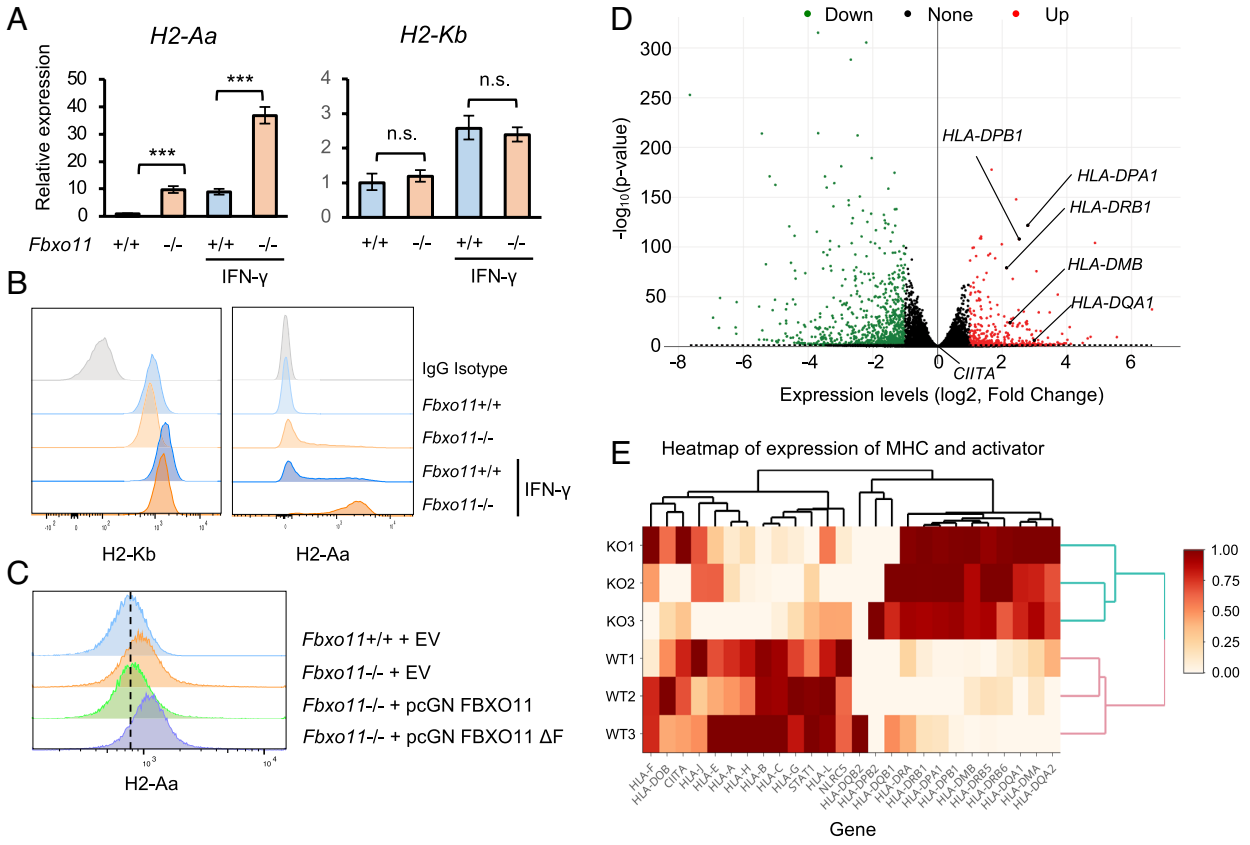

Fig. 3. Increased MHC-II expression in *FBXO11*-deficient cells. (*A* and *B*) WT and *Fbxo11*-deficient RAW264.7 cells were treated with or without IFN-γ (100 U/mL). (*A*) The expression of MHC class II (*H2-Aa*) and MHC class I (*H2-kb*) was assessed by quantitative real-time–PCR. (*B*) The surface expression of MHC class II and class I was measured by flow cytometry. (*C*) *Fbxo11*-deficient RAW264.7 cells were transfected with either HA-empty, FBXO11 WT, or FBXO11 ΔF mutant expression vectors. The surface expression of MHC class II was measured by flow cytometry and compared to *Fbxo11*+/+ cells. (*D*) Volcano plot of gene expression levels measured by RNA-seq in *FBXO11*-deficient MDS-L cells. The data were calculated in fold changes and compared to WT MDS-L cells. Increased expression levels of more than twofold are indicated by red dots and decreased expression levels are indicated by green dots. (*E*) Heatmap for the expression of MHC class I– and class II–related genes compared between *FBXO11*-deficient and WT MDS-L cells. 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.

