HOKKAIDO UNIVERSITY

Title	Mechanisms of NLRC5 nuclear import and retention for enhanced MHC class I transactivation
Author(s)	朱, 宝慧
Description	配架番号：2865
Degree Grantor	北海道大学
Degree Name	博士(医学)
Dissertation Number	甲第16056号
Issue Date	2024-06-28
DOI	https://doi.org/10.14943/doctoral.k16056
Doc URL	https://hdl.handle.net/2115/92773
Type	doctoral thesis
File Information	ZHU_Baohui.pdf, この論文の全文

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

学　位　論　文

Mechanisms of NLRC5 nuclear import and retention for enhanced MHC class I transactivation
（NLRC5の核内輸送および保持によるMHCクラスI転写活性の増強メカニズム）

2024 年 6 月

北　海　道　大　学

バォホィ・ジュ

Baohui Zhu

学　位　論　文

Mechanisms of NLRC5 nuclear import and retention for enhanced MHC class I transactivation
（NLRC5の核内輸送および保持によるMHCクラスI転写活性の増強メカニズム）

2024 年 6 月

北　海　道　大　学

バォホィ・ジュ

Baohui Zhu

Page#

List of Publications and Presentations 1
Summary 2
List of Abbreviations 5
Introduction 6
Methods 8
Results 13
Discussion 30
Conclusion.....33
Acknowledgments.....35
Disclosure of Conflict of Interest.....35
References 36

List of Publications

1. Shuai Hou, Dajun Qu, Yue Li, Baohui Zhu, Dapeng Liang, Xinyue Wei, Wei Tang, Qian Zhang, Jiaojiao Hao, Wei Guo, Weijie Wang, Siqi Zhao, Qi Wang, Sikandar Azam, Misbah Khan, Haidong Zhao, Liye Zhang, and Haixin Lei. XAB2 depletion induces intron retention in POLR2A to impair global transcription and promote cellular senescence. Nucleic Acids Research, 2019 Sep 5; 47(15): 8239–8254.

2. Sikandar Azam, Shuai Hou, Baohui Zhu, Weijie Wang, Tian Hao, Xiangxue Bu, Misbah Khan, and Haixin Lei. Nuclear retention element recruits U1 snRNP components to restrain spliced lncRNAs in the nucleus. RNA Biology, 2019; 16(8): 1001–1009.

3. Ji-Seung Yoo, Michihito Sasaki, Steven X Cho, Yusuke Kasuga, Baohui Zhu, Ryota Ouda, Yasuko Orba, Paul de Figueiredo, Hirofumi Sawa, Koichi S Kobayashi. SARS-CoV-2 inhibits induction of the MHC class I pathway by targeting the STAT1-IRF1-NLRC5 axis. Nature Communication, 2021 Nov 15;12(1):6602.

4. Yusuke Kasuga, Baohui Zhu, Kyoung-Jin Jang, Ji-Seung Yoo. Innate immune sensing of coronavirus and viral evasion strategies. Experimental & molecular medicine, 2021 May;53(5):723-736.

5. Baohui Zhu, Ryota Ouda, Paul de Figueiredo, Koichi S. Kobayashi. ORF6, a repressor of the MHC class I pathway: New molecular target for SARS-CoV-2 drug discovery? Expert Opinion on Therapeutic Targets, under review.

6. Baohui Zhu, Ryota Ouda, Ning An, Koichi Kobayashi. The balance between nuclear import and export of NLRC5 regulates MHC class I transactivation. Journal of Biological Chemistry, under review.

List of Presentations

1. Baohui Zhu. SARS-CoV-2 immune evasion mechanism by targeting MHC class I pathway. 55th Hokkaido Summer Symposium of the Japanese Society of Virology, July 3, 2022, Sapporo, Japan.

2. Baohui Zhu, Ryota Ouda, Ning An, Koichi Kobayashi. Activation of MHC class I by controlling NLRC5 nucleocytoplasmic trafficking. Japanese Society of Immunology, December 7, 2022, Kumamoto, Japan.

Summary

Background and Purpose:
Major histocompatibility complex (MHC) molecules are critical to present antigenic peptides to T cells to activate adaptive immunity. Recent research has identified NLRC5 as the protein responsible for MHC class I transactivation. NLRC5 is an IFN-γ-inducible gene and belongs to the NLR or nucleotide-binding domain (NBD), leucine-rich repeats (LRRs) family. Similar to other NLR proteins, NLRC5 has a tripartite domain structure; a CARD at its N-terminal contains a bipartite nuclear localization signal (NLS) which is required for nuclear translocation. The centrally located NBD contains a nucleotide-binding (Walker A) motif that is involved in the nuclear importation of NLRC5 and the transactivation of target genes. Furthermore, NLRC5 has the longest C-terminal LRR among NLR proteins. Although most of the NLRs family proteins are known to locate only in the cytoplasm, NLRC5 shuttles between the cytosol and nucleus by its nuclear location signals (NLSs) and act as an MHC class I transactivator. The importin α family is capable of recognizing the NLS of cargo proteins, allowing them to enter the nucleus through the nuclear pore complex (NPC). However, the exact mechanism of how importin carries NLRC5 is still unclear. In addition to nuclear import, proper regulation of export is important for optimal protein localization in the nucleus. CRM1, also known as exportin-1, is a nuclear export receptor that binds to leucine-rich nuclear export signals (NES) present in cargo proteins to facilitate their export from the nucleus. It has been reported that treatment with leptomycin B (LMB), a well-characterized inhibitor of CRM1-mediated nuclear export, results in a significant increase in the nuclear localization of NLRC5, suggesting that NLRC5 export is a CRM1-dependent process. Histone acetyltransferases (HATs) are enzymes that catalyze the transfer of an acetyl group from acetyl-CoA to specific lysine residues on histone proteins. This process is known as histone acetylation and can regulate gene expression. Moreover, some HATs have been reported to play an important role in regulating protein localization. In this study, we investigate the regulatory mechanisms underlying the reloalization of NLRC5 and show the relationship between NLRC5 localization and its function, showing that NLRC5 nuclear retention activates the MHC class I pathway. Furthermore, we identified one of the HATs which can retain NLRC5 in the nucleus to promote its function.

Subjects and Methods:
An NLRC5-deficient MCF7 cell line, a human breast cancer cell line, was generated by CRISPR-Cas9 system to study the function of NLRC5. Immunofluorescence analysis was performed to study the altered localization of NLRC5 between the cytoplasm and the nuclear by treatment with IFN-γ or LMB, or co-transfection with HATs. Subcellular protein

fractionation was carried out to study the relocalization of NLRC5 from the cytoplasm to the nucleus by the treatment with or without IFN-γ in the presence of LMB. Mass spectrometry analysis was utilized to identify the NLRC5-specific binding proteins, which are confirmed by co-immunoprecipitation and western blotting. In addition, co-immunoprecipitation and western blotting were performed to study the association of NLRC5 with KPNA6, CRM1 and GCN5, and the domain related to the NLRC5 nuclear-cytoplasmic transport. Luciferase reporter assay, quantitative reverse-transcription PCR (RT-qPCR) analysis, and flow cytometry analysis were utilized to study the correlation of localization in NLRC5 and the promoter activity, RNA level expression and cell surface expression of MHC class I genes, respectively.

Results:

Immunofluorescence and subcellular protein fractionation analysis showed that IFN-γ treatment induced the nuclear accumulation of NLRC5 which was further enhanced by co-treatment with LMB, a CRM1 inhibitor. Our study use luciferase reporter assay, RT-qPCR analysis, and flow cytometry analysis also showed that the nuclear retention of NLRC5 induced by LMB treatment, can further enhance the promoter activity and the expression of MHC class I genes. Mass spectrometry analysis showed that NLRC5 specifically binds to one of the importin members, KPNA6, which was confirmed by co-immunoprecipitation. Co-immunoprecipitation demonstrated that the nuclear localization signal (NLS) within the CARD domain and Walker A motifs within the NACHT domain are both essential for the recruitment of KPNA6. Additionally, we observed that the length of the leucine-rich repeat (LRR) domain affected NLRC5 nuclear import, with shorter LRR domains showing more nuclear accumulation. Furthermore, we studied the effect of three histone acetyltransferases (HATs) on the cellular relocalization of NLRC5. GCN5 was identified as a novel regulator that induces NLRC5 nuclear retention. Co-immunoprecipitation using co-transfection with NLRC5, CRM1 and GCN5 showed that GCN5 induced a decrease in the association of NLRC5 and CRM1 and an increase in the accumulation of NLRC5 in the nuclear and an activity of the MHC class I pathway.

Discussion:

Our previous work showed the NLRC5 import is crucial for its function. In this study, our findings highlight a novel mechanism of translocation in NLRC5, in which three domains of NLRC5 (CARD, NACHT and LRR) were required. Besides revealing the roles of the three domains in NLRC5 nuclear localization, we identified a novel regulator of NLRC5 nuclear retention. We found that GCN5, a member of the histone acetyltransferase (HAT) family, plays a crucial role in regulating NLRC5 nuclear retention. Specifically, GCN5 impedes the binding between NLRC5 and CRM1, leading to enhanced retention of NLRC5 in the nucleus. In addition, IFN-γ, a potent inducer of MHC class I, induces nuclear accumulation of NLRC5.

Although the underlying mechanism of IFN-γ for NLRC5 nuclear localization is not clear, this may suggest that nuclear import and retention of NLRC5 evolved as an additional regulatory mechanism of the MHC class I expression level in addition to the transcriptional upregulation. Among all NLR proteins, NLRC5 and CIITA are the most similar. Regulatory mechanisms of nuclear localization of these two proteins also seem similar. We found that HATs may play a crucial role in the translocation of both NLRC5 and CIITA. Interestingly, the nuclear retention of NLRC5 and CIITA is regulated by different HATs via distinct mechanisms. While GCN5 has been shown to induce nuclear retention of NLRC5 by inhibiting its association with CRM1, the HAT PCAF has been implicated in the nuclear accumulation of CIITA through inducing CIITA acetylation. Specifically, our findings suggest that targeting the CRM1-dependent export pathway of NLRC5 could be a promising strategy for promoting MHC class I expression and enhancing anti-viral and anti-tumor immune responses. Further investigation into specific inhibitors of this pathway may therefore have important therapeutic implications.

Conclusion:

In conclusion, our findings uncovered the molecular mechanisms underlying the nuclear import and retention of NLRC5. The nuclear retention of NLRC5 enhances the expression of MHC class I genes, suggesting that the intervention of the shuttling mechanism of NLRC5 may serve as a potential therapeutic target for virus infections and cancers.

List of Abbreviations

B2M	beta-2-microglobulin
CARD	caspase recruitment domain
CIITA	class II Major Histocompatibility Complex Transactivator
CITA	class I Major Histocompatibility Complex Transactivator
CRM1	chromosome region maintenance 1
GCN5	general control non-repressed 5 protein
HATs	histone acetyltransferases
IVM	ivermectin
KPNA	karyopherin Subunit Alpha
LMB	leptomycin B
LRR	leucine-rich repeats
MHC	major histocompatibility complex
NBD	nucleotide-binding domain
NES	nuclear export signal sequence
NLR	Nod-like receptor
NLRC5	NLR family, CARD domain-containing 5
NLS	nuclear localization signal
NPC	nuclear pore complex
PCAF	P300/CBP-associated factor
TAP1	Transporter associated with antigen processing 1

Introduction

Major histocompatibility complex (MHC) molecules are critical to present antigenic peptides to T cells to activate adaptive immunity (Pamer and Cresswell (1998); Shastri et al., 2005). MHC class I mainly presents intracellular antigens to CD8 T cells whereas MHC class II mostly presents antigens from extracellular sources to CD4 T cells (van den Elsen et al., 1998a; van den Elsen et al., 1998b). Since the expression level of MHC molecules is important to elicit the optimum immune responses against pathogens or cancer (Dhatchinamoorthy et al., 2021; Hewitt, 2003), their expression is tightly regulated by certain mechanisms.

Recent research has identified NLRC5 as the MHC class I transactivator (CITA) (Meissner et al., 2012a). NLRC5 is an IFN-γ-inducible gene that belongs to the NLR or nucleotide-binding domain (NBD), leucine-rich repeats (LRRs) family of proteins (Kobayashi and van den Elsen, 2012). Similar to other NLR proteins, NLRC5 has a tripartite domain structure (Ting et al., 2008; Wilmanski et al., 2008); a CARD (caspase recruitment domain) at its N-terminal contains a bipartite nuclear localization signal (NLS) which is required for the nuclear translocation (Meissner et al., 2010). The centrally located NACHT contains a nucleotide-binding (Walker A) motif that is involved in the nuclear importation of NLRC5 (Meissner et al., 2012b). The LRRs at the C-terminus are generally known to mediate protein–protein interactions or ligand binding (Braun et al., 1991; Kobe and Deisenhofer, 1993). Among the NLR family proteins, NLRC5 is the largest member, possessing unusually long LRRs. However, the function of these LRRs remains poorly understood (Motyan et al., 2013).

