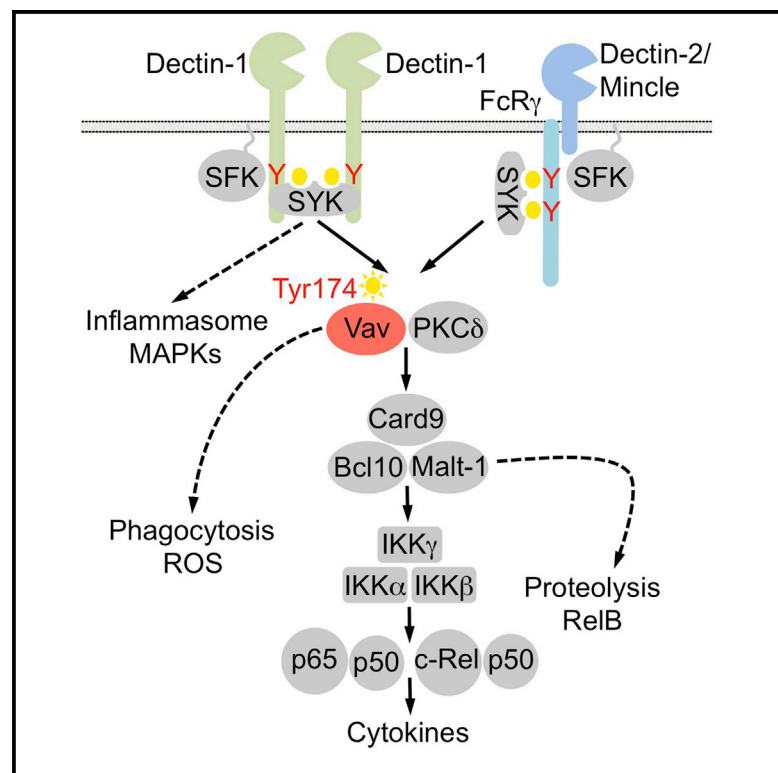


## Vav Proteins Are Key Regulators of Card9 Signaling for Innate Antifungal Immunity

### Graphical Abstract



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### In Brief

CLR/Card9 signaling is essential for antifungal immunity. Roth et al. have identified the Vav family of proteins as key activators of the Card9/NF-κB pathway, essential for CLR-induced inflammatory responses and host defense against fungi.

### Highlights

- Vav proteins control CLR-mediated inflammatory responses
- CLR-induced NF-κB activation is regulated by Vav proteins
- Vav/Card9 signaling is critical for antifungal host defense

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# Vav Proteins Are Key Regulators of Card9 Signaling for Innate Antifungal Immunity

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## SUMMARY

Fungal infections are major causes of morbidity and mortality, especially in immunocompromised individuals. The innate immune system senses fungal pathogens through Syk-coupled C-type lectin receptors (CLRs), which signal through the conserved immune adaptor Card9. Although Card9 is essential for antifungal defense, the mechanisms that couple CLR-proximal events to Card9 control are not well defined. Here, we identify Vav proteins as key activators of the Card9 pathway. Vav1, Vav2, and Vav3 cooperate downstream of Dectin-1, Dectin-2, and Mincle to engage Card9 for NF- $\kappa$ B control and proinflammatory gene transcription. Although Vav family members show functional redundancy, *Vav1/2/3*<sup>-/-</sup> mice phenocopy *Card9*<sup>-/-</sup> animals with extreme susceptibility to fungi. In this context, Vav3 is the single most important Vav in mice, and a polymorphism in human *VAV3* is associated with susceptibility to candidemia in patients. Our results reveal a molecular mechanism for CLR-mediated Card9 regulation that controls innate immunity to fungal infections.

## INTRODUCTION

Cells of the innate immune system sense microbial components or cell damage-associated structures via germline-encoded pattern recognition receptors (PRRs) that subsequently signal

for host defense and tissue homeostasis (Takeuchi and Akira, 2010). One important family of signaling PRRs on myeloid cells are the Syk-coupled C-type lectin receptors (Kerrigan and Brown, 2011; Sancho and Reis e Sousa, 2012). These PRRs play a broad role in innate immunity and are particularly important for host defense against fungal infections (Ferwerda et al., 2009; Robinson et al., 2009; Saijo et al., 2007, 2010; Sato et al., 2006; Taylor et al., 2007; Wells et al., 2008), which constitute an increasing health threat because of growing numbers of patients at risk mainly because of immunosuppressive medical interventions and AIDS. In the context of antifungal immunity, the C-type lectin receptor (CLR) family member Dectin-1 senses  $\beta$ -glucans in fungal cell walls (Brown and Gordon, 2001), whereas Mincle and Dectin-2 detect  $\alpha$ -mannose, glycolipids, and  $\alpha$ -mannans, respectively (Sancho and Reis e Sousa, 2012). Agonist binding by Dectin-1 leads to the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM)-like motifs in its cytoplasmic tail by Src family kinases, resulting in activation of the tyrosine kinase Syk. Likewise, Dectin-2 and Mincle also activate Syk, indicating that these CLR signals engage common effector mechanisms (Mócsai et al., 2010; Sancho and Reis e Sousa, 2012). The innate immune adaptor protein Card9 is critical for CLR signaling. It assembles signaling complexes that also contain Bcl10 and Malt1 (Card9-Bcl10-Malt1 [CBM] complexes) and that serve as scaffolds for activation of the canonical nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway (Roth and Ruland, 2013). This mechanism activates the inhibitor of kappa B ( $\kappa$ B) kinase (IKK) complex, which phosphorylates inhibitory  $\kappa$ Bs, leading to their proteasomal degradation and the release of NF- $\kappa$ B dimers to the nucleus to activate gene transcription (Vallabhapurapu and Karin, 2009). Malt1 can also function as a

