INTRODUCTION

Fungal infections are major public health threats, especially HIV patients, immune-compromised people with hematopoietic stem cell transplantation (HSCT) and chemotherapy, or primary immune deficiencies. Recent studies also found that lots of fungal species bloom in the gut of COVID-19 patients. Many species of fungi are responsible for invasive fungal infections (IFIs), and about 1.5 million people die from IFI yearly. Candida albicans are among the most prevalent opportunistic fungal pathogens of humans. Although several anti-fungal drugs are effective, the mortality rate of Candida infections exceeds 40%. Current antifungal drugs for IFI treatment are limited and may cause undesirable side effects. Moreover, the rapid emergence of drug resistance is a growing problem. Therefore, a better understanding of how the host immune system counteracts fungal infections is vital for the development of novel therapeutic strategies to combat candidiasis.

During fungal infection, CLRs and the Toll-like receptor (TLR) play essential roles in host defense against fungal pathogens by recognizing various fungal surface components. The CLRs are mainly expressed on neutrophils, macrophages, and dendritic cells such as Dectin-1, Dectin-2, Dectin-3, and Mincle, and they recognize β-glucan, α-mannan, and glycolipids of fungi, respectively. Upon recognition of respective ligands, CLRs initiate the phosphorylation of the Tyr-X-X-leu motif (termed ITAM) in the Dectin-1 cytoplasmic tail the adaptor FcRγ and recruitment of FcRγ to Dectin-2 or Mincle, which serves as a docking site for SYK. These events promote SYK translocation and activation, which then activates PLCγ and PKCδ. The central adaptor protein caspase recruitment domain-containing protein 9 (CARD9), B cell leukemia-lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue 1 (MALT1) then form the CARD9–BCL10–MALT1 (CBM) complex to activate mitogen-activated protein (MAP) kinase, along with NF-κB and NFAT transcription factors, leading to the production of proinflammatory cytokines and chemokines. Moreover, these cytokines and chemokines promote neutrophil infiltration, macrophage maturation, and T cell differentiation. The Th1 and Th17 subsets of T helper cells have been demonstrated to participate in host defense against fungal infection via IFN-γ, IL-17A, and IL-17F production, which further activate and recruit macrophages and neutrophils.

SYK is a non-receptor tyrosine kinase that contains two Src homology 2 (SH2) domains and a carboxy-terminal kinase domain. Further, there are two domains (termed interdomains A and B) located, respectively, between the two SH2 domains and between the kinase domain and the second SH2 domain. SYK exists in an autoinhibited structure due to the combination of interdomain A and interdomain B with the kinase domain, which blocks the substrates of access to the catalytic domain in resting cells. As an essential adaptor and kinase of the CLR signaling complex proximal to the plasma membrane, SYK activity must be fine-tuned during fungal infection. Previous study suggested that the activation of this autoinhibited confirmation may be determined by interaction partners or post-translational modifications rather than by intramolecular interactions. For instance, ZAP70, one of the SYK tyrosine kinase family, is ubiquitinated by NRD1, which

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dephosphorylates Zap70 and terminates TCR signaling through recruiting the phosphatase-like proteins STS1 and STS2. It has also been reported that the ubiquitination of SYK plays a critical role in innate antifungal immunity. SYK can be catalyzed for Lys48- or K63-linked polyubiquitination by E3 ubiquitin ligase CBL-B, leading to the degradation of phosphorylated SYK. However, whether SYK activation is being modified by other E3 ubiquitin ligases besides CBL-B is completely unknown.

Members of the tripartite motif (TRIM) family proteins are well known as E3 ubiquitin ligases which contain a conserved RING-finger domain, one or two B-boxes, and a coiled-coil domain. The TRIM family participates in various biological processes including fighting against HIV and tumor progression by modulating the K48- or K63-linked polyubiquitination to the target. Several members, including TRIM10, TRIM15, TRIM26, TRIM27, TRIM31, TRIM34, TRIM38, TRIM39, and TRIM40, are shown to be involved in innate immune responses, possibly due to the encoding genes locating in the locus which encoding the major histocompatibility complex (MHC) class I proteins. Our laboratory has previously reported that TRIM31 is involved in RIG-I-like receptors (RLR) signaling during RNA virus infection. TRIM31 promotes the catalyzation of K63-linked polyubiquitination of mitochondrial antiviral signaling protein (MAVS), and this modification facilitates MAVS aggregation which further induces Interferon-β (IFN-β) production to inhibit virus replication. We also found that TRIM31 can promote proteasomal degradation of NLR Family Pyrin Domain Containing 3 (NLRP3) and therefore inhibits NLRP3 inflammasome activation and attenuates the DSS-induced colitis inflammation. Until now, the role of the TRIM family during fungal infection had rarely been reported.