Though most NLR proteins are known to be located in the cytoplasm, two members of the family, CIITA and NLRC5, have been reported to be translocated into the nucleus. CIITA (MHC class II transactivator) is a master regulator of MHC class II gene expression (Boss and Jensen, 2003; LeibundGut-Landmann et al., 2004; Masternak et al., 2000; Steimle et al., 1994), and the localization of CIITA between the cytoplasm and nucleus is balanced by the presence of NLS and NES (nuclear export signal sequences) (Cressman et al., 2001). NLRC5 shuttles between the cytosol and nucleus, and its entry into the nucleus is essential for the transactivation of MHC class I genes (Meissner et al., 2010). However, the regulatory mechanism of NLRC5 shuttling remains unclear. A thorough understanding of the transactivating function of NLRC5 on MHC class I requires a detailed examination of its mechanism for nuclear-cytoplasmic shuttling.

Functional nuclear proteins require to be transported into the nucleus through the nuclear pore

complex (NPC) by a process mediated by NLS signals. The importin α family, which includes KPNA1 (karyopherin alpha 1), KPNA2 (karyopherin alpha 2), KPNA3 (karyopherin alpha 3), KPNA4 (karyopherin alpha 4), KPNA5 (karyopherin alpha 5), KPNA6 (karyopherin alpha 6), and KPNA7 (karyopherin alpha 7), is capable of recognizing the NLS (Lange et al., 2007), and imports the proteins with NLS into the nucleus through the NPC by association with or without importin β (Chi et al., 1995; Gorlich and Mattaj, 1996; Miyamoto et al., 2002). However, the mechanism to import NLRC5 is unclear.

In addition to nuclear import, proper regulation of export is important for optimal protein localization in the nucleus. CRM1 (chromosome region maintenance 1), also known as exportin 1, is a nuclear export receptor that recognizes and binds to NES present in cargo proteins to facilitate their export from the nucleus (Fornerod et al., 1997; Fukuda et al., 1997). It has been reported that treatment with leptomycin B (LMB) (Wolff et al., 1997), a well-known inhibitor of CRM1-mediated nuclear export, results in a significant increase in the nuclear localization of NLRC5 (Meissner et al., 2012b), suggesting that NLRC5 export is a CRM1-dependent process.

In this study, we investigated the regulatory mechanisms underlying the import and export of NLRC5 utilizing molecular biology techniques. Our study revealed that each domain (CARD, NACHT and LRR) of NLRC5 contributes to the regulation of nuclear import and export through distinct mechanisms and identified a novel HAT that regulates the nuclear retention of NLRC5, resulting in increased MHC class I transactivation.

Methods

Cell lines

The HeLa, MCF7, and HEK293T cell lines were purchased from ATCC. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Nichirei, 175012) and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively, Nacalai Tesque, 09367-34) in a humidified incubator at 37°C, 5% CO2. A human NLRC5-deficient stable cell line was generated by the CRISPR/Cas9 dual-gRNA knockout system. Two sgRNAs (gRNA-Fw: ATCCTTAGACACTCCGGAGGGGG, gRNA-Rv: CAGGCGACTTGGCACAGTGCGGG) targeting the NBD domain of NLRC5 were amplified together with the pH1-scaffold template and inserted into lentiCRISPR V2 vector to construct the lenti-gRNA plasmid. HEK293T cells were transfected with lenti-gRNA plasmid, pCMV-VSV-G and psPAX2 plasmids to generate the lentivirus. The supernatant was collected 48 h after transfection and filtered through a 0.45 μm filter (Sartorius Stedim, S7598-FXOSK). MCF7 cells on one well in a 12-well plate were incubated with 1 ml of the supernatant and 5 μg polybrene (Nacalai Tesque, 12996-81) for another 48 h, followed by selection with 3 μg/mL puromycin (Invivogen, 14861-71). The absence of NLRC5 expression in the generated cell line was confirmed by qPCR using NLRC5-specific primers (hNLRC5-Fw: CTGGCCAGTCTCACCGCACAA, hNLRC5-Rv: CCAGGGGACAGCCATCAAAATC).

Plasmids

GFP-tagged NLRC5 was generated as described previously (Meissner et al., 2010). FLAG-tagged full-length NLRC5, CARD domain, NACHT domain, LRR domain, and LRR deletion mutants (C+N+LR1-20, C+N+LR1-15, C+N+LR1-10, C+N+LR1-5, NLRC5-ΔLRR) were generated by amplifying DNA fragments from GFP-tagged NLRC5 using PCR (primers: NLRC5-Fw: ATATCTAGAATGGACCCCGTTGGCCTCCAGCT, NLRC5-Rv: AAAGTCGACTCAAGTACCCCAAGGGGCCTGGG, CARD-Fw: ATATCTA-GAATGGACCCCGTTGGCCTCCAGCT, CARD-Rv: AAAGTCGACCTACGGGCCCTT-GTTAACCCTGG, NACHT-Fw: GGGTCTAGAATGAGGGTGACCGTGCTTTTGGGGAA, NACHT-Rv: ATAGTCGACCTACACCTGCACTACAGCAGCCTGCT, LRR-Fw: CAC-TCTAGAATGTTGAAGAAGTTGGCCACCCGCAA, LRR-Rv: ATAGTCGACCTACAC-CTGCACTACAGCAGCCTGCT, LRR-deletion-Fw: ATATCTAGAATGGACCCCGTT-GGCCTCCAGCT, C+N+LR1-20-Rv: AAAGTCGACCTACAGCCCAGGCAGGATGG-TAGCTA, C+N+LR1-15-Rv: AAAGTCGACCTAGGACAGCAGGAGGCTCTGCAGCA, C+N+LR1-10-Rv: AAAGTCGACCTAGGGTCCCGGGCAGTCCTTCAGAGT, C+N+LR1-5-Rv: AAAGTCGACCTACTGGGATGCAGCCTCTGCCATCA, NLRC5-ΔLRR-Rv: ATAGTCGACCTACACCTGCACTACAGCAGCCTGCT), and then

cloning the fragments into p3xFLAG-CMV7.1 vector (Invitrogen) with XbaI and SalI. The HLA promoter pGL3-HLA-B (HLA-B250) was kindly gifted from Dr. van den Elsen (Leiden University, Netherlands) (Gobin et al., 2001). FLAG-tagged C+N+LR1-4, C+N+LR1-3, C+N+LR1-2, C+N+LR1, NLRC5-ΔCARD, NLRC5-ΔNACHT, NLRC5-NLSI-m, NLRC5-NLSII-m, and NLRC5-WAm were generated by Q5® Site-Directed Mutagenesis Kit (New England Biolabs, M0554S) using FLAG-tagged full length NLRC5 as a template (primers: NLRC5-muta-Fw: ATGTACCCATACGATGTTCC, C+N+LR1-4-Rv: TTCCTG-GAGCAGGGCTAT, C+N+LR1-3-Rv: GTGAGGGAGAACCTCCAC, C+N+LR1-2-Rv: GAGAGGCAAAGCTTTCAC, C+N+LR1-Rv: TGTCGGCAAGCTCCTGGA, NLRC5-ΔCARD-Fw: AGGGTGACCGTGCTTTTG, NLRC5-ΔCARD-Rv: CATTCTA-GAGCGGCCGCC, NLRC5-ΔNACHT-Fw: TATGTTACCCTCCATTCCCG, NLRC5-ΔNACHT-Rv: CGGGCCCTTGTTAACCCT, NLSIm-Fw: GGCGCAGTGCAA-GAAGCAGCAGC, NLSIm-Rv: GCCGCGGGTGAGGACCCACAGCT, NLSIIm-Fw: CCATGGCCTGGCGGCGCCACATCAGAG, NLSIIm-Rv: TGGAGCTGAGAT-TCAGGTTG, WAm-Fw: TGGCATGGGCgcgACCACGCTGG, WAm-Rv: GCCTTCCCCAAAAGCACG). HA-tagged KPNA6 was generated by amplifying a DNA fragment from pCMVTNT-T7-KPNA6 (Addgene plasmid number: 26682) and cloning it into a pcGN-HA vector (primers: KPNA6-Fw: AAAGAATTCGAGAC-CATGGCGAGCCCAGGGAAAGACAA, KPNA6-Rv: AAATCTAGA TTATAGCTGGAA-GCCCTCCATGGGGGCCT), which was kindly provided by Dr. Shigetsugu Hatakeyama (Hokkaido University, Japan), using EcoRI and XbaI. PRC/RSV-FLAG-hPCAF and pcDNA3.1-HA-hGCN5 were kindly provided by Dr. Peter van den Elsen. FLAG-CRM1 (#17647) and HA-p300 (#89094) were purchased from Addgene.

Flow cytometry

To analyze the surface expression level of HLA, 3×10^5 HEK293T cells were treated with 0, 50, 100, or 150 nM of LMB for 8 h. The single-cell suspensions were then stained with either PE-conjugated anti-human HLA-A, B, and C antibody (Biolegend, 311406) or PE-conjugated isotype control (Biolegend, 400213). After 30 minutes of incubation on ice, the cells were washed and resuspended in FACS buffer (2% FBS/PBS) for analysis using a BD FACS Canto II flow cytometer. Data analysis was performed using FlowJo software.

Immunoprecipitation and western blotting

For immunoprecipitation, 1.2×10^6 HEK293T cells were harvested 48 h after transfection with FuGENE HD (Promega, E2312) according to the manufacturer's protocol. The cells were incubated and sonicated in lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% NP40, 1 mM PMSF, protease inhibitor cocktail (Roche), and phosphatase inhibitor (Nacalai Tesque)), and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. For immunoprecipitation of the FLAG-tagged protein complex, the cell extracts were incubated

overnight with anti-FLAG M2 affinity gel (Sigma, F3165-1MG) at 4 °C. For immunoprecipitation of GFP-tagged protein complex, the cell extracts were gently mixed with anti-GFP antibody (Proteintech, 66002-1-Ig) overnight at 4 °C and then incubated with protein A/G agarose (Pierce) for 4 h at 4 °C. The immunoprecipitants were washed 3 times in ice-cold NP40 lysis buffer (0.1% NP40, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1mM Na3VO4, 1mM PMSF), resuspended in SDS sample buffer, boiled for 4 min at 95°C. SDS-PAGE and blotting were performed as described below. Quantitative comparison of the band intensity was analyzed by ImageJ. Western blotting images were converted into "Grayscale" picture mode by ImageJ software, and bands of interest were selected using the "rectangle" tool. The integrated density was measured for each region of interest and the graphs were generated using GraphPad Prism 9 software (GraphPad Software, USA).

Western blotting

For western blotting, samples were resolved by SDS-PAGE and transferred to PVDF membranes (Merck, IPVH00010). After blocking with a blocking buffer (5% nonfat dried milk diluted in TBST), the membrane was incubated overnight with the following primary antibodies: anti-FLAG (Sigma, SL05105), anti-HA (B&D SYSTEMS, MAB0601), anti-GFP (Proteintech, 66002-1-Ig), anti-Lamin B1 (Cell Signaling, 12586S), and anti-alpha Tubulin (Proteintech, 11224-1-AP), each at 1:1000. After washing 3 times in TBST, 5 min each, the membrane was reacted with the following secondary antibodies: ECL™ Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (GE Healthcare NA931V) and Cytiva's Amersham ECL Rabbit IgG, HRP-linked whole Ab (GE Healthcare NA934). The signal was visualized and scanned with ImageQuant™ LAS 4000 (GE Healthcare). Quantitative comparison of the band intensity was analyzed by ImageJ.

Subcellular protein fractionation

HeLa cells (6×10^5) were transfected with the GFP-NLRC5 expression vector for 24 h followed by IFN-γ stimulation for another 24 h. Before harvesting, the cells were treated with 100 nM of LMB for 8 h. At 48 h post-transcription, the cells were washed twice with phosphate-buffered saline (PBS) and collected in cold lysis buffer (1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1.5 mM MgCl2, 20 mM HEPES [pH 7.4], protease inhibitor cocktail (Roche, 11836170001), 1 mM dithiothreitol [DTT]). After incubation for 15 minutes on ice, the cells were homogenized by passing through a 26-gauge syringe ten times. Following incubation for 20 minutes on ice, the homogenate was centrifuged at 3,000 g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube as a cytosolic fraction, and the nuclear pellet was washed with a fractionation buffer and then dispersed with a pipette and passed through a 25-gauge syringe ten more times. The resuspended nuclear pellet was resuspended in a nuclear buffer (standard lysis buffer with 10% glycerol and 0.1% SDS added). The supernatant was collected after centrifuging at 10,000 g and used as a nuclear fraction. The

cytoplasmic and nuclear fractions were then analyzed by SDS-PAGE.