protease upon CBM complex assembly that cleaves a set of NF- $\kappa$ B regulators, including RelB, to fine-tune immune gene expression (Hailfinger et al., 2011; Jaworski et al., 2014). These Card9 signaling complexes operate downstream of all tested Syk-coupled CLRs (Roth and Ruland, 2013) and are essential for innate antifungal immunity. Indeed, Card9-deficient mice are highly susceptible to infection with *Candida* (Gross et al., 2006; Jia et al., 2014), *Aspergillus* (Jhingran et al., 2012), and *Cryptococcus* (Yamamoto et al., 2014) species. Moreover, loss-of-function mutations in human *CARD9* have been identified as causes of mucocutaneous and invasive fungal infections (Glocker et al., 2009; Pérez de Diego et al., 2015). Nevertheless, despite the critical role for CLR-triggered Card9 signaling in innate immunity and mammalian host defense, the molecular mechanisms that link CLR ligation to Card9-dependent effector mechanisms are not well understood.

Here, we used a mass spectrometry-based proteomic approach and identified Vav proteins as regulators of Card9 signaling. Vav1, Vav2, and Vav3 cooperate downstream of Dectin-1, Dectin-2, and Mincle to engage Card9 complexes for NF- $\kappa$ B control and proinflammatory gene transcription. Like Card9-deficient mice, Vav1/2/3 triple-deficient mice are severely impaired in inflammatory responses to *Candida albicans* infection and host defense against the fungus. Moreover, we report a human polymorphism in *VAV3* that is associated with susceptibility to candidemia. Thus, our results establish Vav proteins as essential regulators of CLR-mediated Card9 control in innate antifungal immunity.

## RESULTS

### Fungal Infection Induces Tyrosine Phosphorylation of Vav in Myeloid Cells

To investigate the mechanisms of Syk-coupled CLR signaling, we stimulated wild-type murine bone marrow-derived dendritic cells (BMDCs), comprising conventional DCs and monocyte-derived macrophages (Helft et al., 2015), for 10 min with zymosan, a yeast cell wall preparation that is highly enriched in Dectin-1 and Dectin-2 agonists, and subsequently affinity-purified tyrosine-phosphorylated proteins for mass spectrometric analysis (Strasser et al., 2012). Under these conditions, we observed signal-induced tyrosine phosphorylation of Vav1 and Vav3, which are cytosolic signaling scaffolds and guanine nucleotide exchange factors that can play context-specific roles in immune receptor pathways (Bustelo, 2014). To validate these findings, we stimulated BMDCs with *C. albicans* hyphae and specifically analyzed Vav1 phosphorylation by western blot analysis. Indeed, Vav1 was tyrosine-phosphorylated after *C. albicans* infection (Figure 1A). These data are in line with previously published results that demonstrated that stimulation with  $\beta$ -glucan or zymosan triggers tyrosine phosphorylation of Vav1 also in microglial cells or neutrophils (Li et al., 2011; Shah et al., 2009). Further analysis with a phosphospecific antibody raised against the regulatory Tyr174 residue of Vav1 additionally revealed that this specific residue is phosphorylated upon *C. albicans* detection, indicating Vav1 activation (Aghazadeh et al., 2000), but not upon LPS stimulation (Figure 1B).

Recent work has demonstrated that Vav phosphorylation downstream of different ITAM-containing receptors is mediated