In this study, we screened several important members of the TRIM family to investigate the possible roles of this family in regulating SYK activity during fungal infections. We identified that TRIM31 is an essential positive regulator for SYK. Particularly, TRIM31 catalyzed the K27-linked polyubiquitination at Lys375 and Lys517 of SYK, which subsequently promoted SYK translocation and binding to CLR(s), and decreased the association of SYK with the phosphatase SHP-1. This therefore up-regulated SYK kinase phosphorylation and activity. Consequently, TRIM31 protected hosts from the lethal systemic infection with C. albicans in the mouse model by increasing inflammatory cytokines and chemokines production, which aided in clearing the pathogen more efficiently. Overall, our study first reported that SYK was modified through K27-linked polyubiquitination by TRIM31 upon fungal infection, and also uncovered its significance in positively regulating the activation of SYK-associated signaling cascades and the resultant anti-fungal immunity.

RESULTS
TRIM31 facilitates K27-linked polyubiquitination of SYK to regulate…

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Notably, mutation at both loci (K375R, K517R) substantially attenuated the K27-linked ubiquitination of SYK to the basal level (without TRIM31 catalyzation) (Fig. 1f). Taken together, these results suggest that TRIM31 directly binds with and catalyzes the K27-linked polyubiquitination of SYK at Lys375 and Lys517.

TRIM31 deficiency impaired anti-fungal immune responses in vivo. Others and we have demonstrated that TRIM31 plays an essential role in the regulation of innate antiviral immunity.39,47,48 However, whether TRIM31 plays a critical role in innate antifungal immunity is not known. Given that TRIM31 catalyzes the polyubiquitination...
of SYK, we proposed that TRIM31 might influence anti-fungal immunity.

To confirm the in vivo function of TRIM31 anti-fungal immune responses, we intravenously infected Trim31−/− and Trim31+/+ mice with a lethal dose of C. albicans. The result showed that Trim31−/− mice were more sensitive to fungal infection than Trim31+/+ mice (Fig. 2a). While Trim31−/− mice manifested a massive loss of weight after infection with C. albicans and finally died of infection, Trim31+/+ mice showed moderate loss of weight, with 45% survival and recovery (Fig. 2a).

To further elucidate the function of TRIM31 in anti-fungal immunity, we assessed the fungal loads in mice at 5 days after infection. We found Trim31−/− mice exhibited more C. albicans colony forming units (CFU) in the kidney, spleen, and liver, compared to that in Trim31+/+ mice (Fig. 2b). Histopathologic analysis of kidneys demonstrated that Trim31−/− mice exhibited increased renal inflammation and lots of C. albicans yeast cells and hyphae (Fig. 2c, d). Besides, the neutrophils infiltrated into the kidneys of infected Trim31−/− mice were significantly higher than those in Trim31+/+ mice (Fig. 2e). Further, we assessed the innate immune response of C. albicans-infected Trim31−/− and Trim31+/+ mice. At early time points of C. albicans infection, Trim31−/− mice showed significantly reduced secretion of IL-6, TNF-α, IL-12, CXCL1, and CXCL2 in their serum at 24 h post-infection as compared with Trim31+/+ mice (Fig. 2f). Consistently, Trim31−/− mice also had lower levels of IL-6 and TNF-α in the homogenates of kidney and liver after 5 days of infection with C. albicans (Fig. 2g).

To investigate the cellular basis of TRIM31 in anti-fungal protective function, we reconstructed lethally irradiated Trim31−/− mice with syngeneic Trim31+/− or Trim31+/+ bone marrow (BM) to generate BM-chimeric mice. Trim31+/+ mice reconstituted with Trim31−/− BM displayed a phenotype similar to that of mice with total Trim31 deficiency after fungal infection (Fig. 2h), indicating that the radiosensitive hematopoietic cells were involved in the anti-fungal immune response. Taken together, these results suggest that TRIM31 positively regulates the anti-fungal immune response in vivo.

TRIM31 regulates anti-fungal Th1 and Th17 responses

Upon sensing fungal PAMPs, CLRs can induce or modulate Th1 and Th17 responses. We next sought to determine whether TRIM31 regulates Th1 and Th17 responses after C. albicans infection.