Immunofluorescent analysis

HeLa cells (3×10^5) were plated on coverslips, incubated overnight, and transfected with DNA constructs using FuGENE HD. Twenty-four hours after transfection, the cells were treated with IFN-γ (100 U/ml) (Peprotech), 100nM of LMB or 20ul Ivermectin alone or their collaboration for certain hours. The cells were then fixed with 4% PFA for 20 minutes at 4°C, permeabilized with 0.05% Triton X-100 in PBS for 5 minutes at room temperature, blocked with 5 mg/ml BSA in PBST (0.04% Tween20 in PBS) for 30 minutes, and incubated with primary antibodies (anti-FLAG (Sigma, SL05105), anti-HA (B&D SYSTEMS, MAB0601), anti-GFP (Proteintech, 66002-1-Ig)) overnight at 4°C. Subsequently, the cells were incubated with secondary antibodies (Goat anti-Mouse IgG (H&L) - Alexa Fluor™ 488 (Invitrogen, A-11001), Goat anti-Rabbit IgG (H&L) – Alexa Fluor® 594 (Invitrogen, A-11012)) for 1 h at room temperature. The nuclei were stained with 1 μg/ml of Hoechst 33342 and analyzed using a confocal laser microscope (Olympus). Quantitative comparison of the nuclear NLRC5 signal intensity (% of nuclear signal intensity/total cell signal intensity) was analyzed by ImageJ, the whole cell or the cell nucleus was selected using the “freehand” tool, and the integrated density was measured for each region of interest.

RNA isolation and RT-qPCR

For the evaluation of mRNA expression, RNA was reverse transcribed into cDNA using ReverTra Ace™ qPCR RT Master Mix reagent (Toyobo) according to the manufacturer’s protocol, and RT-qPCR was performed using THUNDERBIRDTM SYBRTM qPCR Mix reagent (Toyobo, Japan) with the specific primer sets targeting human NLRC5 (hNLRC5-F: CTGGCCAGTCTCACCGCACAA, hNLRC5-R: CCAGGGGACAGCCATCAAAATC), HLA-A (hHLA-A-F: AAAAGGAGGGAGTTACACTCAGG, hHLA-A-R: GCTGTGAGGGACACATCAGAG), HLA-B (hHLA-B-F: CTACCCTGCGGAGATCA, hHLA-B-R: ACAGCCAGGCCAGCAACA), TAP1 (hTAP1-F: AG-GGCTGGCTGGCTGCTTTGA, hTAP1-R: ACGTGGCCCATGGTGTTGTTAT) and hGAPDH-F: GAAGGTGAAGGTCGGAGT, hGAPDH-R: GAAGATGGTGATGGGAT-TTC to determine the expression of each mRNA relative to GAPDH using the ΔΔCt method. Fold-change compared to control cells in the expression of the indicated genes represents the mean (±SD) of triplicate reactions.

Luciferase reporter assays

For the luciferase assays, 1 × 104 HEK293T cells were plated in one well in a 48-well plate. The cells were transfected with a mixture of 150 ng of pGL3-HLA-B and 10 ng of pRL-TK using the FuGENE HD Transfection Reagent following the manufacturer's protocol. After 48 h of transfection, the cells were treated with 100 nM of LMB for 8 h and then harvested for

luciferase activity measurements.

Statistics

Statistical analyses were performed by a Two-tailed unpaired t-test. The result significance was indicated as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns, not significant using data from three independent experiments. The error bars represent mean values ± SD. Analyses were carried out with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

IFN-γ stimulation induces NLRC5 nuclear accumulation.

IFN-γ stimulation is a most potent inducer of the expression of NLRC5 and MHC class I genes (Baldeon et al., 1997; Fruh and Yang, 1999; Fujimaki et al., 1996; Meissner et al., 2010; Zhou, 2009). Consistent with the previous studies by our and other groups, treatment with IFN-γ resulted in a significant increase in the expression of MHC class I and related genes, *HLA-A*, *HLA-B*, and *TAP1* (Transporter associated with antigen processing 1), in *NLRC5*+/+ MCF7 (WT) cells while this induction was impaired in *NLRC5*-/- MCF7 (KO) cells, suggesting that the upregulation of MHC class I genes by IFN-γ treatment was largely dependent on NLRC5 (Fig. 1A). Although this NLRC5 dependency is most likely due to the transcriptional upregulation of NLRC5 by IFN-γ stimulation (Meissner et al., 2010), we also observed that NLRC5 transfection and IFN-γ stimulation cooperate to induce the expression of MHC class I genes (Fig. 1B), suggesting a possible mechanism to potentiate the activity of NLRC5 in the presence of IFN-γ. Considering the critical role of the nuclear import of NLRC5 in the activity as a CITA, we sought to investigate whether the IFN-γ-induced MHC class I genes could be partially attributed to changes in the nuclear localization of NLRC5. The cellular localization of GFP-tagged NLRC5 upon IFN-γ treatment was analyzed by immunofluorescence microscopy using the cells treated with a CRM1 inhibitor, LMB, which has been reported to inhibit the nuclear export of NLRC5 (Meissner et al., 2010). For this, HeLa cells transfected with GFP-tagged NLRC5 were fixed, permeabilized, and the nuclei were stained with 1 μg/ml of Hoechst 33342 and analyzed using a confocal laser microscope. Treatment with IFN-γ induced the nuclear accumulation of NLRC5, and this accumulation was further enhanced by co-treatment with LMB (Fig. 2A). To further investigate the effects of IFN-γ on NLRC5 localization, we performed western blotting to confirm the accumulation of NLRC5 in the nucleus following IFN-γ treatment. HeLa cells transfected with GFP-tagged NLRC5, after 24 h transfection, cells were treated with IFN-γ for an additional 24 h and were harvested and analyzed by western blotting using anti-GFP, anti-α-tubulin, and anti-Lamin B antibodies. Cytoplasmic and nuclear protein fractions were isolated using a cell fractionation technique. Treatment with IFN-γ induced a decrease in NLRC5 in the cytosol and an increase in the nucleus compared to untreated control cells. (Fig. 2B). These results suggest that the increase in the activity of NLRC5 as a CITA upon IFN-γ treatment was not only caused by transcriptional upregulation but also by elevated recruitment of NLRC5 into the nucleus.

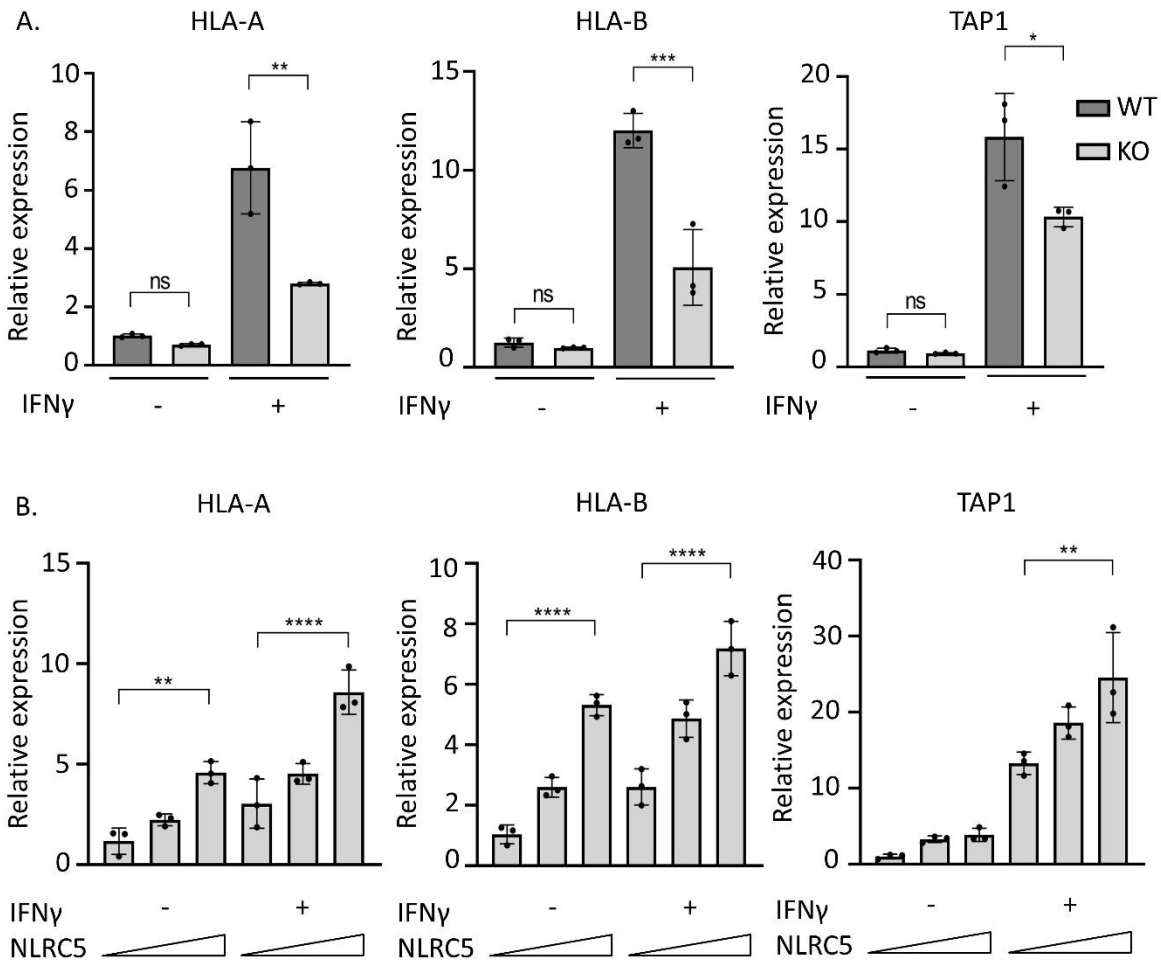


Figure 1. NLRC5 is required for IFN-γ-induced MHC class I-related genes expression. ***A***, The mRNA level of MHC class I and MHC class I-related genes in *NLRC5*+/+ (WT) MCF7 or *NLRC5*-/- MCF7 (KO) cells treated with or without IFN-γ (200U/ml) for 24 h were quantified by RT-qPCR. ***B***, The *NLRC5*-/- MCF7 (KO) cells were transfected with *NLRC5*-encoding plasmid at 0, 0.5, or 1 μg, cultured for 24 h, and subsequently treated with 200 U/ml IFN-γ for an additional 24 h. The mRNA levels of MHC class I and MHC class I-related genes were quantified by RT-qPCR. Each experiment was performed three times independently, and the values shown are mean ± SD. P values were calculated using Student's t test. ns: not significant, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