short half-life of CIITA, but no recovery was observed with ΔF mutant (Fig. 4*D*). We further examined whether the degradation of CIITA related to overexpression of FBXO11 is mediated by the proteasomal pathway. The expression vectors for CIITA and FBXO11 were cotransfected into HEK293T, and the protein levels of CIITA were assessed in the presence or absence of MG132. Proteasome inhibition prevented the degradation of CIITA upon FBXO11 overexpression (Fig. 4*E*). Taken together, these results demonstrate that FBXO11 regulates CIITA protein level by promoting the ubiquitination and the proteasome-dependent degradation.

Expression of FBXO11 Is Negatively Correlated with MHC-II Expression in Cancers and Affect Cancer Patient Survival. We have shown that FBXO11 plays a role in ubiquitination and degradation of CIITA, negatively regulating the expression of MHC-II. FBXO11 is expressed in a variety of human tissues and was confirmed to be expressed in immune cells involved in antigen presentation (*SI Appendix*, Fig. S3*A*). To validate these findings in large human cohorts, we investigated the correlation between *FBXO11* and MHC-II or *CIITA* expression in human peripheral blood mononuclear cells (PBMCs) using the Immuno-Navigator database and found that the *FBXO11* expression was negatively correlated with MHC-II, but not with *CIITA* (Fig. 5*A*) (41). In addition, a similar negative correlation was observed in peripheral blood B cells and macrophages that constitutively express MHC-II (Fig. 5*B* and *SI Appendix*, Fig. S3*B*). We also examined the role of FBXO11 in MHC-II expression in cancer patients. The aberrant expression of MHC-II is observed in a wide variety of cancers including solid tumors where the original cell types do not display MHC-II and its high expression is frequently associated with favorable prognosis (4, 5, 42). In breast cancer, it was also shown that MHC-II expression is associated with a clinical outcome, and indeed, our own Kaplan–Meier analysis showed that breast cancer patients with high MHC-II expression displayed better survival than patients with low MHC-II (Fig. 5*C* and *SI Appendix*, Fig. S4*A*) (42, 43). The patients with high *CIITA* expression also had prolonged overall survival, although the relationship was not as strong as for MHC-II expression (Fig. 5*D*). We therefore hypothesized that in addition to *CIITA* transcript level, the expression of *FBXO11* might contribute to the expression of MHC-II in breast cancers and eventually to the survival of patients. The expression of *FBXO11* was negatively correlated with MHC-II, but not with *CIITA,* in breast cancer (*SI Appendix*, Fig. S4*B*). The patient cohort was categorized into four groups based on the expression levels of *CIITA* and *FBXO11* divided at the median for each gene: $CIITA_{high}$ $FBXO11_{low}$, $CIITA_{high}$ $FBXO11_{high}$, $CIITA_{low}$ $FBXO11_{low}$, and $CIITA_{low}$ $FBXO11_{high}$. We found the highest expression of MHC-II genes in $CIITA_{high}$ $FBXO11_{low}$ group and the lowest expression in $CIITA_{low}$ $FBXO11_{high}$ group, suggesting that high *CIITA* expression and low *FBXO11* level cooperatively induce MHC-II expression (Fig. 5*E*). We further performed Kaplan–Meier survival analysis in breast cancer patients based on the expression of *FBXO11*. While survival was slightly prolonged in patients with low *FBXO11* expression (Fig. 5 *F*, *Top*), $CIITA_{high}$ $FBXO11_{low}$ group