After 5 days of infection with C. albicans, real-time PCR assay showed that expression of the genes encoding Ifng, Il17a, and Il17f was attenuated in the kidneys from Trim31−/− mice compared to Trim31+/+ mice (Fig. 3a). Further, we isolated spleen cells from Trim31−/− and Trim31+/+ mice infected with or without C. albicans. We found re-stimulation of the spleen cells with HKCA-Y from Trim31+/+ mice infected with C. albicans greatly increased the secretion of IL-17A and IFN-γ (Fig. 3b). While the secretion of IL-17A and IFN-γ was greatly attenuated in the spleen cells from infected Trim31−/− mice upon re-stimulation with HKCA-Y (Fig. 3b). We also investigated the T-cell differentiation in the spleen at day 5 after C. albicans infection. The percentage of Th1 and Th17 cells in the spleen was greatly decreased in Trim31−/− mice compared to that in the spleen from Trim31+/+ mice (Fig. 3c, d).

Antibody responses to fungal infection are identified as playing an important role in immune protection.49 C. albicans also is the major inducer of anti-fungal immunoglobulin G (IgG).50 We found that Trim31−/− mice showed significantly reduced production of IgG in their serum after 5 days infection as compared with Trim31+/+ mice. Together, these results demonstrate that TRIM31 is required for the Th1 and Th17 differentiation after C. albicans infection.

TRIM31 regulates anti-fungal innate immunity

SYK is a key adaptor of anti-fungal innate immune response via CLRs. Upon activation, SYK is phosphorylated and modulates the activation of NF-κB and MAPKs, which results in the production of proinflammatory cytokines and chemokines. We next sought to examine the function of TRIM31 in CLR-induced cytokine and chemokine production. Activation CLRs, such as Dectin-1, Dectin-2/3, and Mincl, elicit the production of proinflammatory cytokines and chemokines, which play important roles in anti-fungal innate immunity. We obtained BMDCs from Trim31+/+ and Trim31−/− mice, followed by stimulation with various CLR ligands Zymd (Dectin-1 ligand), α-mannan (Dectin-2/3 ligand), TDB (Mincl ligand), heat-killed C. albicans yeast (HKCA-Y) and heat-killed C. albicans hyphae (HKCA-H). We found that the production of proinflammatory cytokines including IL-6, TNF-α, IL-1β, IL-12, IL-23, and chemokines such as CXCL1 and CXCL2 were markedly reduced in Trim31−/− BMDCs compared to that in Trim31+/+ BMDCs (Fig. 4a and Supplementary Fig. S4a). The deletion of TRIM31 in BMDMs also reduced the production of proinflammatory cytokines including IL-6, TNF-α, IL-1β, IL-12, and chemokines CXCL1 and CXCL2 (Fig. 4b and Supplementary Fig. S4b).

Furthermore, we restored TRIM31 protein expression in Trim31−/− BMDCs and BMDMs through lentiviral infection. WB analysis showed successful restoration of TRIM31 protein expression (Fig. 4c). We found reconstitution of WT TRIM31, rather than the catalytically inactive mutant mTRIM31 (CS2A, CS5A), was able to restore the induction of IL-6 and TNF-α upon stimulation with Zymd or α-mannan stimulation (Fig. 4c), indicating TRIM31 E3 ligase activity was important for CLR-mediated pro-inflammatory cytokines production. Thus, our results indicate that TRIM31 is a positive regulator for cytokine and chemokine production in response to C. albicans infection.

TRIM31 regulates SYK kinase activity

Based on the results above mentioned, we postulated that TRIM31 may affect the CLR pathways by directly regulating SYK kinase activation. Thus, we prepared BMDCs from Trim31+/+ and Trim31−/− mice, followed by stimulation with HKCA-Y and HKCA-H and measured the phosphorylation (active form) of the CLR-induced signaling. We found that the phosphorylation of SYK in Trim31−/− BMDCs was significantly attenuated in response to HKCA-Y and HKCA-H compared to that in Trim31+/+ BMDCs (Fig. 5a, b). SYK-dependent phosphorylation of PLCγ2 and PKCδ were also attenuated in Trim31−/− BMDCs (Fig. 5a, b). Additionally, phosphorylation of the downstream molecule ERK, JNK, and p38...
were down-regulated in Trim31−/− BMDCs as well. Furthermore, activation of NF-κB signaling indicated by p65 phosphorylation was also decreased in Trim31−/− BMDCs (Fig. 5a, b). We obtained similar results in BMDMs (Supplementary Fig. S5a, b). These data indicate that TRIM31 positively regulates SYK-associated signaling cascades in the CLR pathway.

To directly confirm SYK polyubiquitination mediated by TRIM31 is required for SYK activation, Dectin-1, SYK, and WT TRIM31 or TRIM31 (C53A, C56A) were co-expressed in HEK293T cells followed by stimulation with HKCA-Y. Meanwhile, we also transfected Dectin-2, FcRy, SYK, and WT TRIM31 or TRIM31 (C53A, C56A) into HEK293T followed stimulation with HKCA-H. Overexpression of WT
TRIM31 facilitates K27-linked polyubiquitination of SYK to regulate anti-fungal Th1 and Th17 responses.