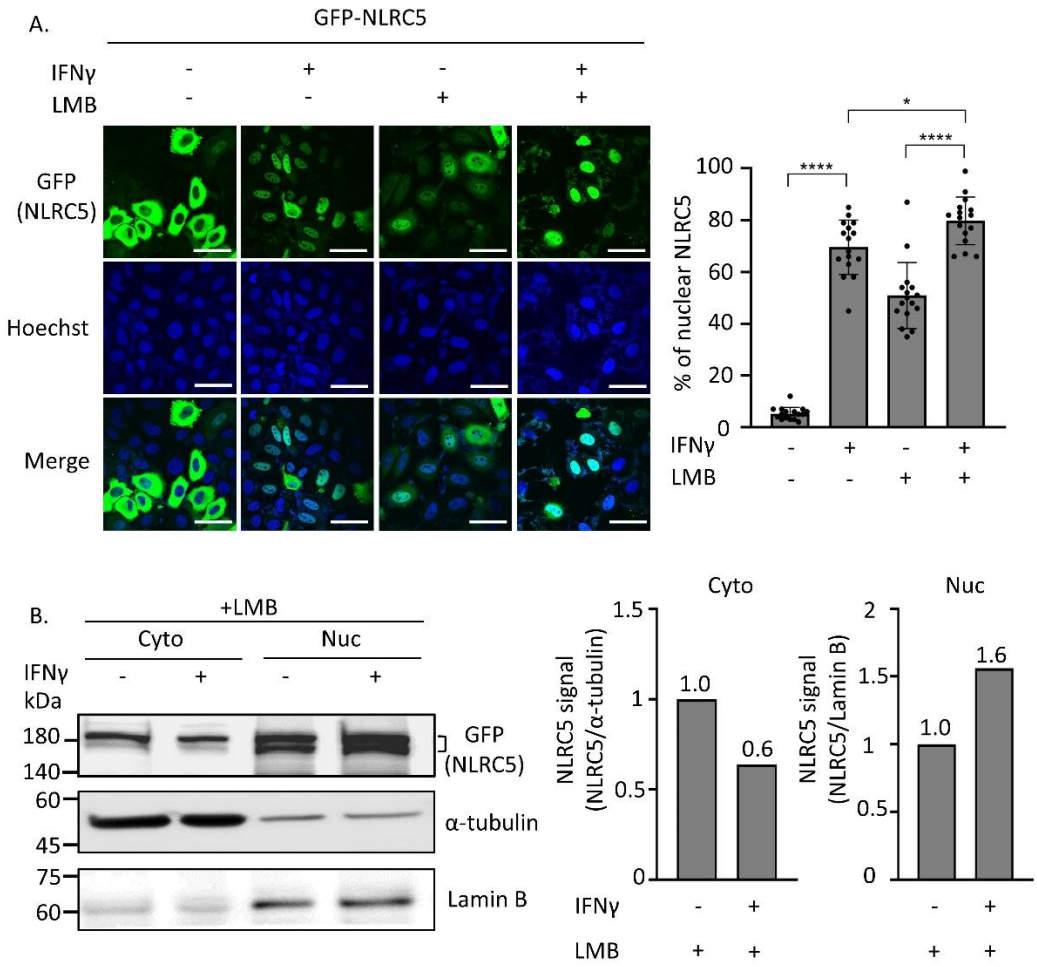
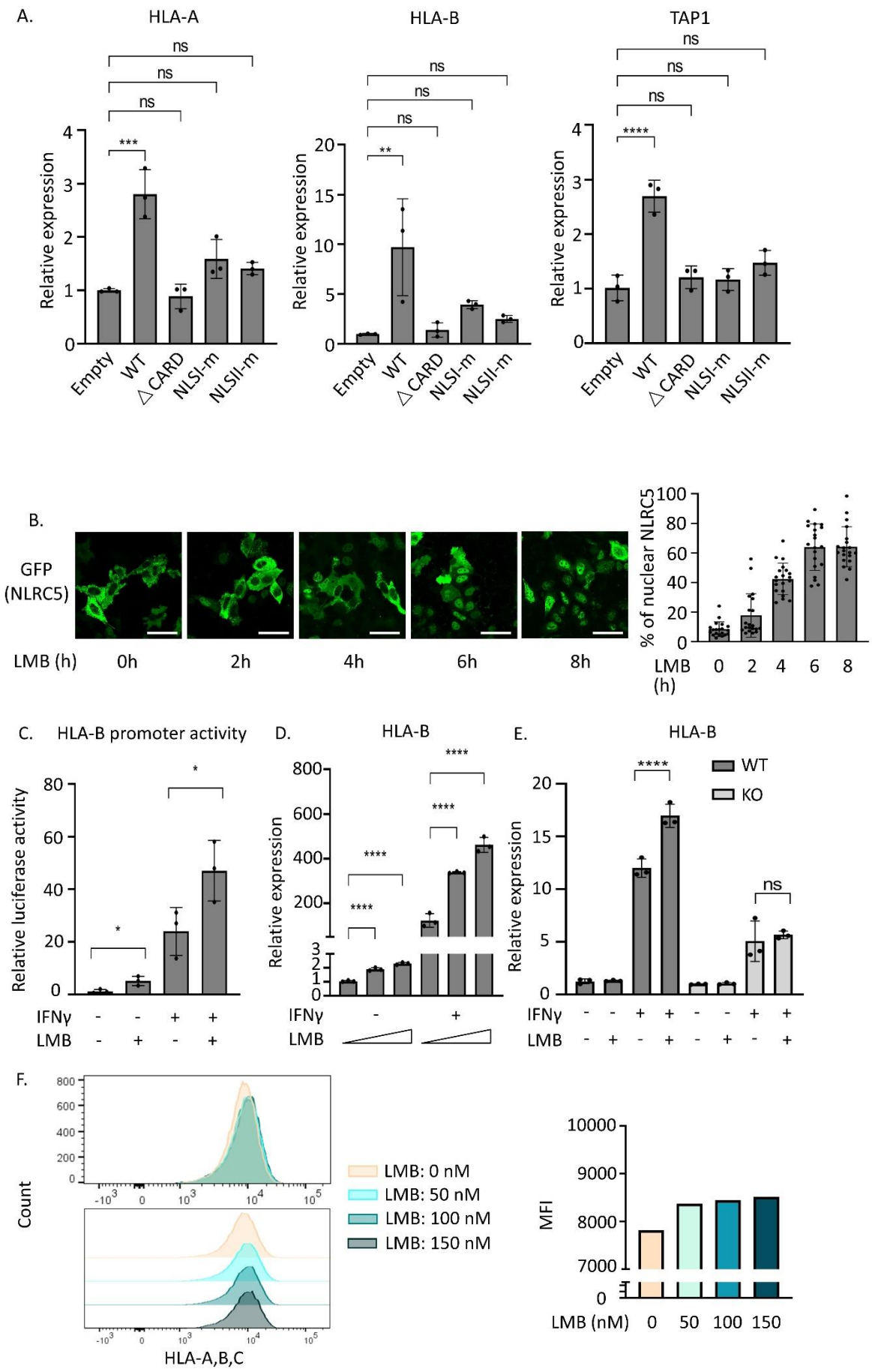


Figure 2. IFN-γ induces nuclear accumulation of NLRC5.

HeLa cells were transfected with a plasmid encoding GFP-tagged NLRC5, and cultured for 24 h. The transfected cells were stimulated with or without 200 U/ml IFN-γ alone for 32 h, or leptomycin B (LMB) alone for 8 h, or treated with 200 U/ml IFN-γ for 32 h in combination with LMB for the last 8 h. ***A***, (Left panel) Representative images of the cellular localization of NLRC5. Cell nuclei were stained with Hoechst 33342. (Right panel) The bar graph indicates a quantitative comparison of the nuclear NLRC5 signal intensity, which was calculated as a percentage of the total cell signal intensity using ImageJ. The analysis was performed with 20 cells for each group. ***B***, (Left panel) The nuclear (Nuc) and cytoplasmic (Cyto) fractions were analyzed by western blotting using anti-GFP, anti-α-tubulin, and anti-Lamin B antibodies. (Right panel) The bar graph shows a quantitative comparison of the cytoplasmic or nuclear GFP-NLRC5 signal intensity normalized by the intensity of α-tubulin (for cytoplasmic NLRC5) or Lamin B (for nuclear NLRC5) using ImageJ. Each experiment was performed three times independently, and the values shown are mean ± SD. *P*-values were calculated using Student's t test. *$p < 0.05$, ****$p < 0.0001$.

NLRC5 nuclear importation and retention enhance MHC class I induction.

To elucidate the significance of the localization of NLRC5 in the nucleus, we asked if blocking the nuclear importation or exportation of NLRC5 may impact the MHC class I gene transactivation. First, we examined the significance of nuclear localization of NLRC5 by using three mutant NLRC5 expression vectors; 1) CARD deleted mutant which lacks entire NLS, 2) mutant NLS-I in which the first half of bipartite NLS is mutated, and 3) mutant NLS-II in which the second half of bipartite NLS is mutated. HeLa cells were transfected with wild-type NLRC5 or the mutant NLRC5 constructs. After 48 h transfection, Total RNA was isolated from the transfected cells and the expression levels of the target genes were quantified using RT-qPCR. An increase in *HLA-A*, *HLA-B* and *TAP1* mRNA expression was observed in wild-type NLRC5 overexpressing cells, but not in all of these mutant NLRC5 overexpressing cells (Fig. 3A), suggesting that NLRC5 translocation into the nucleus was required to elevate the transactivation of MHC class I genes. Second, the effect of elevated retention of NLRC5 in the nucleus on the transactivation of MHC class I genes was investigated by the blockage of NLRC5 exportation from the nucleus. Treatment GFP-NLRC5 expressing cells with LMB showed the increase in nuclear retention of NLRC5 in a time-dependent manner (Fig. 3B). Treatment with LMB induced the increase in *HLA-B* promoter activity in HEK293T cells (Fig. 3C), and its transcription in HeLa cells (Fig. 3D), and augmented surface expression of HLA-A, B, C in HEK293T cells (Fig. 3F and G), indicating that nuclear retention of NLRC5 may enhance MHC class I transactivation. Co-treatment of LMB and IFN-γ resulted in further increased HLA-B promoter activity in HEK293T cells (Fig. 3C) and its transcription in *NLRC5* WT MCF7 cells (Fig. 3E), while no increase of them was found in *NLRC5*-deficient MCF7 cells (Fig. 3E). Collectively, these findings emphasize the importance of NLRC5 localization in the nucleus for the effective induction of MHC class I genes. Both the translocation of NLRC5 into the nucleus and its subsequent retention in this compartment are critical for the efficient activation of the MHC class I pathway.


A.
HLA-A
HLA-B
TAP1
ns
***
**
****
Relative expression
Empty
WT
△CARD
NLSI-m
NLSII-m
B.
GFP
(NLRC5)
LMB (h)
0h
2h
4h
6h
8h
% of nuclear NLRC5
LMB
(h)
C.
HLA-B promoter activity
Relative luciferase activity
IFNγ
LMB
D.
HLA-B
E.
HLA-B
WT
KO
F.
Count
HLA-A,B,C
LMB: 0 nM
LMB: 50 nM
LMB: 100 nM
LMB: 150 nM
MFI
LMB (nM)

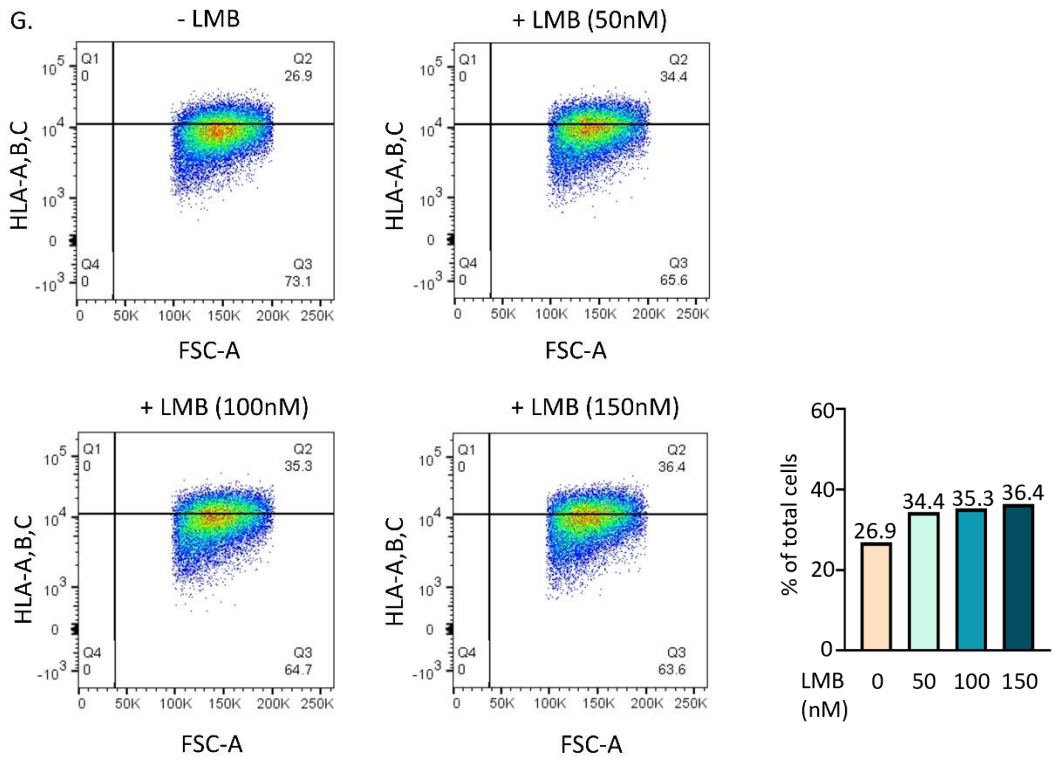


Figure 3. NLRC5 nuclear importation and retention contribute to MHC class I activation.

A, The mRNA level of MHC class I (HLA-A and -B) and MHC class I-related genes in HEK293T cells transfected with plasmids encoding wild type NLRC5, CARD deleted NLRC5 or NLS mutated NLRC5-encoding were quantified by RT-qPCR. ***B***, HeLa cells were transfected with the plasmid encoding GFP-tagged NLRC5, cultured for 48 h, and treated with 100 nM leptomycin B (LMB) for additional 0, 2, 4, 6 or 8 h. (Left panel) Representative image of the cellular localization of NLRC5. (Right panel) Quantification of the nuclear NLRC5 signal intensity (% of nuclear signal intensity/total cell signal intensity) is shown with a bar graph analyzed by ImageJ. Twenty cells were evaluated in each sample. ***C***, HeLa cells were transfected with an NLRC5-dependent MHC class I promoter (HLA-B250)-luciferase reporter construct. Twenty-four hours after transfection the cells were stimulated with IFN-γ (200 U/ml, 32 h) or LMB alone (100 nM, 8 h), or treated with IFN-γ for 32 h in combination with LMB for the last 8 h. The luciferase activity was measured by dual-luciferase assay. Relative change to reporter-transfected, untreated HeLa cells was plotted. ***D***, HeLa cells were stimulated with LMB (0, 50 or 100 nM, 8 h) alone, or co-treated with LMB (0, 50 or 100 nM, 8 h) following treatment with IFNγ (200 U/ml) alone for 24 h. HLA-B mRNA level was quantified using RT-qPCR. ***E***, The HLA-B mRNA expression in NLRC5+/+MCF7 (WT) or NLRC5-/-MCF7 (KO) cells stimulated with or without IFNγ (200 U/ml, 32 h) alone, LMB (100 nM, 8 h) alone or their combination was quantified using RT-qPCR. ***F***, The surface expression of HLA-A/B/C on HEK293T cells co-treated with

LMB (0, 50, 100 or 150 nM) and IFNγ (200 U/ml) for 8 h following treatment with IFNγ (200 U/ml) for 24 h was analyzed by flow cytometry. (Left panel) A histogram indicates the HLA-A/B/C expression on the cells treated with the indicated dose of LMB. (Right panel) The quantification of the mean fluorescence intensity (MFI) of HLA-A/B/C. ***G***. The surface expression of HLA- A, B, and C in HEK293T cells co-treated with IFNγ (200U/ml) and LMB (0, 50, 100 or 150nM) for 8 h following treatment with IFNγ (200U/ml) for 24 h was measured and shown with a bar graph. Each experiment was performed three times independently, and the representative result was shown. The values shown are mean ± SD. P-values were calculated using Student's t test. ns: not significant, *$p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The bipartite NLS in CARD of NLRC5 is required for KPNA6 recruitment.