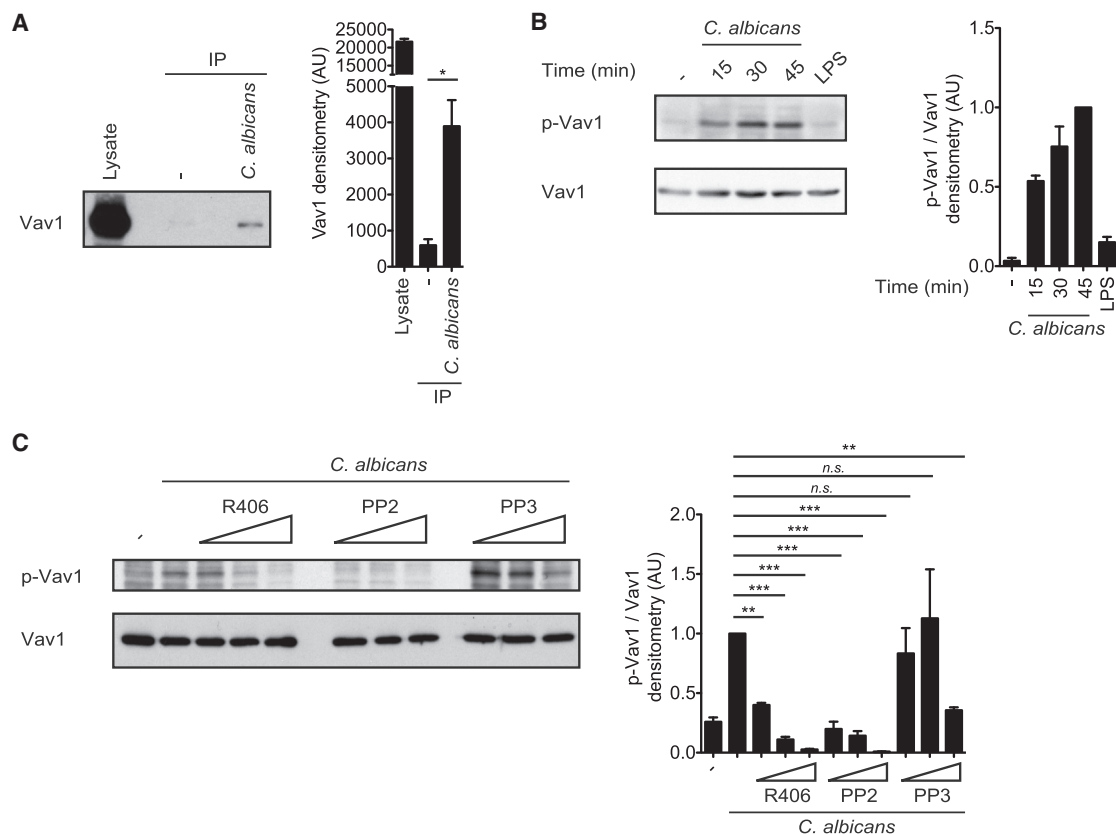
by Src and Syk kinases (Bustelo, 2014). To determine the importance of the CLR-proximal Src and Syk kinases in Vav phosphorylation, we pretreated BMDCs with the Src inhibitor PP2 or the Syk inhibitor R406 prior to the addition of *C. albicans*. Both Src and Syk inhibition blocked Vav1-Tyr174 phosphorylation in a dose-dependent manner, whereas pretreatment with PP3, an inactive analog of PP2, did not affect *C. albicans*-induced phosphorylation of Vav1 at Tyr174 (Figure 1C). Together, these results indicate that Vav1 is activated in myeloid antigen-presenting cells specifically after fungal sensing via an Src and Syk kinase-dependent mechanism.

### CLR-Mediated Inflammatory Responses Are Critically Dependent on Vav Proteins

Next, we studied the functional relevance of Vav proteins in *C. albicans*-induced inflammatory responses. Because dendritic cells express Vav2 (Spurrell et al., 2009) in addition to Vav1 and Vav3, which have partially redundant functions in immune cells (Fujikawa et al., 2003; Tybulewicz, 2005), we investigated BMDCs from mice that lack each individual Vav isoform, two Vav isoforms, or all three Vav family members. Notably, the lack of any one or all Vav molecules did not impair BMDC differentiation (data not shown; Graham et al., 2007; Spurrell et al., 2009). However, BMDCs from Vav1/2/3 triple-deficient mice were severely defective in *C. albicans*-induced TNF production, whereas tumor necrosis factor (TNF) secretion in response to Toll-like receptor (TLR) stimulation with LPS or Pam3CSK4 was unaffected in these cells (Figure 2A). Moreover, the production of interleukin-2 (IL-2) and IL-10 was also defective in BMDCs from Vav1/2/3<sup>-/-</sup> mice (Figure 2A). The finding that Vav1, Vav2, or Vav3 single-deficient or Vav2/3<sup>-/-</sup> myeloid antigen-presenting cells are only partially impaired in *C. albicans*-induced TNF, IL-2, and IL-10 generation (Figure 2B) indicates redundancy for Vav proteins in fungus-induced cytokine responses.

One important inflammatory cytokine for antifungal immunity is IL-1 $\beta$  (Vonk et al., 2006), the generation of which is controlled by NF- $\kappa$ B-dependent pro-IL-1 $\beta$  gene transcription followed by NLRP3 inflammasome-mediated Caspase-1-dependent pro-IL-1 $\beta$  processing (Gross et al., 2009; Hise et al., 2009). Stimulation of wild-type BMDCs with *C. albicans* induced the robust secretion of mature IL-1 $\beta$  into the culture supernatant (Figure 2C). In contrast, Vav1/2/3-deficient cells were almost completely defective in *Candida*-induced IL-1 $\beta$  production (Figure 2C). This defect was caused by the selective impairment of pro-IL-1 $\beta$  gene transcription in Vav1/2/3<sup>-/-</sup> cells (Figure 2D), whereas the deficiency of Vav proteins did not affect NLRP3 inflammasome-dependent Caspase-1 activation (Figure 2E). Together, these experiments demonstrate that Vav proteins are critical for cytokine production by myeloid cells following the detection of whole fungal cells.

Next, we studied the specific requirement of Vav proteins for Dectin-1-, Dectin-2-, and Mincle-induced cytokine responses using selective agonists for each individual receptor. Stimulation of Dectin-1 with curdlan, a purified particulate  $\beta$ -glucan (LeibundGut-Landmann et al., 2007), induced the robust production of TNF in wild-type BMDCs, whereas Vav1/2/3-deficient cells failed to produce TNF in response to Dectin-1 triggering (Figure 2F). Likewise, stimulation of BMDCs with agonistic



**Figure 1. Vav1 Is Tyrosine-Phosphorylated in Response to *Candida***

(A) BMDCs were untreated (–) or stimulated with *C. albicans* hyphae (MOI 5) for 15 min. Proteins from cell lysates were immunopurified with anti-phosphotyrosine antibodies and analyzed by immunoblot with a Vav1 antibody (left). Also shown is quantification of Vav1 by densitometry (right).