Fig. 3 TRIM31 is indispensable for anti-fungal Th1 and Th17 responses. a qRT-PCR analysis of Il17a, Il17f from kidneys of Trim31+/+ (n = 6) and Trim31−/− (n = 6) mice infected with C. albicans (2 × 105 fungal cells per mouse) for 5 days. b ELISA analysis of IFN-γ (left) and IL-17A (right) in the supernatant of splenic cells obtained from Trim31+/+ and Trim31−/− mice (n = 6 per group) infected with C. albicans (2 × 105 fungal cells per mouse) for 5 days, followed by stimulation with HKCA-Y (MOI, 1) for 2 days. c, d Splenic cells isolated from the Trim31+/+ or Trim31−/− mice and stimulated as in (b). Intracellular staining of IFN-γ (Th1) and IL-17A (Th17) were determined with flow cytometry. The representative figure is shown in (e), and the results are summarized in (d). e ELISA analysis of IgG in serum from Trim31+/+ and Trim31−/− mice (n = 6 per group) infected with C. albicans (2 × 105 fungal cells per mouse) for 5 days. *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001 (Student’s t-test in a, b, d, e). Data are from one experiment representative of three independent experiments (a, b, d, e; mean ± s.d.)

TRIM31, but not TRIM31 (C53A, C56A), greatly enhanced SYK phosphorylation at the basal level, and fungal stimulation further elevated SYK phosphorylation (Fig. 5c and Supplementary Fig. S5c). To determine whether polyubiquitination of SYK at K375 and K517 sites is required for SYK activation, we overexpressed Dectin-1, SYK, SYK (K375R, K517R) mutant, and TRIM31 into HEK293T cells transfected with Dectin-2, FcR, and SYK before or after stimulation with HKCA-Y (Supplementary Fig. S5d). These results suggest that the K375/S17-linked polyubiquitin chains mediated by TRIM31 is critical for SYK activation.

To investigate whether TRIM31-mediated SYK ubiquitination is involved in antifungal immune responses, we further reconstituted Syk−/− BMDCs with WT SYK or SYK (K375R, K517R) mutant through lentiviral infection. WB analysis showed the successful reconstitution of SYK protein in Syk−/− BMDCs (Fig. 5e). As expected, SYK deficiency attenuated Zymd- or α-mannan-induced IL-6 and TNF-α production (Fig. 5e). Reintroduction of SYK or SYK (K375R, K517R) efficiently restored the induction of IL-6 and TNF-α by Zymd or α-mannan in Syk−/− BMDCs (Fig. 5e). Notably, TRIM31 greatly increased the production of IL-6 and TNF-α in Syk−/− BMDCs reconstituted with WT SYK, whereas SYK (K375R, K517R) mutant-mediated induction of IL-6 and TNF-α was not further increased in the presence of TRIM31 (Fig. 5e). Taken together, these data demonstrate that SYK polyubiquitination at K375 and K517 mediated by TRIM31 is essential for SYK activation and the subsequent pro-inflammatory cytokines production.

TRIM31-mediated polyubiquitination promotes recruitment of SYK to CLRs Ligation with CLR agonist and subsequent recruitment of SYK to the receptors leads to the phosphorylation at Y525/526 (Y519/520 in mice) and activation of SYK to initiate the downstream CLR signaling.5 To investigate the sequential order of the ubiquitina- tion and phosphorylation of SYK during the process of SYK activation, we constructed an SYK mutant (Y525F, Y526F), in which the phosphorylation tyrosine sites at positions 525 and 526 were mutated to phenylalanine. We found that WT SYK and SYK (Y525F, Y526F) had similar binding capacity with TRIM31 (Supplementary Fig. S5a). Of note, TRIM31 could still mediate the ubiquitination of SYK (Y525F, Y526F) similar to that of WT SYK (Supplementary Fig. S6b). These results suggest that SYK phosphorylation is not required for TRIM31-mediated SYK ubiquitination.