NLS in the CARD domain was required to import NLRC5 into the nucleus as shown above. Since the cargo proteins carrying NLSs are frequently transported into the nucleus through the nuclear pore protein complex mediated by an importin-dependent mechanism, we hypothesized NLRC5 nuclear transport was also processed by the importins. Immunofluorescence analysis showed that treatment with ivermectin, an inhibitor of the importin alpha/beta-dependent import pathway, resulted in a loss of nuclear localization of GFP-tagged NLRC5 in a dose-dependent manner, indicating that NLRC5 was translocated into the nucleus by an importin-dependent mechanism (Fig. 4A). Previous study reported that siRNA deletion of KPNA6, one of importin α members, resulted in impaired NLRC5 nuclear importation compared to control siRNA treated-cells (Yoo et al., 2021). To study the significance of the NLS within the CARD domain of NLRC5 for the recruitment of KPNA6, HeLa cells were first co-transfected with expression vectors of HA-tagged KPNA6 and FLAG-tagged CARD, NACHT, or LRRs. Consistent with our hypothesis, a strong association between KPNA6 and the CARD was observed by immunoprecipitation analysis, but not NACHT or LRRs (Fig. 4B), suggesting that CARD is a crucial domain in the recruitment of KPNA6 to NLRC5. To further investigate the role of the NLSs of NLRC5 in its association with KPNA6, HeLa cells were transfected by expression vectors for HA-tagged KPNA6 together with FLAG-tagged wild-type NLRC5, NLSI-mutated NLRC5 (NLSI-m) or NLSII-mutated NLRC5 (NLSII-m). While wild-type NLRC5 bound to KPNA6, NLSI-m and NLSII-m were associated with KPNA6 to a lesser extent, approximately at 60% and 30% respectively in comparison to wild-type (Fig. 4C). Collectively, these results demonstrate that both upstream and downstream amino acids clusters in the NLS within NLRC5 CARD domain were essential for the recruitment of KPNA6.

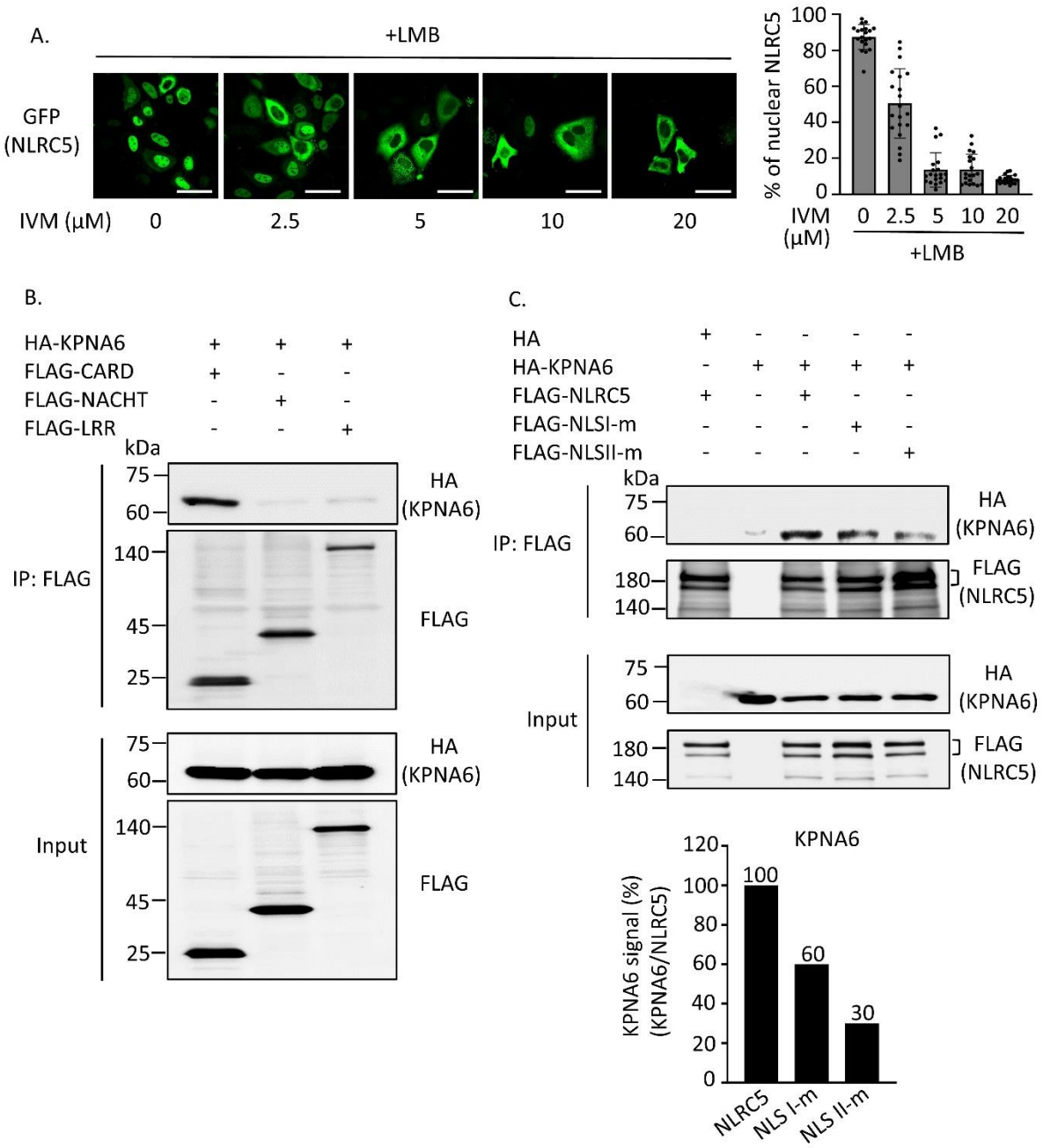


Figure 4. A bipartite NLS in CARD of NLRC5 is required for association with KPNA6. ***A***. HeLa cells were transfected with plasmid encoding GFP-tagged NLRC5, cultured for 24 h, and co-treated with ivermectin (0, 2.5, 5, 10 or 20 μM) and LMB (0, 50, 100 or 150 nM) for 8 h following treatment with ivermectin (0, 2.5, 5, 10 or 20 μM) alone for 24 h. (Left panel) The cellular localization of NLRC5 was analyzed by confocal microscopy. (Right panel) Quantification of the nuclear NLRC5 signal intensity (% of nuclear signal intensity/total cell signal intensity) is shown with a bar graph analyzed by ImageJ. Twenty cells were evaluated for each sample. ***B***, HEK293T cells were co-transfected with a plasmid encoding HA-tagged KPNA6 and either a plasmid encoding FLAG-CARD, FLAG-NACHT or FLAG-LRR. The transfected cells were harvested after 48 h, immunoprecipitated with anti-FLAG beads, and

subjected to western blotting using anti-HA and anti-FLAG antibodies. ***C***, HEK293T cells were transfected with plasmids encoding FLAG-NLRC5, FLAG-NLSI-m or FLAG-NLSII-m along with HA-tagged KPNA6. (Upper panel) Cell lysates were prepared after 48 h, immunoprecipitated with anti-FLAG beads, and subjected to western blotting using anti-HA and anti-FLAG antibodies. (Lower panel) Quantitative comparison of the KPNA6 signal intensity to that of NLRC5 in the immunoprecipitated complex was analyzed by ImageJ. Each experiment was performed three times independently.

Walker A motif of NLRC5 is important for efficient recruitment of KPNA6

The Walker A motif, which is highly conserved among ATP-binding proteins, plays a crucial role in the binding and hydrolysis of ATP. It is located within the NACHT domain of NLRC5, a domain known for its involvement in protein-protein interactions and nucleotide binding. A schematic diagram shows the position of the Walker A motif within the NACHT domain of NLRC5 (Fig. 5A). Previous reports show that the Walker A motif of NLRC5 is critical for ATP binding and is critical for both nuclear importation and transactivation of the MHC class I promoters (Ting et al., 2008; Wilmanski et al., 2008). Consistent with the reports, GFP-tagged mutant NLRC5 lacking either the NACHT domain or the Walker A motif failed to translocate into the nucleus (Fig. 5B). To investigate the potential role of the Walker A motif in the association of NLRC5 with importin proteins, we conducted immunoprecipitation experiments using HA-tagged KPNA6 with FLAG-tagged wild-type NLRC5 and a FLAG-tagged NLRC5 with the mutated Walker A motif. The results of the immunoprecipitation experiments revealed a strong binding between wild-type NLRC5 and KPNA6, indicating their robust association. However, NLRC5 with the mutant Walker A motif (WAm) exhibited a weak binding to KPNA6 (Fig. 5C), suggesting that the ATP binding to the Walker A motif may be necessary for conformational changes in NLRC5, leading to its activation and efficient recruitment and association with KPNA6. These findings suggest a functional link between the ATP-binding ability of the Walker A motif in NLRC5 and its interaction with importin proteins, particularly KPNA6. It implies that ATP hydrolysis and the resulting conformational changes in NLRC5 may be essential steps for its proper nuclear importation and subsequent transactivation of target genes, such as MHC class I promoters.

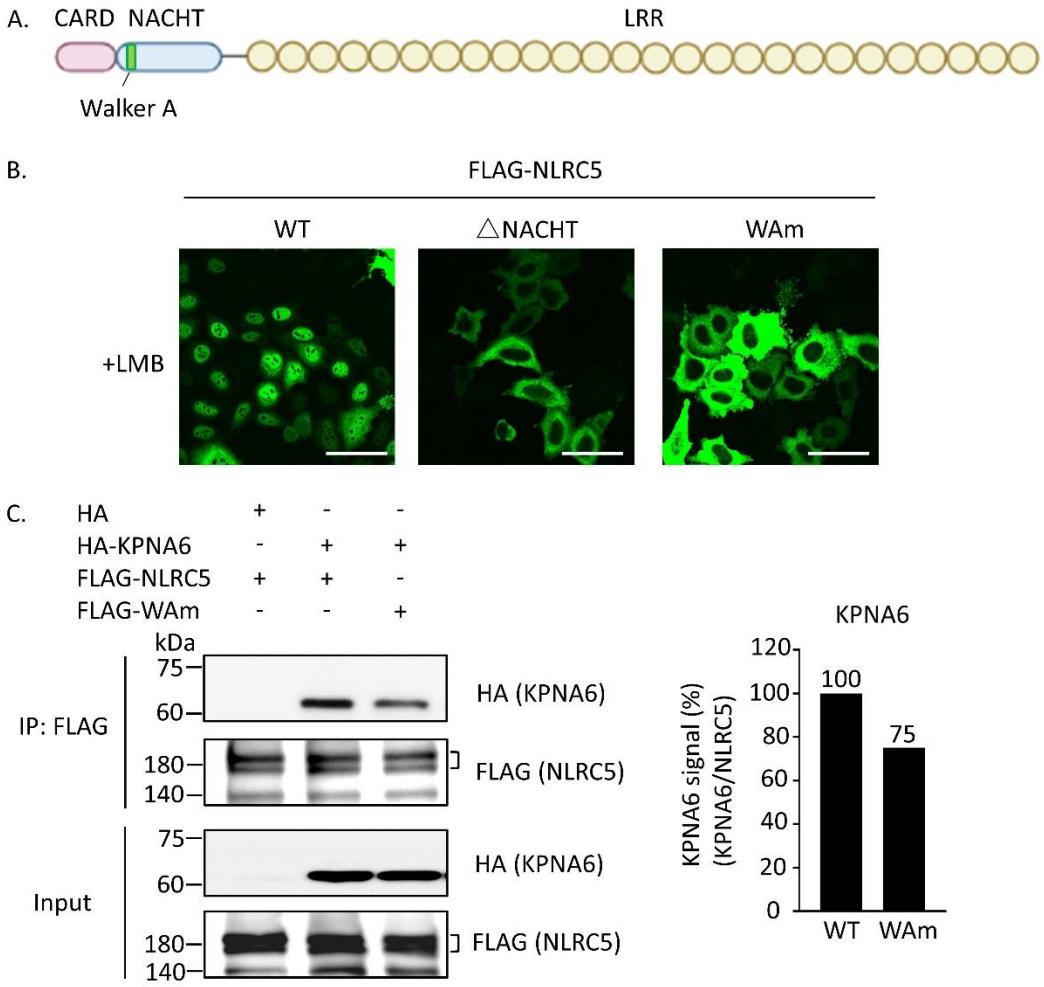


Figure 5. ATP binding is important for the efficient association of KPNA6 with NLRC5. ***A***, Schematic structure of NLRC5, indicating the CARD, NACHT, and LRRs domains of NLRC5. The Walker A motif in the NACHT domain is represented by the green box. ***B***, Representative image of the cellular localization of indicated NLRC5 protein. HeLa cells were transfected with the plasmids encoding FLAG-tagged wild-type NLRC5 (WT), NACHT deleted NLRC5 (△NACHT) or walker A motif mutated NLRC5 (WAm), cultured for 24 h, and then treated with 100 nM LMB for 8 h. ***C***, HEK293T cells were transfected with plasmids encoding FLAG-tagged wild-type NLRC5 (FLAG-NLRC5) or walker A motif mutated NLRC5 (FLAG-WAm) along with the HA-tagged KPNA6 vector. (Left panel) The cell lysates were prepared 48 h post-transfection, immunoprecipitated with anti-FLAG M2 beads, and subjected to western blotting using anti-HA and anti-FLAG antibodies. (Right panel) Quantitative comparison of the KPNA6 signal intensity to that of NLRC5 is shown with a bar graph analyzed by ImageJ. Each experiment was performed three times independently.