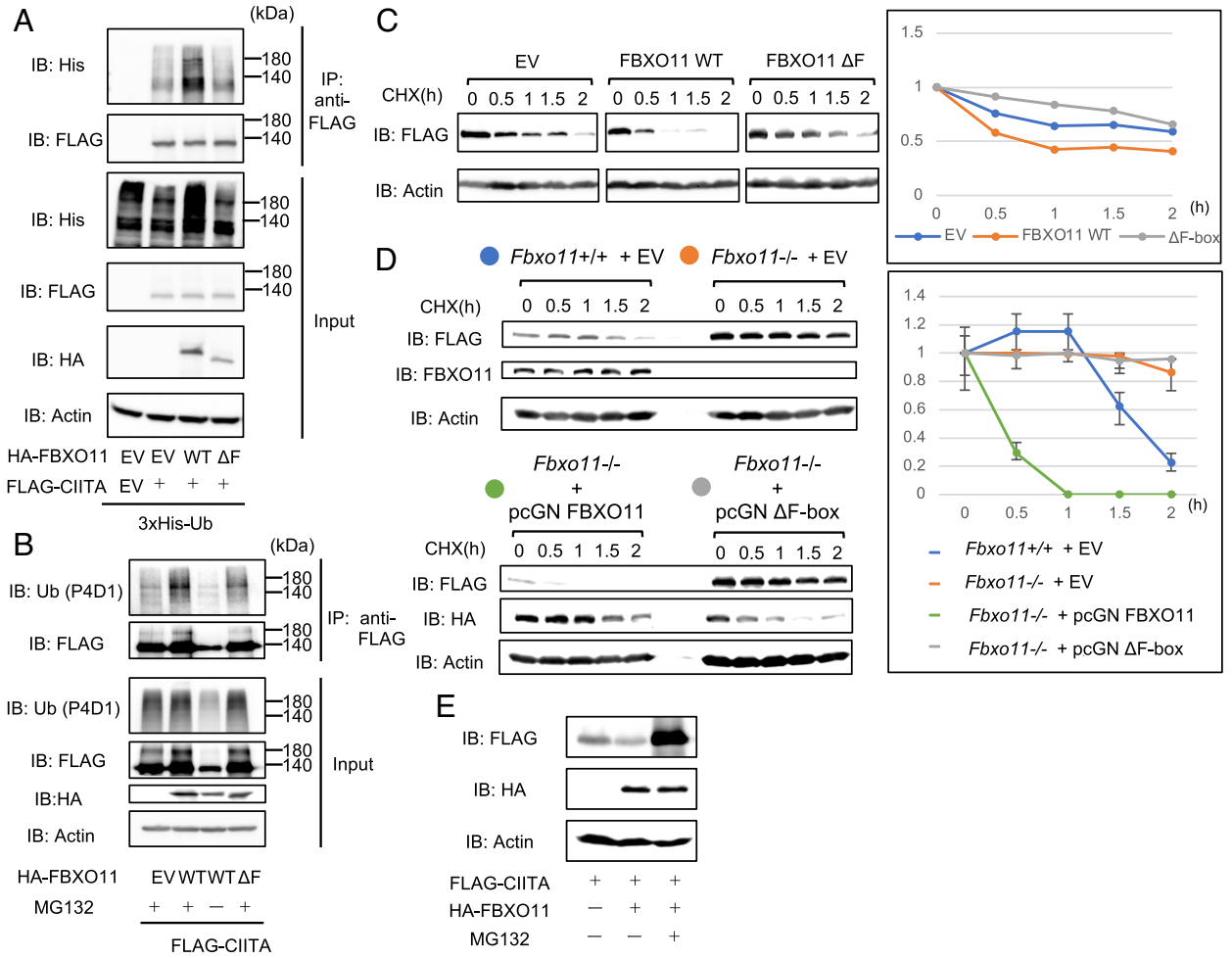

Fig. 4. FBXO11 mediates ubiquitination and degradation of CIITA. (*A*) HEK293T cells were transfected with the plasmids expressing FLAG-CIITA and His-Ubiquitin (His-Ub) along with either HA-empty, FBXO11 WT, or FBXO11 ΔF mutant expression vectors and treated with MG132 (20 μM) for 6 h before harvesting. Cell lysates were boiled in 1% SDS, denatured, and immunoprecipitated with anti-FLAG antibody. Western blotting was performed with indicated antibodies. (*B*) HEK293T cells were transfected with the expression vectors encoding FLAG-CIITA along with either HA-empty, FBXO11 WT, or FBXO11 ΔF mutant expression vectors and treated with or without MG132 (20 μM) for 6 h before harvesting. The cell lysates were boiled in 1% SDS, denatured, and immunoprecipitated with anti-FLAG antibody. Western blotting was performed with indicated antibodies. (*C*) HEK293 cells in 10 cm dishes were transfected with the plasmid expressing FLAG-CIITA along with either HA-empty, FBXO11 WT, or FBXO11 ΔF mutant expression vectors. After 6 h of transfection, the cells were divided into six-well dishes and incubated for another 18 h followed by cycloheximide (CHX) treatment at a concentration of 50 μg/m for indicated periods. The cells were then harvested and lysed for western blotting analysis. (*D*) WT or *Fbxo11*-deficient RAW264.7 cells in 10 cm dishes were transfected with the plasmid expressing FLAG-CIITA and treated with CHX as in Fig. 4*C*. *Fbxo11*-deficient RAW264.7 cells were further transfected with HA-FBXO11 WT or FBXO11 ΔF mutant expression vectors. After 6 h of transfection, the cells were divided into six-well dishes and incubated for another 18 h followed by cycloheximide (CHX) treatment at a concentration of 100 μg/m for indicated periods. The cells were then harvested and lysed for western blotting analysis. The graph illustrates the quantification over time of CIITA protein levels normalized to β-actin based on band intensities from the gel. The band intensity of the CIITA protein at 0 h of CHX treatment was defined as 100%. (*E*) HEK293T cells were transfected with plasmid expressing FLAG-CIITA and either HA-empty or FBXO11 expression vector and treated with DMSO or MG132 for 6 h before harvest. Cells were lysed and followed by western blotting analysis.