Because the binding of SYK to CLRs promotes its phosphoryla- tion/activity, we hypothesized that K27-linked polyubiquitination might affect SYK recruitment to CLRs. To confirm this, HEK293T cells were transfected with Dectin-1, SYK, and TRIM31 or TRIM31(C53A, C56A), then the cells were treated with HKCA-Y or left untreated. Overexpression of WT TRIM31, but not TRIM31 (C53A, C56A), greatly increased the interaction of Dectin-1 and SYK, and fungal stimulation further elevated the association between Dectin-1 and SYK (Fig. 6a). Similarly, the interaction between SYK and FcRγ was also increased in the presence of WT TRIM31, but not TRIM31 (C53A, C56A) in HEK293T cells transfected with Dectin-2, FcRγ, and SYK before or after stimulation with HKCA-H (Supplementary Fig. S6c). These data suggest that the elevated interaction between SYK and Dectin-1/Dectin-2-FcRγ is dependent on TRIM31 catalytic activity.

Because TRIM31-mediated polyubiquitination of SYK at K375 and K517 (Fig. 1f), we hypothesized that K375 and K517 within SYK are important for the recruitment of SYK to Dectin-1. We then transfected Dectin-1, TRIM31, WT SYK, or SYK (K375R, K517R) into HEK293T cells, followed by fungal stimulation or left untreated. We found that TRIM31 greatly increased the interaction between WT SYK and Dectin-1. However, this increased interaction is not
observed when SYK was mutated at K375 and K517 (Fig. 6b). Similarly, the interaction between WT SYK and FcRγ was increased in the presence, while the interaction between SYK (K375R, K517R) and FcRγ was unchanged in the presence of TRIM31 (Supplementary Fig. S6d). Further, we showed that Zymd and α-mannan stimulation in BMDCs increased the interaction between SYK and Dectin-1 or FcRγ (Fig. 6c, d). However, Zymd-induced and α-mannan-induced interactions between SYK and Dectin-1/FcRγ were decreased in Trim31−/− BMDCs compared to that in Trim31+/+ BMDCs (Fig. 6c, d). Thus, our results highlight the important role of TRIM31 in modulating the interaction between SYK and CLR receptors.

TRIM31 promotes SYK translocation to the membrane

CLRs are a family of transmembrane proteins, but SYK resides predominantly in the cytosol in resting BMDCs. SYK is translocated to the cell membrane, where it interacts with CLRs upon CLR ligation. To independently verify whether TRIM31 promotes SYK recruitment to cell membrane followed stimulation, we prepared cell membrane and cytosolic fractions from BMDCs after stimulation with Zymd or α-mannan. We found these stimulations could induce SYK translocation to the membrane, while SYK membrane translocation was greatly decreased in Trim31−/− BMDCs (Fig. 6e, f). To directly visualize the translocation of SYK to the cell membrane, confocal microscopy was performed. In resting cells, SYK was found to be diffusely distributed in the cell cytosol of BMDCs, and Zymd or α-mannan stimulation recruited SYK to the cell membrane and colocalized with Dectin-1 or FcRγ. However, the colocalization was decreased in Trim31−/− BMDCs (Fig. 6g, h). Previous studies have shown that Dectin-1 agonists could induce a rearrangement of the cell membrane and trigger cellular phagocytosis.52 We also found Zymd induced fewer phagosomes in Trim31−/− BMDCs (Fig. 6g). In conclusion, these data suggest that TRIM31-mediated polyubiquitination promotes SYK recruitment to Dectin-1 or Dectin-2-FcRγ.

TRIM31 inhibits SHP-1-mediated SYK dephosphorylation

The non-receptor protein tyrosine phosphatases SHP-1 is found to regulate SYK activation negatively by dephosphorylating SYK.53–55 To investigate whether TRIM31-mediated SYK ubiquitination
affected the binding between SYK and SHP-1, we prepared BMDCs from Trim31+/+ and Trim31−/− mice and stimulated with Zymd or α-mannan. We found the interaction between SYK and SHP-1 was increased in Trim31−/− BMDCs compared to that in Trim31+/+ BMDCs (Fig. 7a, b). Meanwhile, we co-expressed SYK, SHP-1, and WT TRIM31 or TRIM31 (C53A, C56A) in HEK293T cells. WT TRIM31, but not TRIM31 (C53A, C56A) attenuated the interaction between SYK and SHP-1 (Fig. 7c). This suggests that the enzymatic activity of TRIM31 is vital for preventing the interaction between SYK and SHP-1.
To further explore whether TRIM31 influences the dephosphorylation of SYK by SHP-1, we co-expressed the abovementioned proteins in HEK293T cells. As expected, transfection of SHP-1 greatly attenuated SYK phosphorylation, while, transfection of WT TRIM31, but not TRIM31 (CSA, CSA6A), greatly inhibited SHP-1-mediated SYK dephosphorylation (Fig. 7d). This indicated that TRIM31 is essential for sustaining SYK phosphorylation. To study whether polyubiquitination of SYK at K375 and K517 sites is required for SHP-1-mediated dephosphorylation. We also co-expressed Dectin-1, WT SYK, SYK (K375R, K517R), SHP-1, and TRIM31 in HEK293T cells. WT SYK and SYK (K375R, K517R) mutant were dephosphorylated by SHP-1 as expected (Fig. 7e). Overexpression of TRIM31 attenuated SHP-1-mediated WT SYK dephosphorylation, while, TRIM31 did not prevent SHP-1-mediated dephosphorylation of SYK (K375R, K517R) (Fig. 7e). Together, these data highlight that TRIM31-mediated polyubiquitination is essential in sustaining SYK phosphorylation by inhibiting interaction with SHP-1.