LRRs suppress the nuclear accumulation of NLRC5.

NLRC5 is characterized by having the longest LRRs among all members of the NLR (NOD-like receptor) protein family. The specific function of NLRC5 and the significance of its LRRs are still not fully understood. However, previous studies have revealed that the LRRs of another protein called CIITA (Class II transactivator) are associated with its exportation from the nucleus to the cytoplasm (Hake et al., 2000), we hypothesized that LRRs of NLRC5 were also involved in the regulation of its cellular localization. To test this hypothesis, FLAG-tagged expression vectors for NLRC5 with different lengths of LRRs were generated using molecular cloning techniques (Fig. 6A). This involved designing specific primers and employing PCR to amplify NLRC5 fragments with desired deletions. HeLa cells were then transiently transfected with the NLRC5 deletion mutant constructs. After transfection, the cells were treated with leptomycin B (LMB) (Wolff et al., 1997), a well-characterized inhibitor of CRM1-mediated nuclear export, that is reported to induce a significant increase in the nuclear localization of NLRC5 (Meissner et al., 2012b). Since treatment with LMB over 4 h results in the nuclear localization of even wild-type NLRC5 (Fig. 3B), the cells were fixed, permeabilized, and subjected to immunofluorescence staining after transfection of those deletion mutant plasmids, followed by LMB treatment for 4 h. Antibodies against the FLAG tag was used to visualize NLRC5 localization. Fluorescent images were captured using immunofluorescence microscopy. ImageJ was used to quantify the nuclear and cytoplasmic fluorescence intensity of NLRC5 in the transfected cells. The results demonstrated that NLRC5 lacking LRRs exhibited the most prominent localization in the nucleus. However, as the length of the LRRs increased, the nuclear localization of NLRC5 decreased (Fig. 6B). These results suggest the LRRs of NLRC5 play an important role in manipulating NLRC5 cellular localization by suppressing its nuclear accumulation.

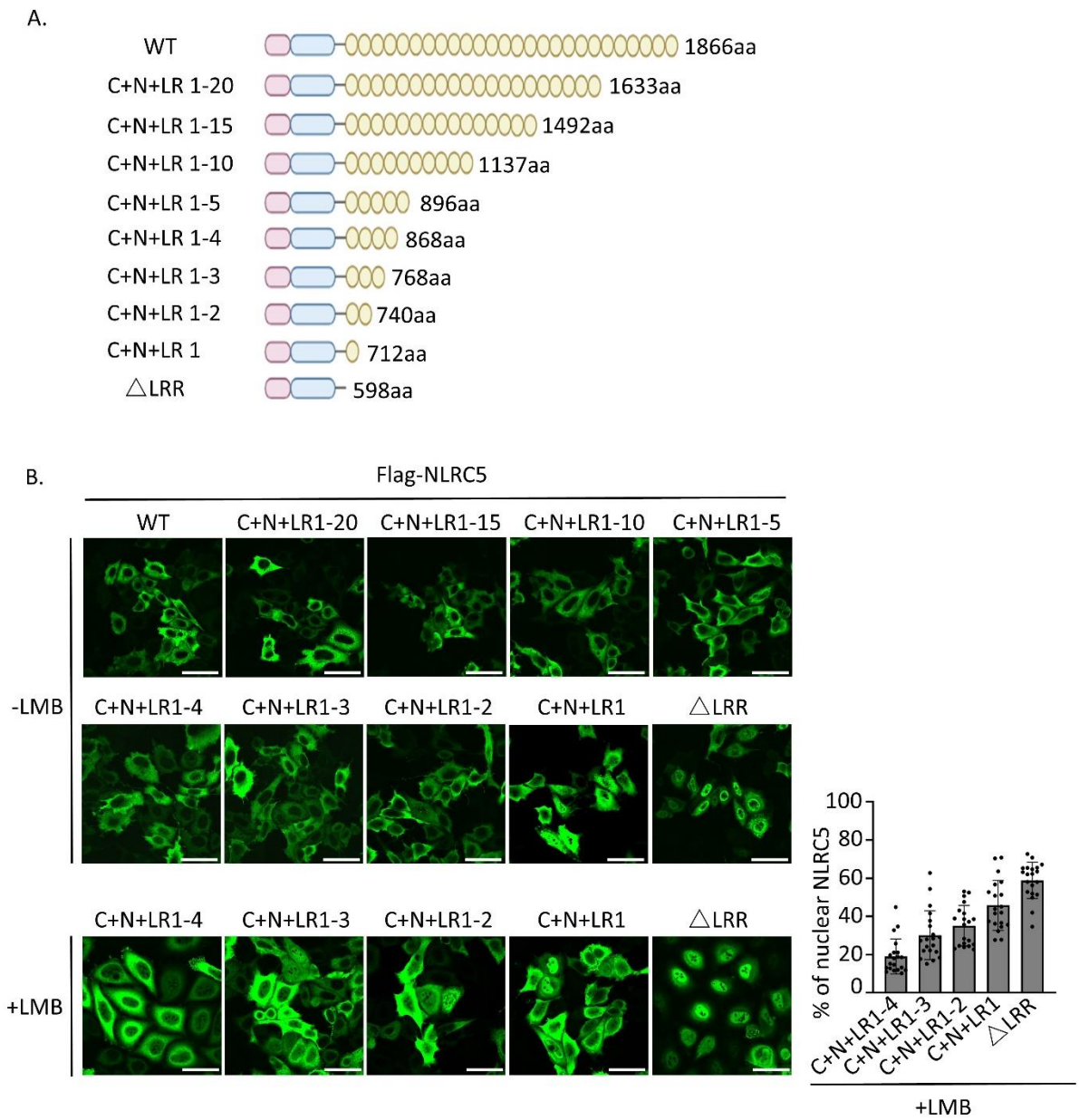
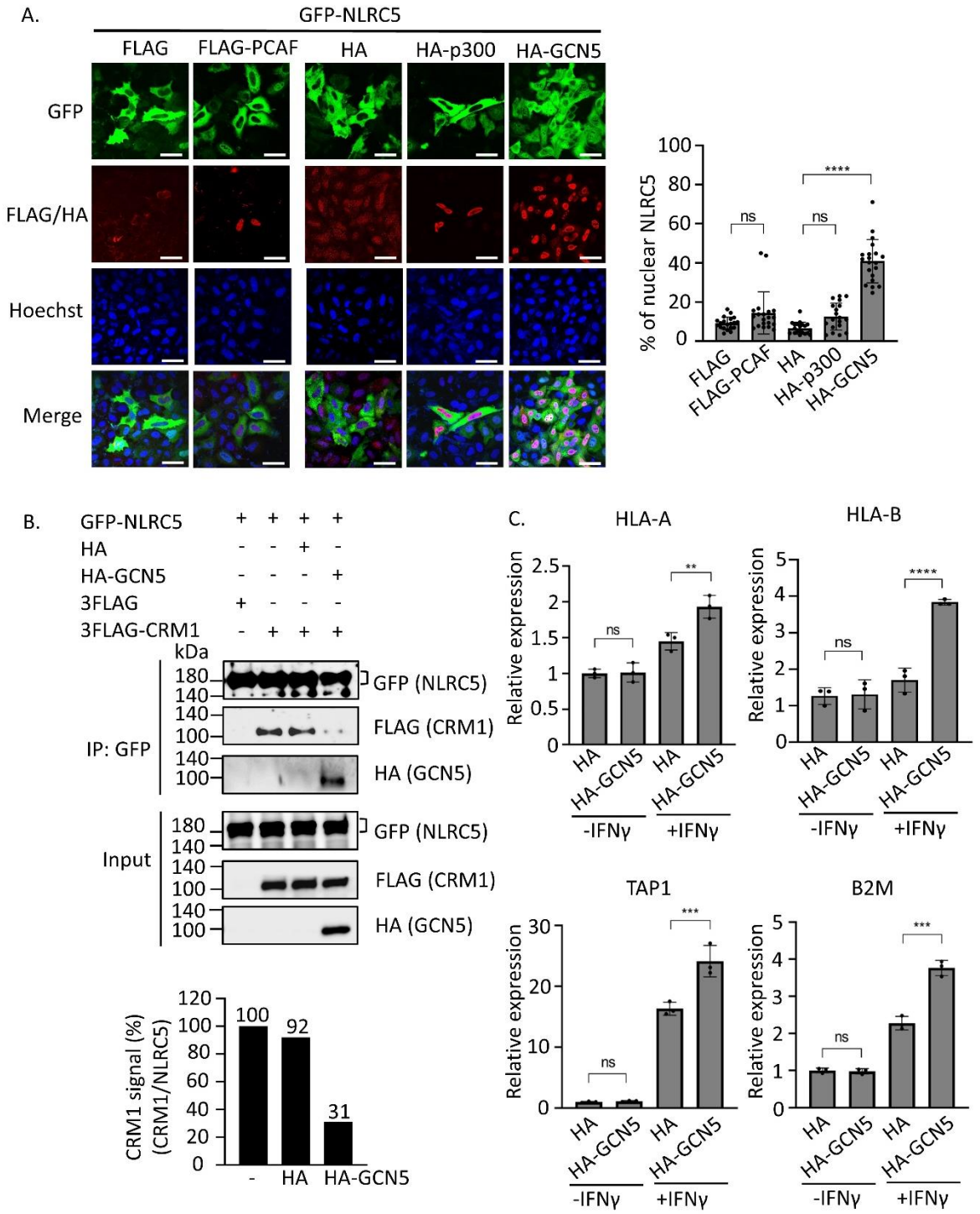


Figure 6. A shorter LRR results in the accumulation of NLRC5 in the nucleus.

A, Schematic image of NLRC5, indicating the CARD (C), NACHT (N) and full-length/dissected LRR (LR) domains of NLRC5. ***B***, HeLa cells were transfected with indicated constructs, cultured for 48 h, and treated with 100 nM LMB for 4 h. (Left panel) Cellular localization of NLRC5 was indicated by immunofluorescence analysis. (Right panel) Quantitative comparison of the nuclear NLRC5 signal intensity (% of nuclear signal intensity/total cell signal intensity) is shown with a bar graph analyzed by ImageJ. Twenty cells were evaluated for each sample.

GCN5 promotes the nuclear accumulation of NLRC5 by blocking CRM1-dependent NLRC5 exportation.