(B) Immunoblot analysis of BMDCs unstimulated (–), treated with *C. albicans* hyphae (MOI 0.5) for the indicated times, or stimulated with LPS (100 ng/ml) for 45 min and probed with antibodies against phospho-Vav1 (Tyr174) or Vav1 (left). Also shown is densitometrical quantification of phospho-Vav1 relative to total Vav1, normalized to BMDCs treated with *C. albicans* for 45 min (right).

(C) BMDCs were untreated (–) or preincubated with the Syk inhibitor R406 (0.5, 1, and 2 μM) or the Src-kinase inhibitor PP2 (1.5, 3, and 6 μM) or its inactive analog PP3 (1.5, 3, and 6 μM) and then stimulated with *C. albicans* hyphae (MOI 1) for 30 min. Lysates were immunoblotted with anti-phospho-Vav1 (Tyr174) or anti-Vav1 antibodies (left). Also shown is densitometrical quantification of phospho-Vav1 relative to total Vav1, normalized to BMDCs treated with *C. albicans* without any inhibitors (right). Data of at least three independent experiments are shown as mean ± SEM.

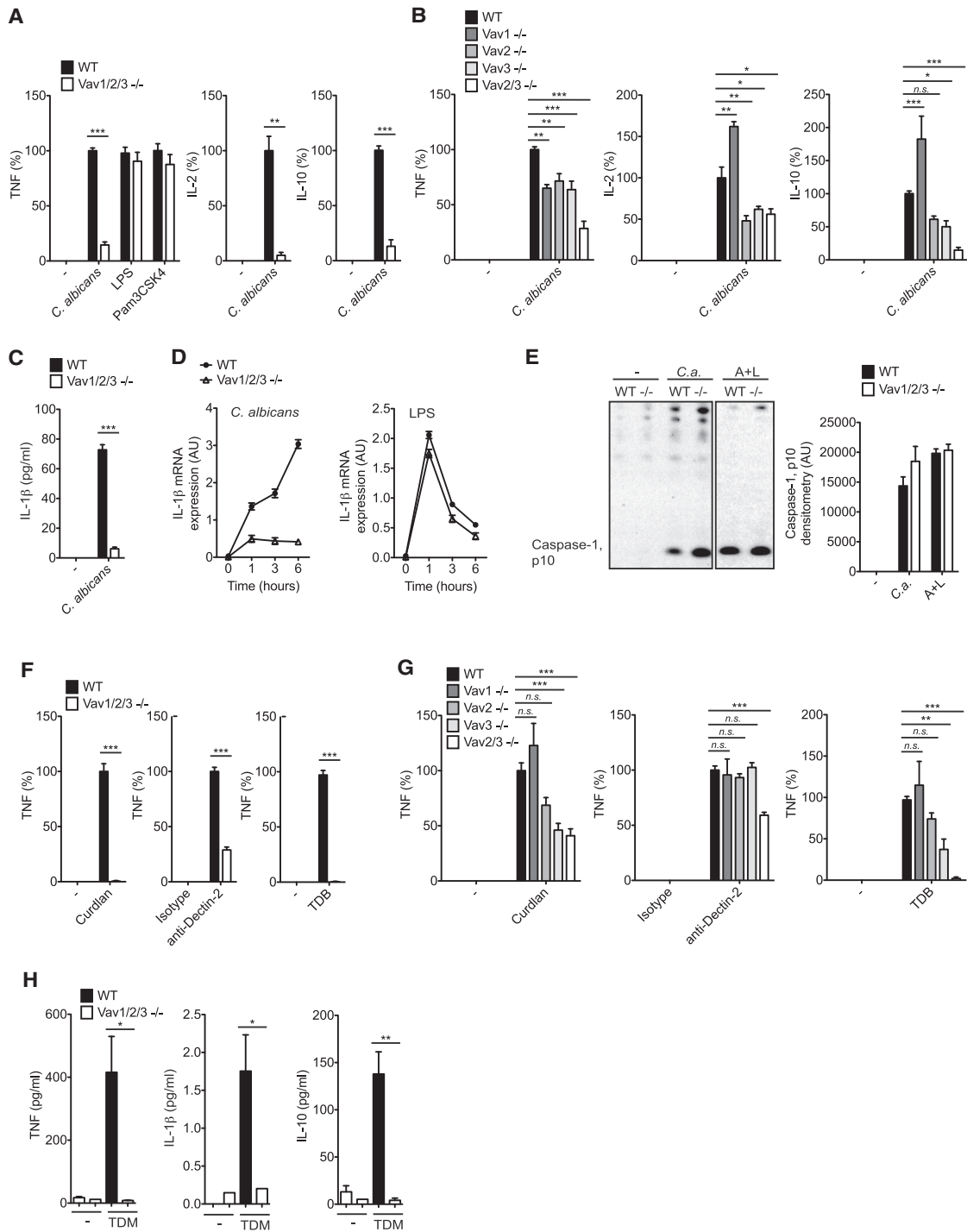
\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; Student's t test (A) and one-way ANOVA and post hoc Tukey-Kramer test (C). n.s., not significant.