**DISCUSSION**

As a key regulator in mounting immune responses against fungal infection, SYK activation is tightly regulated by phosphorylation of the dual tyrosine residues in the linker and kinase domains. In resting state, the two N-terminal SH2 domains of SYK are distorted in a way preventing its binding with the ITAM motif of Dectin-1/FcγR. Thus SYK is kept in an inactive status. Upon the engagement with β-glucan-containing particles, the cognate receptor Dectin-1 becomes phosphorylated immediately at its ITAM motif, which is believed to be initiated through the Src tyrosine kinase. Then SYK is recruited to the ITAM motif, and further phosphorylates other SYK molecules. The downstream CLR-signaling further activates the downstream CLR-signaling cascades. However, whether SYK activity is modulated by other post-translational modifications, such as ubiquitination, has not been studied extensively yet.

Here, we identified that TRIM31 functions as an essential regulator for SYK action and the resultant innate antifungal immunity. Mechanistically, TRIM31 specifically catalyzed SYK at K375 and K517 for K27-linked polyubiquitination, which promoted SYK binding to the ITAM motifs of Dectin-1 and FcγR, and decreased the association with SHP-1, thus facilitated SYK phosphorylation. In addition, Trim31-deficient BMDCs and BMDMs showed significantly reduced production of pro-inflammatory cytokines and decreased CLR-mediated signaling activation (Fig. 8). Consistently, Trim31-deficient mice were more sensitive to fungal infection than Trim31+/− mice. To our knowledge, this is the first report defining the critical role of SYK polyubiquitination in the regulation of SYK activation and antifungal immunity.

Protein ubiquitination is a way involved in regulating the proteolysis of SYK. The E3 ligases CBL-B associates with SYK and ubiquitinates SYK for K48-linked polyubiquitination, the ubiquitinated SYK is subsequently recruited to lysosomes for degradation. However, only the phosphorylated SYK can be ubiquitinated and degraded by CBLB as reported. Instead, our study identified that the E3 ligase TRIM31 interacts and catalyzes K27-linked polyubiquitination of SYK at K375 and K517 upon fungal stimulation. Interestingly, SYK ubiquitination occurs ahead of phosphorylation.

And the K27-linked SYK polyubiquitination promotes its translocation to the plasma membrane, which may further amplify the SYK phosphorylation. Thus, our results demonstrate that TRIM31 functions are completely different from CBLB by catalyzing K27-polyubiquitination, and this modification positively regulates SYK-associated downstream signaling activation.

We identified K375 and K517 within SYK as the ubiquitination sites mediated by TRIM31. Ubiquitination at these two lysine residues is important for SYK interaction with CLRs, membrane translocation, and inhibition of binding with SHP-1. K375 localizes close to Tyr348 and Tyr352 within the interdomain B, whose phosphorylation is essential for the disruption of the inactive conformation of SYK and results in kinase activation. K517 is close to Tyr525 and Tyr526, which localize in the activation loop of SYK. Since K27-linked polyubiquitination of SYK at these two sites (K375, K517) promotes its phosphorylation/kinase activity, we propose that polyubiquitination might facilitate the shift of SYK conformation from an inactive status to the active status with the kinase domain exposed.

Previous researches have shown that the members of the TRIM family are greatly involved in a broad range of biological processes, such as the life cycle of HIV, tumor development, and progression. However, the role of the TRIM family in anti-fungal immunity is less reported. Trim62 is the only member found to be involved in antifungal immunity through catalyzing CARD9 polyubiquitination at K125 and impacting its activation and the subsequent fungal clearance. Notably, our study extends the current appreciation of this family in antifungal responses by elucidating the significance of TRIM31 in modulating SYK activation. We performed a screening of this family on the role of ubiquitinating SYK and found that TRIM31 specifically interacts and ubiquitinates SYK. Interestingly, several members such as TRIM26, TRIM40, TRIM65, which have previously been reported to be responsible for anti-viral responses by regulating Interferon-β production, are not involved in SYK post-modification. In innate antiviral immune, TRIM31 has been reported as an enhancer to promote aggregation and activation of MAVS. Based on our findings, TRIM31 is also as a positive factor to regulate innate antifungal immune. So, we think the protective effect of TRIM31 in antiviral and antifungal is consistent. TRIM31 can bind with different adaptor proteins to induce more pro-inflammatory cytokines or interferon through different pathways against fungal pathogens or virus infection in innate immune cells. Collectively, our results indicate that members of the TRIM family play distinct and exclusive roles in combating various pathogens, and TRIM31 is essential for fighting against fungal pathogens via catalyzing K27-linked SYK polyubiquitination.