exhibited much better survival rate than $CIITA_{low}$ $FBXO11_{high}$ group (Fig. 5 *F*, *Bottom*), indicating that these gene expression levels collaborate for survival. This collaboration between FBXO11 and CIITA was also observed in breast cancers with HER2, progesterone receptor, and estrogen receptor–positive breast cancers, which are known clinical prognosis features of breast cancer, suggesting that CIITA and FBXO11 affect prognosis independently of these receptor expression status (*SI Appendix*, Fig. S4*C*). Interestingly, collaboration between FBXO11 and CIITA was impactful in the patients at early clinical stages (stage I and II) but not advanced stages (stage III and IV), although the molecular mechanisms behind this are unclear (*SI Appendix*, Fig. S4*D*). Finally, to consider potential confounding factors, we asked whether the expression of known oncogenic substrates of FBXO11 affected the survival of breast cancer patients. We found no difference in the survival of breast cancer patients grouped by high or low expression of BCL6, TP53, or SNAI1. We found modest association between prognosis and expression levels of *BLIMP1* (*SI Appendix*, Fig. S4*E*), and there was more significant difference in prognosis between $FBXO11_{low}$ $BLIMP1_{high}$ group and $FBXO11_{high}$ $Blimp1_{low}$ group (*SI Appendix*, Fig. S4*F*). While the roles of BLIMP1 in breast cancer and MHC are only partly understood (44), these observations may suggest that BLIMP1 expression may also play a role in the prognosis of breast cancer patients, although the effects of BLIMP1 seemed less than those by MHC-II. Taken together, these data indicate that the expression levels of *CIITA* and *FBXO11* cooperate for the expression of MHC-II and the prognosis of breast cancer patients.

Discussion

CIITA has been known as a master regulator of MHC-II gene expression since its discovery in 1993 (6). Although both