antibodies against Dectin-2 or with trehalose-6,6-dibehenate (TDB), a synthetic analog of the mycobacterial cord factor trehalose 6,6'-dimycolate (TDM), which specifically activates Mincle (Lobato-Pascual et al., 2013; Miyake et al., 2013; Schoenen et al., 2010), induced TNF in wild-type cells, but this response was severely impaired in Vav1/2/3-deficient BMDCs (Figure 2F). Therefore, although Vav single- or double-mutant cells exhibited a partially impaired cytokine response upon selective Dectin-1, Dectin-2, or Mincle stimulation (Figure 2G), the Vav family as a whole is essential for cytokine production after Syk-coupled CLR ligation in vitro.

### Vav Proteins Regulate CLR-Induced Inflammatory Responses In Vivo and Are Essential for Antifungal Defense

To investigate the function of Vav proteins in CLR-mediated inflammatory responses in vivo, we intravenously injected the

Mincle ligand TDM (Ishikawa et al., 2009; Miyake et al., 2013) into wild-type and Vav1/2/3<sup>-/-</sup> mice. Consistent with previously published results (Ishikawa et al., 2009), TDM injection resulted in the strong systemic production of TNF, IL-1β, and IL-10 in wild-type mice (Figure 2H). However, these responses were defective in Vav1/2/3 triple-deficient animals (Figure 2H). Next, we studied the role of Vav proteins in inflammatory cytokine responses after fungal infection in vivo. To this end, we intravenously injected wild-type or Vav1/2/3 triple knockout mice with live *C. albicans* and measured serum TNF and IL-6 levels after 6 hr. Notably, the production of both inflammatory cytokines was almost completely abolished in Vav1/2/3<sup>-/-</sup> mice (Figure 3A). Subsequently, we studied the importance of individual Vav proteins in host protection against fungi by infecting wild-type or Vav single-, double-, or triple-deficient mice with 1 × 10<sup>5</sup> colony-forming units (CFUs) of *C. albicans*. Four days later, we assessed intravital fungal growth in the kidneys, the main



**Figure 2. Vav Proteins Are Essential for Syk-Coupled CLR-Induced Proinflammatory Responses**

(A and B) TNF, IL-2, and IL-10 levels in the supernatants of wild-type (WT) and *Vav1/2/3*<sup>-/-</sup> BMDCs (A), or Vav single- or double mutant BMDCs of the indicated genotypes (B) that were untreated (–) or stimulated with *C. albicans* hyphae (MOI 0.3), LPS (500 ng/ml), or Pam3CSK4 (50 ng/ml) for 16 hr were analyzed by ELISA. (C) IL-1 $\beta$  in the supernatants of WT and *Vav1/2/3*<sup>-/-</sup> BMDCs left untreated (–) or stimulated with *C. albicans* hyphae (MOI 1) for 6 hr. (D) Quantitative real-time PCR analysis of IL-1 $\beta$  transcripts in WT and *Vav1/2/3*<sup>-/-</sup> BMDCs left untreated or stimulated with *C. albicans* hyphae (MOI 1) (left) or LPS (100 ng/ml) (right) for the indicated times; results are relative to those of  $\beta$ -actin mRNA. (E) Immunoblot analysis of mature Caspase-1 (p10) in cell culture supernatants of WT and *Vav1/2/3*<sup>-/-</sup> (–/–) BMDCs left untreated (–), stimulated with *C. albicans* hyphae (MOI 1) for 4 hr (C.a.), or pre-stimulated with LPS (50 ng/ml) for 6 hr prior to the addition of ATP (5 mM) for 45 min (A+L). Quantification of mature Caspase-1 p10 by densitometry is shown to the right of the western blot.

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target organ of the fungus (Brieland et al., 2001). Although wild-type mice cleared the fungus readily, the kidneys of all investigated *Vav1/2/3*<sup>-/-</sup> mice were enlarged and infiltrated with macroscopically visible fungal colonies (Figure 3B). Quantitatively, we detected more than 100-fold higher titers of *C. albicans* in the kidneys of *Vav1/2/3* triple-deficient animals compared with the wild-type (Figure 3C), and histopathology confirmed intravital fungal growth (Figure 3D). The fungal burdens in *Vav3* single knockout and *Vav2/3* double knockout mice were increased by trend compared with the wild-type, but a selective loss of *Vav1* or *Vav2* did not result in higher fungal titers compared with wild-type mice (Figure 3C). Finally, we studied the role of Vav signaling in the survival of mice after fungal infection. In line with the incapacity to control fungal invasion of the organs, *Vav1/2/3*-deficient mice died rapidly after injection of  $1 \times 10^5$  CFU of *C. albicans*, whereas the wild-type control animals survived this challenge (Figure 3E). These experiments reveal that Vav proteins play essential roles in fungus-induced cytokine production and antifungal immunity in vivo, with *Vav3* being the single most important Vav family member for protection against *C. albicans* infection.