The previous report has shown that TRIM31 promotes Atg5/Atg7-independent autophagy in a palmitoylation-dependent manner, and eliminates the invading bacteria in intestinal cells. Similarly, autophagy is a critical tool for the killing of fungal pathogens. Fungal pathogens are recognized by CLRs and are engulfed, thus trigger activation of SYK, ultimately result in the recruitment of the microtubule-associated protein 1A/1B-light chain 3 (LC3)-conjugation machinery consisting of the Atg5 and Atg12. Moreover, fungal pathogens also trigger the LC3-associated phagocytosis pathway. As noted previously, TRIM31...
may promote the elimination of invading fungal through autophagy, besides promoting K27-linked polyubiquitination of SYK. Of course, we need to confirm this function in the future.

In summary, we demonstrated the crucial role of TRIM31 in antifungal responses by catalyzing K27-linked polyubiquitination of SYK at K375 and K517. This facilitates the recruitment of SYK to the phosphorylated ITAMs of upstream CLR receptors for SYK activation. It also decreases the association of SYK and SHP-1 to sustain SYK activity. Overall, our work elucidates the mechanisms by which TRIM31 modulates SYK activity, and also highlights the potential therapeutic implications of these findings.
Fig. 6 TRIM31 positively regulates translocation of SYK to the membrane and colocalization with Dectin-1 and FcRγ. a HEK293T cells were cotransfected with Myc-Dectin-1, V5-SYK, Flag-TRIM31, or Flag-TRIM31 (C53A, C56A) by various combinations, cells were unstimulated or stimulated with HKCA-Y (MOI, 2) for 15 min. Then cell lysates were IP with anti-Myc and WCL, respectively, probed with antibodies (left margins). b HEK293T cells were transfected with Myc-Dectin-1, V5-SYK, V5-SYK (K375R, K517R), and Flag-TRIM31 by various combinations, cells were unstimulated or stimulated with HKCA-Y (MOI, 2) for 15 min. Followed by IP with anti-Myc and WCL, respectively, probed with antibodies (left margins). c, d Trim31+/+ and Trim31−/− BMDCs unstimulated (0 min) or stimulated with Zymd (e) or α-mannan (d) for 15 or 30 min, followed by IP with anti-SYK or anti-FcRγ, probed with antibodies (left margins). e, f Trim31+/+ and Trim31−/− BMDCs were stimulated with Zymd (e) or α-mannan (f) for 15 or 30 min, and whole-cell lysates were separated into membrane and cytosolic fractions. Immunoblot analysis with antibodies (left margins). g, h Using SYK-conjugated specific antibody (green), Dectin-1 or FcRγ conjugated specific antibody (red) while the nuclear compartment was observed by DAPI-staining of DNA (blue). Scale bars, 20 µm. Data are from one experiment representative of three independent experiments (a–h).

Fig. 7 TRIM31 inhibits SHP-1 regulation SYK activation. a, b Trim31+/+ and Trim31−/− BMDCs uninfected (0 min) or infected Zymd (a) or α-mannan (b) for 15 min and 30 min, followed by IP with anti-SYK, probed with anti-SHP-1, WCL immunoblot analysis with indicated antibodies (left margins). c HEK293T cells were transfected with Myc-SYK, V5-SHP-1 and Flag-TRIM31, or Flag-TRIM31 (C53A, C56A). Followed by IP with anti-V5, probed with indicated antibodies (left margins). d HEK293T cells were transfected with Myc-SYK, Myc-Dectin-1, V5-SHP-1, Flag-TRIM31, or Flag-TRIM31 (C53A, C56A) by various combinations, cells were unstimulated or stimulated with HKCA-Y (MOI, 2) for 15 min, WCL immunoblot analysis with indicated antibodies (left margins). e HEK293T cells transfected by various combinations of plasmids expressing Flag-SYK, Flag-SYK (K375R, K517R), Myc-Dectin-1, V5-SHP-1, and Flag-TRIM31, cells were unstimulated or stimulated by HKCA-Y (MOI, 2) for 15 min. WCL immunoblot analysis with indicated antibodies (left margins). Data are from one experiment representative of three independent experiments (a–e).
Fig. 8  A hypothetical model of the role of TRIM31 in anti-fungal immunity response. TRIM31 catalyzes the K27-linked polyubiquitination of SYK, which can disrupt the autoinhibited status of SYK, promote SYK binding to CLRs, decrease the association of SYK and SHP-1, leading to the increased SYK kinase activity (left). TRIM31 deficiency leads less SYK translocation to the membrane, produces lower amounts of pro-inflammatory cytokines and chemokine in response to C. albicans infection (right).
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transcribed from total RNA with a PrimeScript RT-PCR Kit (Takara). Supplementary Table S2 included all primers used for RT-PCR assays. Reverse-transcribed samples were amplified by CFX Connect Real-Time PCR Detection System (BIO-RAD) using the SYBR Green PCR Master Mix (Roche). *actin* was used as an internal control, used the 2^{-ΔΔCt} method to calculate relative expression changes.