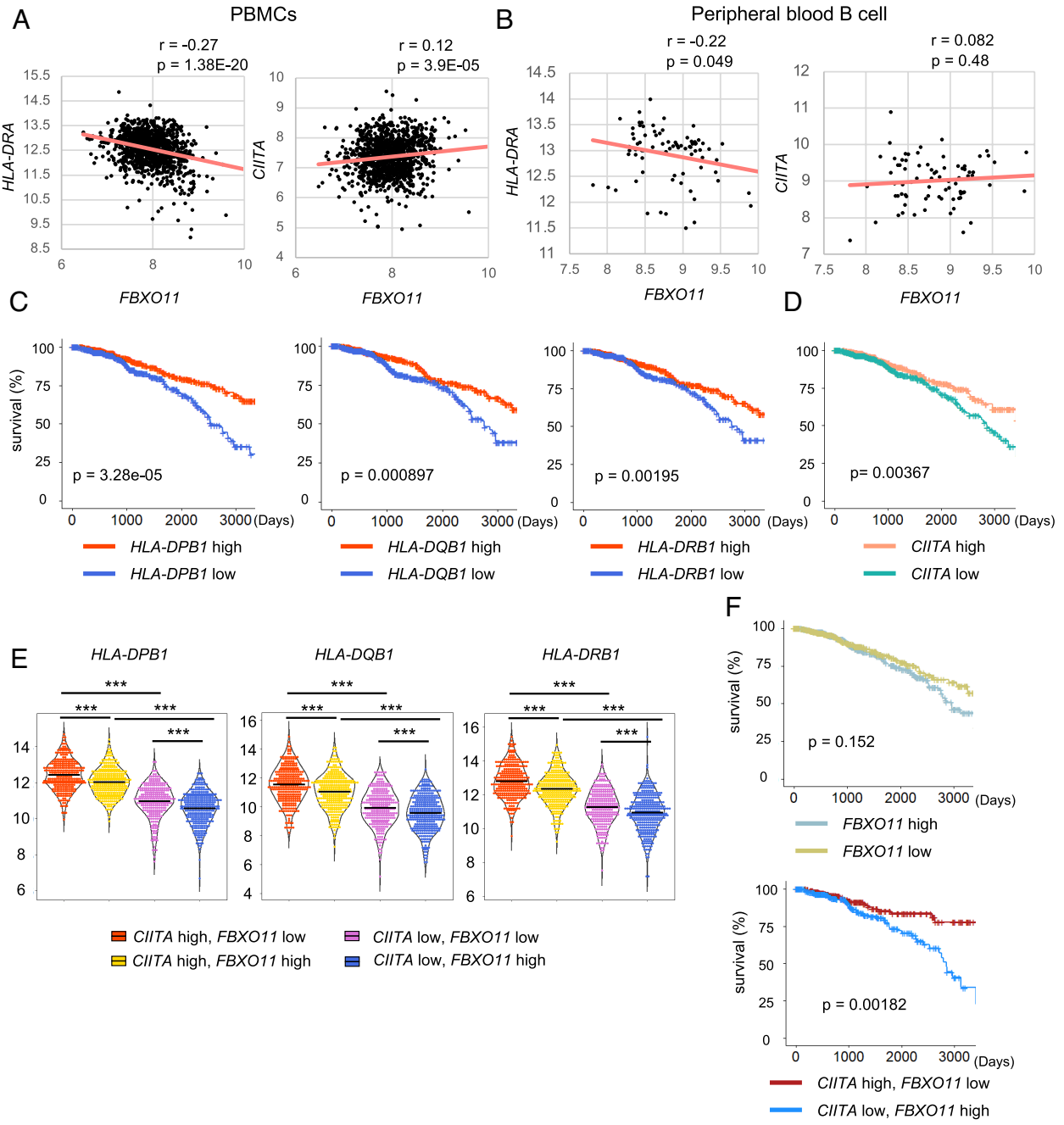

Fig. 5. The expression of *FBXO11* is negatively correlated with the MHC-II expression in normal cells and breast cancer. (*A* and *B*) Scatter plots for the expression of *FBXO11* and *HLA-DRA* or *CIITA* in (*A*) PBMCs or (*B*) peripheral blood B cells. (*C* and *D*) Kaplan–Meier survival curves for breast cancer patients with low and high expression of the indicated (*C*) MHC-II genes or (*D*) *CIITA*. (*E*) MHC-II gene expressions in breast cancer patients classified by a combination of high and low expression levels of *CIITA* and *FBXO11*. (*F*) Kaplan–Meier survival curves for breast cancer patients with low and high expression of *FBXO11* alone or in combination with *CIITA*. For each gene, patients with z-score > 0 were defined as "high" group and those with z-score < 0 were defined as "low" group.The p-values were indicated as *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. The error bars represent mean values ± SD.