To investigate the molecular mechanism underlying NLRC5 nuclear accumulation, we explored the potential involvement of histone acetyltransferases (HATs), given their reported role in NLRC5-induced MHC class I activation (Meissner et al., 2012c). Previous studies have demonstrated that the nuclear accumulation of CIITA can be enhanced by the HAT protein PCAF (P300/CBP-associated factor) (Spilianakis et al., 2000), Based on these findings, we hypothesized that certain HAT proteins might also contribute to the nuclear accumulation of NLRC5. We selected three HAT members (PCAF, p300, and GCN5) based on our previous observation for their ability to increase NLRC5-induced MHC class I gene activation (Meissner et al., 2012c). To assess the impact of these HAT proteins on NLRC5 nuclear accumulation, we co-transfected HeLa cells with GFP-NLRC5 and each HAT expression vector. Remarkably, co-transfection of GCN5 resulted in a significant increase in the nuclear accumulation of GFP-NLRC5, whereas cells transfected with PCAF or p300 did not exhibit a comparable increase in nuclear NLRC5 levels (Fig. 7A). This observation led us to consider the possible involvement of GCN5 for inhibition of CRM1-mediated exportation of NLRC5. To test whether the association between NLRC5 and CRM1 was altered by the expression of GCN5, we performed immunoprecipitation analysis using cells co-transfected with the expression vectors for NLRC5, CRM1, and either the GCN5 expression vector or control. Strikingly, the presence of GCN5 led to a significant decrease in the association between CRM1 and NLRC5 compared to the control condition (Fig. 7B). To further elucidate the functional consequences of GCN5-mediated NLRC5 nuclear accumulation, we examined its impact on MHC class I gene induction. HeLa cells were transfected with either an empty HA tag vector or an HA-tagged GCN5 expression vector, followed by stimulation with or without IFN-γ. Subsequently, we compared the expression levels of MHC class I and related genes using RT-qPCR. The mRNA expression of IFN-γ-induced *HLA-A*, *HLA-B*, *TAP1* and *B2M* (beta-2-microglobulin) were significantly upregulated in the presence of GCN5, indicating its positive effect on MHC class I gene induction (Fig. 7C). Furthermore, we assessed the impact of GCN5 on NLRC5-induced MHC class I promoter activity. Co-transfection of GCN5 with NLRC5 resulted in a substantial boost in MHC class I promoter activity compared to NLRC5 alone. (Fig. 7D). In summary, these results suggest that GCN5 plays a critical role in promoting NLRC5 nuclear accumulation by interfering with the interaction between NLRC5 and CRM1. This disruption of NLRC5 exportation leads to the activation of MHC class I genes, as evidenced by the upregulation of MHC class I gene expression and enhanced MHC class I promoter activity in the presence of GCN5.


A.
GFP-NLRC5
FLAG
FLAG-PCAF
HA
HA-p300
HA-GCN5
GFP
FLAG/HA
Hoechst
Merge
% of nuclear NLRC5
ns
ns
****
B.
GFP-NLRC5
HA
HA-GCN5
3FLAG
3FLAG-CRM1
kDa
IP: GFP
Input
GFP (NLRC5)
FLAG (CRM1)
HA (GCN5)
CRM1 signal (%)
(CRM1/NLRC5)
100
92
31
C.
HLA-A
HLA-B
TAP1
B2M
Relative expression
-IFNγ
+IFNγ
ns
**
****
***

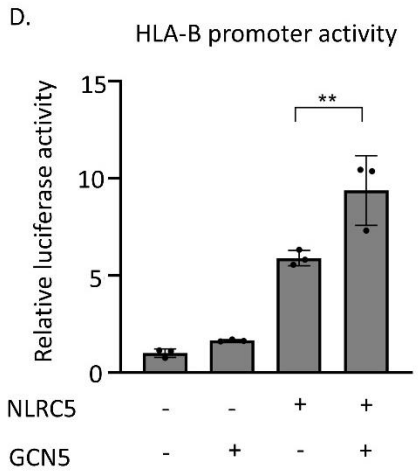


Figure 7. GCN5 induces nuclear retention of NLRC5 by interfering NLRC5-CRM1 association.

A, HeLa cells were transfected with the plasmid encoding GFP-tagged NLRC5 along with plasmids encoding the indicated FLAG- or HA-tagged proteins. (Left panel) Forty-eight hours after transfection the cellar localization was analyzed by a confocal microscopy. Cell nuclei were stained with Hoechst 33342. (Right panel) The bar graph represents a quantitative comparison of the nuclear NLRC5 signal intensity, which was calculated as a percentage of the total cell signal intensity using ImageJ. The analysis was performed on 20 cells for each sample. ***B***, HEK293T cells were transfected with the GFP-tagged NLRC5 encoding vector along with FLAG-tagged CRM1 and/or HA-tagged GCN5 encoding vectors and cultured for 48 h. Cell lysates were incubated with anti-GFP antibody overnight, then immunoprecipitated with protein A/G agarose beads. (Upper panel) Immunoprecipitated protein complexes were subjected to western blotting using anti-GFP, anti-FLAG and anti-HA antibodies. (Lower panel) The relative change of the intensity of CRM1 normalized by NLRC5 was shown with a bar graph. ***C***, HEK293T cells were transfected with HA-empty or HA-GCN5 encoding plasmid, cultured for 24 h, and subsequently treated with 200 U/ml IFNγ for an additional 24 h. The mRNA levels of MHC class I (*HLA-A* and *HLA-B*) and MHC class I-related genes (*TAP1* and *B2M*) were quantified by RT-qPCR. ***D***, HeLa cells were transfected with an NLRC5-dependent MHC class I promoter (HLA-B250)-luciferase reporter construct together with the plasmid encoding either FLAG-tagged NLRC5 or HA-tagged GCN5, and the luciferase activity was measured by dual-luciferase assay. Relative changes to control cells transfected with luciferase reporter construct were analyzed and plotted. Each experiment was performed three times independently, and the values shown are mean ± SD. P-values were calculated using Student's t test. ns: not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Discussion

Effective regulation of the MHC class I pathway by NLRC5 is contingent upon its nuclear import (Meissner et al., 2010; Meissner et al., 2012b). This study demonstrates the mechanism of nuclear import and retention of NLRC5, highlighting the significance of three domains of NLRC5 in the nuclear translocation and revealing a novel regulator of the nuclear retention of NLRC5. Our data demonstrate that NLS located within the CARD and the Walker A motif present in the NACHT domain are both essential for the recruitment of importin (Fig. 4 and Fig. 5). Moreover, we found that LRRs negatively affect the nuclear localization of NLRC5 in a length-dependent manner, suggesting the possible presence of multiple NES in the LRRs (Fig. 6) (Fornerod et al., 1997). Indeed, the NES-binding nuclear export receptor CRM1 associates with NLRC5, further indicating that the potential NES located in the LRRs promotes the nuclear export of NLRC5 (Fig. 7B). The observed relationship between LRR length and nuclear localization implies that the LRRs of NLRC5 may function as regulatory domains involved in controlling its subcellular distribution. The precise mechanism by which LRRs influence NLRC5 localization remains to be elucidated. It is possible that the LRRs directly interact with nuclear import or export factors, such as importins or exportins, or they may affect the overall conformation of NLRC5, thereby influencing its trafficking between the nucleus and cytoplasm. Further investigations are warranted to unravel the underlying molecular mechanisms by which the LRRs of NLRC5 contribute to its cellular localization and to determine the functional consequences of such localization dynamics. Additionally, exploring the interplay between LRRs and other domains within NLRC5 may provide valuable insights into the overall function and regulation of NLRC5 in immune responses and beyond. Besides revealing the roles of the three domains in the nuclear localization, we identified a novel regulator of the nuclear retention. We found that GCN5, a member of the histone acetyltransferase (HAT) family, impedes the binding between NLRC5 and CRM1, leading to enhanced retention of NLRC5 in the nucleus. We therefore propose the following model for the nuclear import and retention of NLRC5 (Fig. 8). Through the NLS, NLRC5 recruits KPNA6, which facilitates its transport through the nuclear pore complex into the nucleus. On the other hand, NLRC5 in the nucleus may be exported to the cytoplasm through the association with CRM1, limiting the transactivation of MHC class I genes (Fig. 8 left panel). It seems that CRM1-mediated export of NLRC5 is counterbalanced by the presence of GCN5, which inhibits the association between NLRC5 and CRM1, leading to the accumulation of NLRC5 in the nucleus (Fig. 8 right panel). While the transcriptional induction of NLRC5 is important for the upregulation of MHC class I (Meissner et al., 2010), it is tempting to speculate that fine-tuning the balance of the nuclear import/export of NLRC5 may be important for maintaining the base level of MHC class I expression at steady state for the sentinel purpose. It is further tempting to

speculate that nuclear import and retention can also play a role in the upregulation of MHC class I at an inflammatory state. Indeed, we found that IFN-γ, known as a potent inducer of MHC class I (Meissner et al., 2010), induced nuclear accumulation of NLRC5 (Fig. 2). Although the underlying mechanism of IFN-γ for NLRC5 nuclear localization is not clear, this may suggest that nuclear import and retention of NLRC5 have been evolved as an additional regulatory mechanism of the MHC class I expression level in addition to the transcriptional upregulation.

NLRC5 and CIITA are both nuclear proteins and share the highest similarity among all NLR proteins (Meissner et al., 2010; Nickerson et al., 2001). Regulatory mechanisms of nuclear localization of these two proteins also seem similar. First, among NLR members, they share phylogenetically the most similar NBD domains that are required for nuclear import (Benko et al., 2010; Meissner et al., 2010). Second, both CIITA and NLRC5 carry NLS, which enables them to associate with importins and translocate into the nucleus (Cressman et al., 2001; Meissner et al., 2010; Spilianakis et al., 2000) Third, both proteins contain LRRs at the C-terminus, which is the common location for the NES, a critical site for the CRM1-dependent nuclear export. (Fornerod et al., 1997; Vijayan et al., 2019). Finally, we found that HATs play a crucial role in the translocation of both NLRC5 and CIITA. Interestingly, the nuclear retention of NLRC5 and CIITA is regulated by different HATs via distinct mechanisms. PCAF and GCN5 are two closely related HATs (Xu et al., 1998) and both are known to acetylate histone proteins to promote transcriptional activation (Xu et al., 1998). In the case of CIITA, PCAF has been identified as a major regulator, enhancing the nuclear accumulation of CIITA by inducing direct acetylation of CIITA (Spilianakis et al., 2000). In the case of NLRC5, PCAF did not affect the nuclear localization (Fig. 7A). However, GCN5 constitutes a major HAT that regulates and induces the nuclear retention of NLRC5 by inhibiting its association with CRM1 (Fig. 7B).

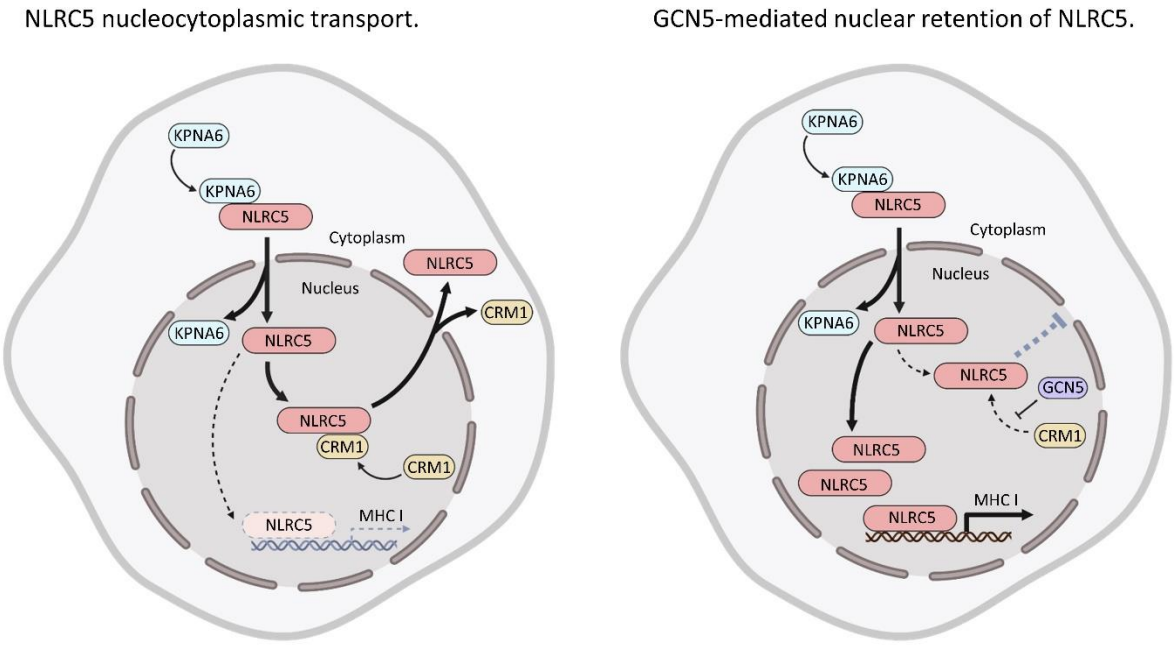


Figure 8. Schematic illustration of the mechanism of NLRC5 nuclear import and retention.

NLRC5 is mainly located in the cytoplasm of the cell. NLRC5 is associated with KPNA6 in the cytoplasm and imported into the nucleus through the nuclear pore. (Left panel) CRM1 binds NLRC5 resulting in export of NLRC5 out of the nucleus through the nuclear pore. (Right panel) GCN5 inhibits the NLRC5-CRM1 binding, preventing NLRC5 export from the nucleus. The nuclear accumulation of NLRC5 induced by these mechanisms results in the transactivation of MHC class I.