**ELISA**
Based on the manufacturer’s instructions, the concentrations of mouse IL-6, TNF-α, IL-12P40, IL-12P70, IL-1β, IL-17A, and IFN-γ in tissue homogenates or cell culture supernatants were determined by ELISA Kits (Dakewe Biotech). The concentrations of mouse IL-23, CXCL1, CXCL2, and IgG in cell culture supernatants or serum were determined by ELISA Kits (Multi sciences).

**Immunofluorescence staining and confocal analysis**
For immunofluorescence staining,-fixed cells were incubated with parafomaldehyde (4%, Beyotime) for 20 min, then permeabilized using Triton X-100 (0.1%, Beyotime) for 20 min and blocked for 1 h in 1% BSA (Beyotime) at room temperature. Then the cells were incubated with indicated primary antibodies (Supplemental Table S1) overnight at 4°C, then cells were washed in wash buffer (Beyotime) four times and incubation with Alexa Fluor 568 (Invitrogen) or Alexa Fluor 488 (Invitrogen) secondary antibodies for 1 h at room temperature, after washing in wash buffer for four times, cells were counterstained with DAPI (Abcam). Cells were imaged with confocal laser microscopy (LSM780, Carl Zeiss).

**Immunoblot, immunoprecipitation, and ubiquitination assay**
For immunoblot analysis, cells were lysed with Cell Lysis Reagent (sigma) supplemented with a phosphatase inhibitor (Roche) and protease inhibitor cocktail (sigma). A bicinchoninic acid assay (Thermo fisher) was used to measure the protein concentrations in the extracts, then the protein concentrations were made equal in different samples with cell lysis. For immunoprecipitation (IP), whole-cell extracts were collected in IP buffer, which contained 1% (vol/vol) NP-40, 50 mM EDTA, 150 mM NaCl, 50 mM Tris–HCl (pH 7.4) and a protease inhibitor cocktail (Sigma). After centrifugation for 15 min at 14,000×g, supernatants were collected and were made equal in different samples, incubated with 1–2 μg of the corresponding antibodies for 6 h, then Protein A/G beads (Santa Cruz Biotechnology) were added to the supernatants at 4°C and incubation for 2 h, beads were washed five times with IP buffer at 4°C. Proteins were eluted with a 2× protein loading buffer from the beads. Then being boiled for 10 min, all the samples were fractionated by 10% SDS–PAGE and were transferred to a PVDF membrane and then probed with the specific antibodies. For analysis of the ubiquitination of SYK or other molecules in HEK293T cells, HEK293T was transfected with HA-Ub (WT) or HA-Ub mutants, Myc-SYK and Flag-TRIM31, and then the cell lysates were immunoprecipitated with anti-Myc and analyzed by immunoblot with anti-HA antibody. For analysis of the ubiquitination of SYK in BMDCs, BMDCs were stimulated with α-mannan or Zymd, then cell lysates were immunoprecipitated with anti-SYK and analyzed by immunoblot with anti-ubiquitin (WT, K27, K48, K63).

In vitro binding assay
SYK and TRIM31 proteins were purchased from (GeneCopoeia), then mixing SYK and TRIM31 together, followed by IP with anti-TRIM31 and WB with anti-SYK.

**Cell fractionation**
BMDCs were stimulated with α-mannan or Zymd, then cells were performed by membrane protein extraction kit (Thermo Scientific), following standard protocols recommended by the manufacturer.
**ADDITIONAL INFORMATION**

**Supplementary Information** The online version contains supplementary material available at [https://doi.org/10.138b/s41392-021-00711-3](https://doi.org/10.138b/s41392-021-00711-3).

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