transcriptional and posttranslational regulations are important for the activity of CIITA, the regulatory mechanism to determine the CIITA protein level has not been understood (8, 45). In the present study, we screened binding proteins for CIITA using an unbiased proteomic approach and identified FBXO11 as a binding partner for CIITA. FBXO11 interacts with CIITA to promote ubiquitination and proteasome-dependent degradation. Several lines of our data indicate that FBXO11 plays critical roles in the regulation of protein level of CIITA and expression level of MHC-II. First, FBXO11 is critical for the ubiquitination and degradation of CIITA as greatly prolonged half-life of CIITA was observed in *Fbxo11*-deficient cells (Fig. 4 *A*, *B*, and *D*). Second, overexpression of FBXO11 decreased CIITA protein level, which was accompanied with reduced transcription and surface expression of MHC-II (Fig. 2). Third, in the absence of FBXO11, the expression of MHC-II was significantly increased in both mouse and human cells (Fig. 3). Lastly, the expression level of FBXO11 was negatively correlated with MHC-II expression in both normal blood cells and cancer cells, indicating that FBXO11 plays significant roles in the regulation of MHC-II in these cells (Fig. 5 and *SI Appendix*, Fig. S4). Strikingly, the regulation by FBXO11 is restricted to MHC-II with no relation

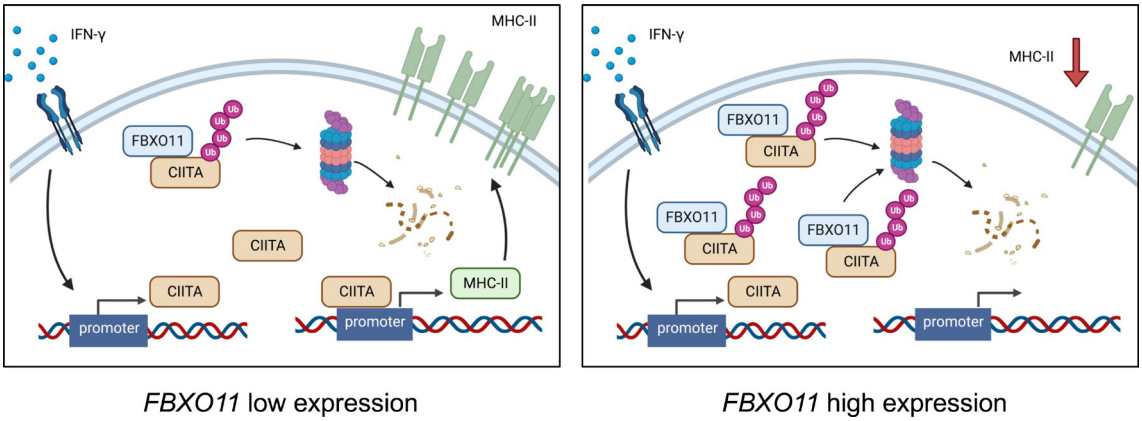

Fig. 6. A schematic model of regulation of the CIITA–MHC-II pathway by FBXO11. (*Left*) In FBXO11 low-expressing cells, degradation speed of CIITA is slow, allowing the high surface expression of MHC class-II. (*Right*) In FBXO11 high-expressing cells, CIITA is rapidly degraded, resulting in reduced CIITA-mediated MHC class-II expression.

to MHC-I. We observed the critical role of FBXO11 in the specific regulation of MHC-II, but not of MHC-I, at protein level of MHC transactivator (Fig. 2*E*), at the promoter activity of MHC (Fig. 2 *A*–*C*), at mRNA level of MHC (Figs.2*H* and 3 *A*, *D*, and *E*), and at surface expression level of MHC (Fig. 3 *B* and *C*). It is reasonable to assume that such specificity is attributed to specific binding of FBXO11 to CIITA but not to NLRC5 (Fig. 1*D* and *SI Appendix*, Table S1). Although the negative regulatory role of FBXO11 in MHC-II expression is consistent in all the data we examined, we observed that MHC-II suppression by overexpression of FBXO11 was at most half, even when following a specific time course (Fig. 2 *A*, *C*, *D*, and *F* and *SI Appendix*, Fig. S1*D*). Consistent with this observation, the ratio of CIITA ubiquitinated by FBXO11 to unmodified CIITA was not very high (Fig. 4 *A* and *B*). These results suggest that only a portion of CIITA may be regulated through FBXO11-mediated degradation pathway and there may be a mechanism that confers resistance to FBXO11.