Conclusion

NLRC5 is the key factor involved in the regulation of major components of the MHC class I pathway and was considered a critical host factor targeted by cancer cells and virus-infected cells to evade anti-cancer and antiviral immunity (Chelbi and Guarda, 2016; Yoshihama et al., 2016; Yoshihama et al., 2017). NLRC5 imports into the nucleus to function as the transactivator of MHC class I, while it was found mostly located in the cytoplasm of the cell, revealing that the cellular shuttling for NLRC5 to activate MHC I could be a rapid process.

Therefore, we hypothesized that inducing the nuclear accumulation of NLRC5 may be a strategy to promote the expression of MHC class I. Consistent with our hypothesis, gene expression studies in HeLa, MCF7, and 293T cell lines showed that NLRC5 nuclear retention induced by leptomycin B upregulated the induction of MHC class I (Fig. 3), indicating that targeting the nuclear translocation of NLRC5 would be an efficient therapeutic intervention for immune evasion.

In addition, our findings uncovered the molecular mechanisms underlying the nuclear import and retention of NLRC5. The implications of our findings extend beyond fundamental cellular biology, as they present exciting possibilities for clinical applications.

One potential clinical application of our research lies in the realm of viral infections. Viruses often employ various strategies to evade the immune system, including downregulating the expression of MHC class I molecules on infected cells. This evasion mechanism enables viruses to evade recognition and destruction by cytotoxic T cells. By targeting the shuttling mechanism of NLRC5, it may be possible to enhance the nuclear retention and subsequent expression of MHC class I genes, thereby bolstering the immune response against viral infections. This approach could potentially limit viral replication, reduce viral load, and facilitate the clearance of infected cells.

Furthermore, the aberrant regulation of MHC class I expression is also observed in certain types of cancers. Tumor cells can evade immune surveillance by downregulating MHC class I molecules, allowing them to evade recognition by cytotoxic T cells and escape immune-mediated destruction. Our findings suggest that targeting the shuttling mechanism of NLRC5 could represent a promising therapeutic strategy to restore MHC class I expression in cancer cells. By doing so, we may enhance the immune recognition of tumor cells, bolstering the efficacy of immunotherapies and promoting anti-tumor immune responses.

However, it is important to note that the clinical applicability of our findings requires further

investigation. Future research efforts should focus on assessing the feasibility and efficacy of targeting the shuttling mechanism of NLRC5 in preclinical and clinical settings. Additionally, it would be valuable to explore the potential interactions and synergies between NLRC5 modulation and existing therapeutic approaches, such as antiviral drugs or immunotherapies.

In conclusion, our study provides crucial insights into the nuclear import and retention of NLRC5, highlighting its impact on MHC class I gene expression. These findings have exciting implications for potential therapeutic interventions in virus infections and cancers. With further research and development, targeting NLRC5 shuttling mechanisms could offer new avenues for combating viral infections and improving the efficacy of cancer treatments.

Acknowledgments

The authors thank C. Matsukawa and K. Ogawa for secretarial assistance, and Dr. Ji-Seung Yoo for invaluable guidance and support for this project. We thank all members of the Department of Immunology of the Graduate School of Medicine at Hokkaido University for their support during this study. We thank Dr. Peter Van den Elsen, and Dr. Shigetsugu Hatakeyama for providing reagents.

Author contributions

Baohui Zhu: Conceptualization, Methodology, Investigation, Visualization, Writing - Original Draft. Ryota Ouda: Investigation, Writing - Review & Editing. Ning An: Investigation. Tsutomu Tanaka: Writing - Review & Editing. Koichi S Kobayashi: Supervision, Writing- Reviewing and Editing.

Funding

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKEN under grant numbers 19K21250, 20K21511, 22H02883, 22KK0112, 19K1668109 and 22K19415, the Japan Agency for Medical Research and Development (AMED) under Grants JP223fa627005 and 23ym0126801j0002, the Japan Science and Technology (JST) START University Ecosystem Promotion Type under the grant number JPMJST2284, Takeda Science Foundation, Bristol Myers Squibb, SENSHIN Medical Research Foundation, Hitachi Global Foundation, Kobayashi Foundation and The Toyo Suisan Foundation. B.Z. is the recipient of a fellowship from China Scholarship Council.

Disclosure of conflict of interest

The authors declare no conflict of interest.

References

Baldeon, M.E., Neece, D.J., Nandi, D., Monaco, J.J., and Gaskins, H.R. (1997). Interferon-gamma independently activates the MHC class I antigen processing pathway and diminishes glucose responsiveness in pancreatic beta-cell lines. Diabetes *46*, 770-778.

Benko, S., Magalhaes, J.G., Philpott, D.J., and Girardin, S.E. (2010). NLRC5 limits the activation of inflammatory pathways. Journal of immunology *185*, 1681-1691.

Boss, J.M., and Jensen, P.E. (2003). Transcriptional regulation of the MHC class II antigen presentation pathway. Current opinion in immunology *15*, 105-111.

Braun, T., Schofield, P.R., and Sprengel, R. (1991). Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. The EMBO journal *10*, 1885-1890.

Chelbi, S.T., and Guarda, G. (2016). NLRC5, a promising new entry in tumor immunology. Journal for immunotherapy of cancer *4*, 39.

Chi, N.C., Adam, E.J., and Adam, S.A. (1995). Sequence and characterization of cytoplasmic nuclear protein import factor p97. The Journal of cell biology *130*, 265-274.

Cressman, D.E., O'Connor, W.J., Greer, S.F., Zhu, X.S., and Ting, J.P. (2001). Mechanisms of nuclear import and export that control the subcellular localization of class II transactivator. Journal of immunology *167*, 3626-3634.

Dhatchinamoorthy, K., Colbert, J.D., and Rock, K.L. (2021). Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. Frontiers in immunology *12*, 636568.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Fruh, K., and Yang, Y. (1999). Antigen presentation by MHC class I and its regulation by interferon gamma. Current opinion in immunology *11*, 76-81.

Fujimaki, H., Hikawa, N., Nagoya, M., Nagata, T., and Minami, M. (1996). IFN-gamma induces expression of MHC class I molecules in adult mouse dorsal root ganglion neurones. Neuroreport *7*, 2951-2955.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-311.

Gobin, S.J., van Zutphen, M., Westerheide, S.D., Boss, J.M., and van den Elsen, P.J. (2001). The MHC-specific enhanceosome and its role in MHC class I and beta(2)-microglobulin gene transactivation. Journal of immunology *167*, 5175-5184.

Gorlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science *271*, 1513-1518.

Hake, S.B., Masternak, K., Kammerbauer, C., Janzen, C., Reith, W., and Steimle, V. (2000). CIITA leucine-rich repeats control nuclear localization, in vivo recruitment to the major histocompatibility complex (MHC) class II enhanceosome, and MHC class II gene transactivation. Molecular and cellular biology *20*, 7716-7725.

Hewitt, E.W. (2003). The MHC class I antigen presentation pathway: strategies for viral immune evasion. Immunology *110*, 163-169.

Kobayashi, K.S., and van den Elsen, P.J. (2012). NLRC5: a key regulator of MHC class I-dependent immune responses. Nature reviews Immunology *12*, 813-820.

Kobe, B., and Deisenhofer, J. (1993). Crystal structure of porcine ribonuclease inhibitor, a

protein with leucine-rich repeats. Nature *366*, 751-756.
Lange, A., Mills, R.E., Lange, C.J., Stewart, M., Devine, S.E., and Corbett, A.H. (2007). Classical nuclear localization signals: definition, function, and interaction with importin alpha. The Journal of biological chemistry *282*, 5101-5105.
LeibundGut-Landmann, S., Waldburger, J.M., Krawczyk, M., Otten, L.A., Suter, T., Fontana, A., Acha-Orbea, H., and Reith, W. (2004). Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. European journal of immunology *34*, 1513-1525.
Masternak, K., Muhlethaler-Mottet, A., Villard, J., Zufferey, M., Steimle, V., and Reith, W. (2000). CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. Genes & Development *14*, 1156-1166.
Meissner, T.B., Li, A., Biswas, A., Lee, K.H., Liu, Y.J., Bayir, E., Iliopoulos, D., van den Elsen, P.J., and Kobayashi, K.S. (2010). NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. Proceedings of the National Academy of Sciences of the United States of America *107*, 13794-13799.
Meissner, T.B., Li, A., and Kobayashi, K.S. (2012a). NLRC5: a newly discovered MHC class I transactivator (CITA). Microbes and infection *14*, 477-484.
Meissner, T.B., Li, A., Liu, Y.J., Gagnon, E., and Kobayashi, K.S. (2012b). The nucleotide-binding domain of NLRC5 is critical for nuclear import and transactivation activity. Biochemical and biophysical research communications *418*, 786-791.
Meissner, T.B., Liu, Y.J., Lee, K.H., Li, A., Biswas, A., van Eggermond, M.C., van den Elsen, P.J., and Kobayashi, K.S. (2012c). NLRC5 cooperates with the RFX transcription factor complex to induce MHC class I gene expression. Journal of immunology *188*, 4951-4958.
Miyamoto, Y., Hieda, M., Harreman, M.T., Fukumoto, M., Saiwaki, T., Hodel, A.E., Corbett, A.H., and Yoneda, Y. (2002). Importin alpha can migrate into the nucleus in an importin beta- and Ran-independent manner. The EMBO journal *21*, 5833-5842.
Motyan, J.A., Bagossi, P., Benko, S., and Tozser, J. (2013). A molecular model of the full-length human NOD-like receptor family CARD domain containing 5 (NLRC5) protein. BMC bioinformatics *14*, 275.
Nickerson, K., Sisk, T.J., Inohara, N., Yee, C.S., Kennell, J., Cho, M.C., Yannie, P.J., 2nd, Nunez, G., and Chang, C.H. (2001). Dendritic cell-specific MHC class II transactivator contains a caspase recruitment domain that confers potent transactivation activity. The Journal of biological chemistry *276*, 19089-19093.
Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. Annual review of immunology *16*, 323-358.
Shastri, N., Cardinaud, S., Schwab, S.R., Serwold, T., and Kunisawa, J. (2005). All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. Immunological reviews *207*, 31-41.
Spilianakis, C., Papamatheakis, J., and Kretsovali, A. (2000). Acetylation by PCAF enhances CIITA nuclear accumulation and transactivation of major histocompatibility complex class II genes. Molecular and cellular biology *20*, 8489-8498.
Steimle, V., Siegrist, C.A., Mottet, A., Lisowska-Grospierre, B., and Mach, B. (1994). Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. Science *265*, 106-109.

Ting, J.P., Lovering, R.C., Alnemri, E.S., Bertin, J., Boss, J.M., Davis, B.K., Flavell, R.A., Girardin, S.E., Godzik, A., Harton, J.A., *et al.* (2008). The NLR gene family: a standard nomenclature. Immunity *28*, 285-287.
van den Elsen, P.J., Gobin, S.J., van Eggermond, M.C., and Peijnenburg, A. (1998a). Regulation of MHC class I and II gene transcription: differences and similarities. Immunogenetics *48*, 208-221.
van den Elsen, P.J., Peijnenburg, A., van Eggermond, M.C., and Gobin, S.J. (1998b). Shared regulatory elements in the promoters of MHC class I and class II genes. Immunology today *19*, 308-312.
Vijayan, S., Sidiq, T., Yousuf, S., van den Elsen, P.J., and Kobayashi, K.S. (2019). Class I transactivator, NLRC5: a central player in the MHC class I pathway and cancer immune surveillance. Immunogenetics *71*, 273-282.
Wilmanski, J.M., Petnicki-Ocwieja, T., and Kobayashi, K.S. (2008). NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. Journal of leukocyte biology *83*, 13-30.
Wolff, B., Sanglier, J.J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chemistry & biology *4*, 139-147.
Xu, W., Edmondson, D.G., and Roth, S.Y. (1998). Mammalian GCN5 and P/CAF acetyltransferases have homologous amino-terminal domains important for recognition of nucleosomal substrates. Molecular and cellular biology *18*, 5659-5669.
Yoo, J.S., Sasaki, M., Cho, S.X., Kasuga, Y., Zhu, B., Ouda, R., Orba, Y., de Figueiredo, P., Sawa, H., and Kobayashi, K.S. (2021). SARS-CoV-2 inhibits induction of the MHC class I pathway by targeting the STAT1-IRF1-NLRC5 axis. Nature communications *12*, 6602.
Yoshihama, S., Roszik, J., Downs, I., Meissner, T.B., Vijayan, S., Chapuy, B., Sidiq, T., Shipp, M.A., Lizee, G.A., and Kobayashi, K.S. (2016). NLRC5/MHC class I transactivator is a target for immune evasion in cancer. Proceedings of the National Academy of Sciences of the United States of America *113*, 5999-6004.
Yoshihama, S., Vijayan, S., Sidiq, T., and Kobayashi, K.S. (2017). NLRC5/CITA: A Key Player in Cancer Immune Surveillance. Trends in cancer *3*, 28-38.
Zhou, F. (2009). Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. International reviews of immunology *28*, 239-260.