### Vav Proteins Are Regulators of CLR-Induced NF- $\kappa$ B Activation

The susceptibility of *Vav1/2/3* triple-deficient animals and the failure to induce CLR-mediated cytokine responses are reminiscent of the severe phenotype observed in *Card9*-deficient mice, suggesting that Vav proteins and *Card9* may operate in a common signaling cascade. Because of the partially overlapping functions of the three Vav isoforms, we focused subsequent biochemical studies on *Vav1/2/3* triple knockout BMDCs. Upon *C. albicans* stimulation, phosphorylation of the tyrosine kinase Syk and of the Dectin-1 signal transducer PLC $\gamma$ 2 (Xu et al., 2009) did not differ between wild-type and *Vav1/2/3*<sup>-/-</sup> BMDCs (Figure 4A), indicating that Vav proteins are not required for the immediate receptor-proximal events. Moreover, because Erk1/2, p38, and Jun N-terminal kinase (JNK) were similarly phosphorylated in *C. albicans*-stimulated wild-type and *Vav1/2/3*<sup>-/-</sup> BMDCs (Figure 4B), we conclude that Vavs are not essential for the regulation of mitogen-activated protein kinase (MAPK) signaling after fungal infection. However, activation of protein kinase C  $\delta$  (PKC $\delta$ ), which controls *Card9* engagement (Strasser et al., 2012), was partially impaired in BMDCs lacking all three Vav isoforms (Figure 4C; Figure S1A). Likewise, PKC $\delta$  deficiency partially compromised *Vav1* phosphorylation in response to *C. albicans* or curdlan stimulation (Figure S1B). Moreover, although *C. albicans* or curdlan stimulation of wild-type BMDCs induces the rapid activation of the IKK complex, as determined by measuring IKK $\alpha/\beta$  phosphorylation (Figure 4C; Figures S1A

and S1B), this response is almost completely abolished in the absence of Vav proteins (Figure 4C; Figure S1A) or PKC $\delta$  (Figure S1B; Strasser et al., 2012). Consistent with these findings, *C. albicans*-induced nuclear translocation of the transcriptional active NF- $\kappa$ B subunits p65, c-Rel, and RelB were also severely defective in *Vav1/2/3*<sup>-/-</sup> BMDCs, as they are in *Card9*-deficient cells (Figures 4D and 4E; Figures S1C and S1D), demonstrating a critical role for Vav proteins in CLR-dependent NF- $\kappa$ B control.

As indicated above, the activation of the *Card9/Bcl10/Malt1* complex not only regulates IKK activation but also induces Malt1 proteolytic activity (Jaworski et al., 2014). Consistently, stimulation of wild-type BMDCs with CLR ligands results in the rapid proteolytic processing of the Malt1 substrate RelB (Figures 4F and 4G; Figures S1E and S1F; Gewies et al., 2014; Hailfinger et al., 2011; Jaworski et al., 2014). Curdlan-induced RelB cleavage was completely defective in BMDCs from *Malt1*<sup>-/-</sup> mice and from mice with gene-targeted inactivation of the Malt1 paracaspase function (*Malt1*<sup>PM/-</sup>) (Gewies et al., 2014; Figure 4F; Figure S1E) and, therefore, can be used as a marker for CBM complex activation. Likewise, Malt1-mediated RelB cleavage upon *C. albicans* stimulation was also defective in *Vav1/2/3* triple knockout cells (Figure 4G; Figure S1F), which, together with the defective IKK activation in *Vav1/2/3*<sup>-/-</sup> BMDCs, indicates that Vav proteins operate upstream of the *Card9/Bcl10/Malt1* signalosome.

Finally, we studied the role of Vav proteins in CLR-induced gene expression in a global manner. To this end, we performed high-throughput cDNA sequencing (RNA sequencing [RNA-seq]) followed by gene set enrichment analysis (GSEA) of curdlan-stimulated and untreated BMDCs. In Dectin-1-stimulated wild-type BMDCs, we detected a significant enrichment of upregulated NF- $\kappa$ B-dependent transcripts compared with *Vav1/2/3*<sup>-/-</sup> cells (Figure 4H), which is consistent with the defective IKK activation described above. These results further validate Vav proteins as essential regulators of CLR-mediated NF- $\kappa$ B control.

### A Human *Vav3* Polymorphism Is Associated with Susceptibility to Candidemia

After identifying Vav proteins as integral regulators of the Dectin-1/*Card9*/NF- $\kappa$ B signaling cascade in murine cells, we were interested in the potential roles for Vav molecules in human antifungal immunity. Therefore, we investigated whether genetic variations linked to any of the *VAV* genes correlate with susceptibility to candidemia in patients by studying a previously described cohort of candidemia patients and an appropriate control group (Jaeger et al., 2015). Interestingly, the analysis of SNPs associated with *VAV3* revealed a significant association with candidemia, with the significant SNPs distributed across the linkage