Although it has been shown that three regions in CIITA are responsible for the protein degradation, FBXO11 associates with CIITA solely via the P/S/T domain (Fig. 1*E*). Indeed, the amount of CIITA protein that could no longer bind to FBXO11 by deleting the P/S/T domain was increased (Fig. 1*E*), indicating that FBXO11 serves as an E3 ligase responsible for CIITA degradation through recognition of degron at the P/S/T domain. Although other two regions in CIITA, containing AAD and the first 10 amino acids of extreme N terminus of isoform III, also play a role in the degradation of CIITA, the P/S/T domain–mediated degradation through FBXO11 seems dominant (19–21). While isoform III CIITA in WT cells was degraded rapidly in the chase experiment using cycloheximide, CIITA in *Fbxo11*-deficient cells was degraded at very modest rate (Fig. 4*D*), indicating that AAD and N terminus of isoform III may provide only ancillary role in the stability of CIITA. We hereby propose the following model for the regulation of MHC-II expression through CIITA. The CIITA expression level is mainly regulated by the transcriptional and posttranslational regulation. The transcriptional upregulation is mediated by STAT1-dependent inflammatory cytokine signals, dominantly elicited by IFN-γ (46). Multiple modifications are made on CIITA, including FBXO11-dependent degradation, that govern the level of CIITA protein. In cells where FBXO11 expression is low, translated CIITA is kept intact for sustaining period of time, ensuring the high level of MHC-II expression (Fig. 6 *Left*). However, CIITA undergoes ubiquitin-mediated degradation in cells with high FBXO11 expression, resulting in low MHC-II expression (Fig. 6 *Right*).

Given the inhibitory role of FBXO11 in the regulation of the MHC class II–dependent antigen presentation pathway, it is tempting to speculate the biological significance of FBXO11-dependent ubiquitination of CIITA. MHC-II expression is generally up-regulated during infection and/or inflammation through inflammatory cytokines in order to provide efficient antigen presentation to CD4 T cells (4). The immune responses should return to the steady-state homeostatic level once infection or other inflammatory events are solved. Perhaps, rapid negative regulatory mechanism via degradation of CIITA has evolved for this purpose. It is even more tempting to speculate that possible mechanisms resistant to FBXO11 may have evolved to sustain the stability of CIITA to maintain a sufficient level of MHC-II expression for host defense while still allowing the downregulation of excessive expression of MHC-II.

We also observed correlation of FBXO11 with cancer patient survival (Fig. 5*F*). Aberrant expression of MHC-II can be observed in multiple solid tumors where MHC-II expression may not usually be present. Although the role of such MHC-II expression has not been elucidated, it has been proposed that MHC-II expression in tumors, called tumor-specific MHC-II (ts-MHC-II), plays a part of significant roles in cancer immunity in a variety of tumor types, and high expression of ts-MHC-II is associated with a favorable prognosis and responsiveness to immune checkpoint inhibitors (3–5, 42). While tumor-related proteins such as BCL6, p53, SNAIL1, and BLIMP1 are known substrates of FBXO11, the role of FBXO11 in cancer is not yet clear (30, 40, 47–52). We examined the relationship between these FBXO11 substrates and survival of breast cancer patients and found that expression levels of *BCL6*, *TP53*, and *SNAI1* had no effect on overall survival (*SI Appendix*, Fig. S4*E*). Patients with high BLIMP1 expression had a modest advantage in patient survival, suggesting that a portion of effects of FBXO11 is possibly mediated by BLIMP1, but the effects of BLIMP1 expression level were smaller than those by MHC-II. Our data of Kaplan–Meier survival of breast cancer indicate that along with MHC-II expression, the combination of expression level of FBXO11 and CIITA is useful as prognostic biomarkers for breast cancer patients (Fig. 5).

In conclusion, we identified that FBXO11 is a bona fide E3 ligase of CIITA. FBXO11 plays critical roles in the regulation of MHC-II expression by determining the CIITA protein level. Future studies of FBXO11-mediated degradation of CIITA may provide potential therapeutic targets for various diseases involving MHC-II and cancer biomarkers.

Materials and Methods

HEK293T and HeLa cells were obtained from RIKEN Bio BRC, Japan. RAW264.7 cells were obtained from American Type Culture Collection. Luciferase assays, quantitative reverse transcription-PCR, immunoprecipitation, and western blotting were performed as described previously (53, 54). Ubiquitination assays were performed in HEK293T cells overexpressing FBXO11 or in *Fbxo11*-deficient RAW264.7 cells. For detailed methods, see *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. The mass spectrometric datasets data have been deposited in jPOST repository: https://repository.jpostdb.org/entry/JPST001898 (PXD037963) (55, 56).

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