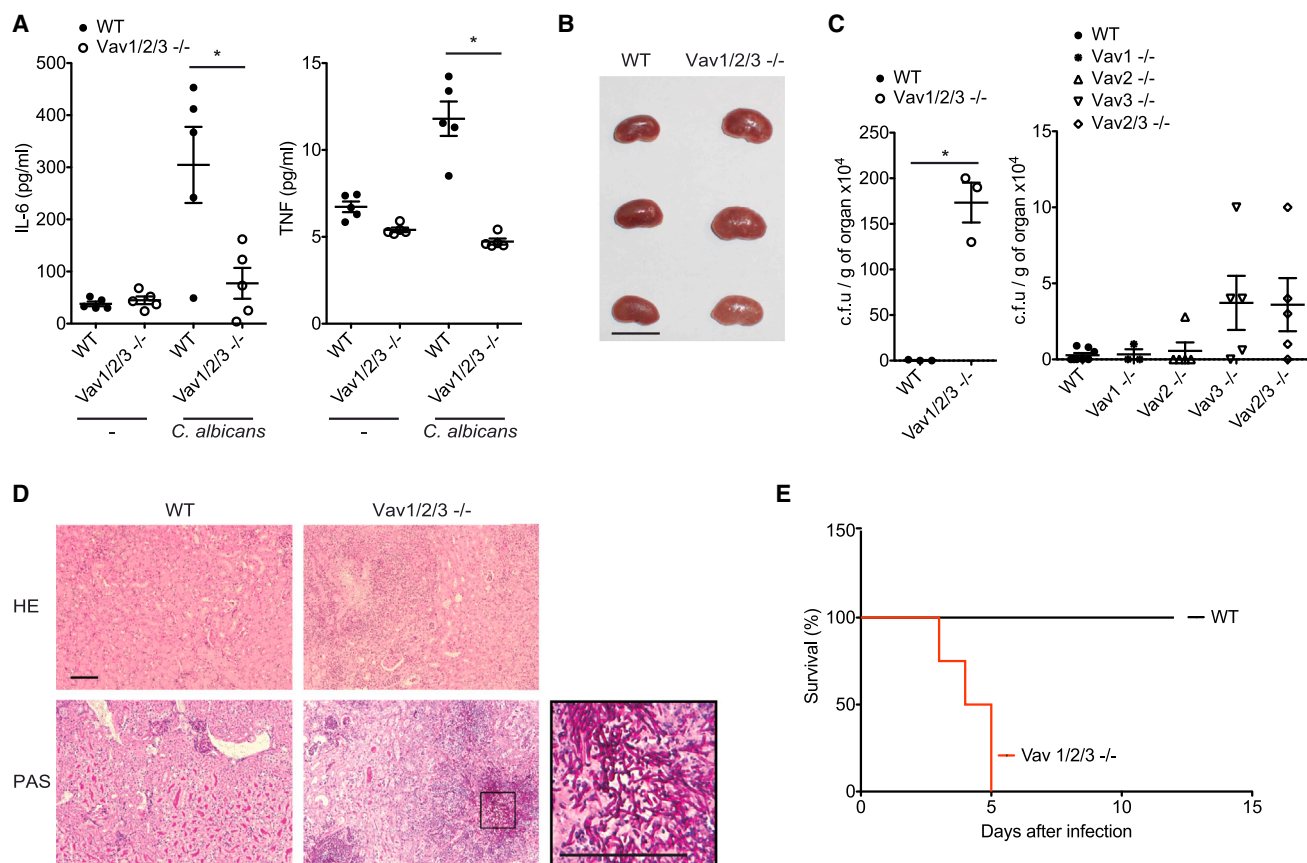
(F and G) BMDCs from WT and *Vav1/2/3*<sup>-/-</sup> mice (F), or Vav single- or double mutant mice of the indicated genotypes (G) were untreated (-), stimulated with the Dectin-1 ligand curdlan (10  $\mu$ g/ml), plate-bound anti-Dectin-2 (12.5  $\mu$ g/ml) or isotype control antibodies (Isotype), or the Mincle ligand TDB (100  $\mu$ g/ml). TNF levels in the cell culture supernatants were quantified by ELISA.

(H) WT and *Vav1/2/3*<sup>-/-</sup> mice were injected intravenously with TDM containing oil-in-water emulsions or with the vehicle control (-). 24 hr post-injection, TNF, IL-1 $\beta$ , and IL-10 levels in sera were determined by CBA.

(A, B, F, and G) Data are expressed as percent of WT  $\pm$  SEM of three independent experiments.

(C-E and H) Data of at least three independent experiments are presented as mean  $\pm$  SEM.

\*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001, Student's t test (A, C, F, and H), and one-way ANOVA and post hoc Tukey-Kramer test (B and G).



### Figure 3. Vav Proteins Are Required for Systemic Antifungal Host Defense

(A–E) Mice of the indicated genotypes were intravenously infected with  $1 \times 10^5$  CFUs of *C. albicans*.

(A) Serum IL-6 and TNF levels in WT and *Vav1/2/3*<sup>-/-</sup> mice were determined by CBA 6 hr after infection.

(B and C) After 4 days, kidneys were macroscopically examined (B; scale bar, 10 mm), and *C. albicans* titers were determined in the kidneys (C).

(D) Kidney sections from *C. albicans*-infected WT and *Vav1/2/3*<sup>-/-</sup> mice were stained with H&E or periodic acid-Schiff (PAS; scale bars, 100  $\mu$ m).

(E) WT (n = 5) and *Vav1/2/3*<sup>-/-</sup> (n = 4) mice were monitored daily for health and survival. Statistical survival analyses were performed using the log-rank test ( $p < 0.005$ ).

(A and C) Each symbol represents an individual mouse; small horizontal lines indicate the mean; error bars indicate the SEM. Data of one experiment in each case are presented. \* $p < 0.05$ , Student's t test (A and C, left) and one-way ANOVA (C, right,  $p = 0.06$ ).

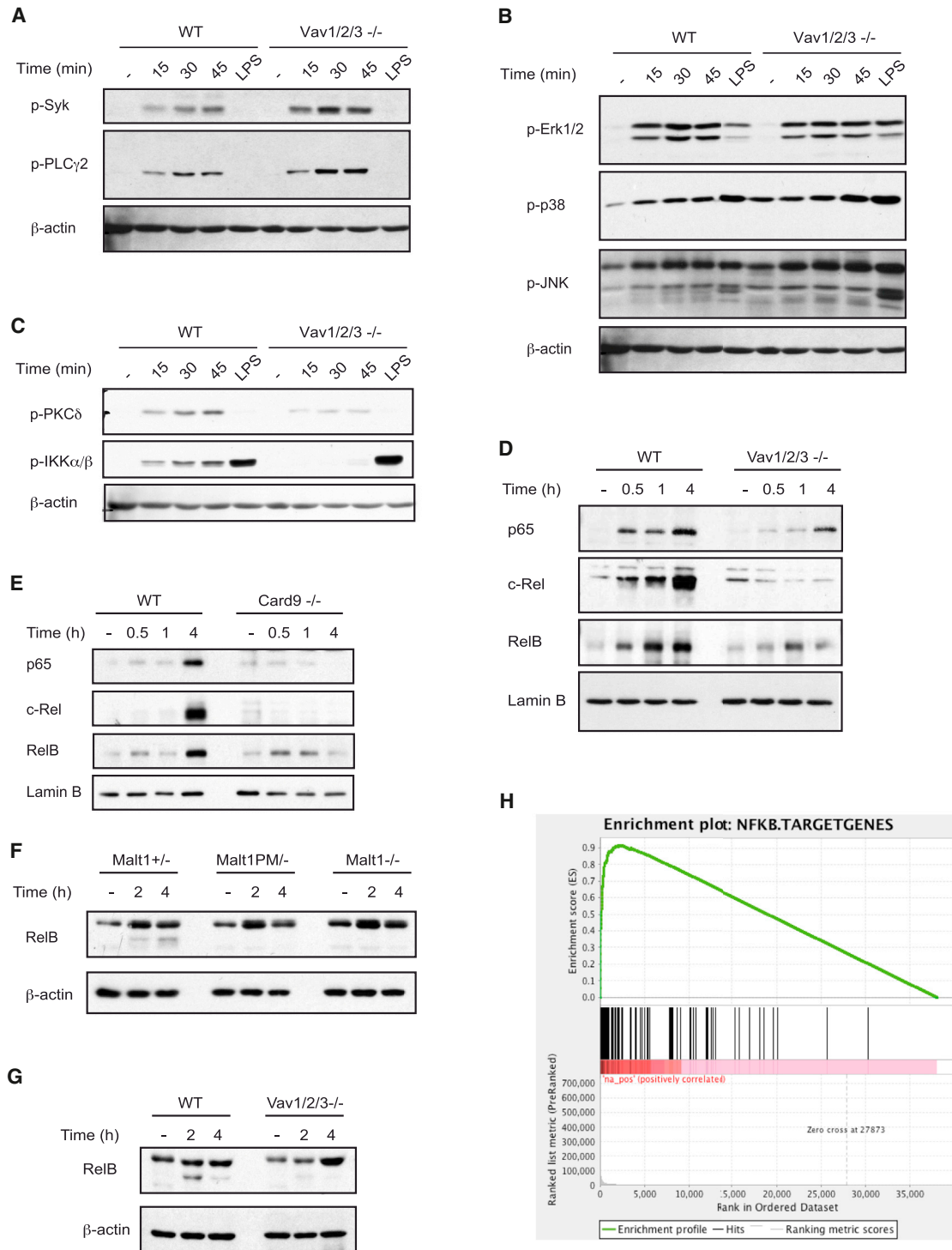
disequilibrium (LD) region of the gene. The association does not extend beyond the LD region, and the strongest association was with the SNP rs4914950 (Figure 5A). The risk genotype of the SNP rs4914950 TT is increased from 10% (control population) to 18% (candidemia patients) (Figure 5B).

## DISCUSSION

Using a complementary approach of immunology, molecular biology, and genetic studies, the results presented in this manuscript define an essential role for Vav proteins in CLR-mediated inflammatory responses and establish these molecules as essential signaling platforms that relay proximal events from Syk-coupled CLRs to the Card9 signaling complex for NF- $\kappa$ B control and antifungal defense (Dambuzza and Brown, 2015; Osorio and Reis e Sousa, 2011; Roth and Ruland, 2013). Although individual Vav isoforms can compensate for each other, their combined absence results in a blockade of CLR-

dependent NF- $\kappa$ B activation similar to Card9 deficiency, with Vav3 being the most important family member in this pathway in mice and, presumably, in humans.

When engaged by fungal particles, Syk-coupled CLRs activate various intracellular signaling pathways that regulate phagocytosis, microbicidal activities, and gene transcription. Using *C. albicans* as a clinically relevant model organism that is sensed by multiple CLRs and induces CLR signaling (Robinson et al., 2009; Taylor et al., 2007; Wells et al., 2008), we observed Vav activation downstream of Syk. In addition, using defined agonists of Dectin-1, Dectin-2, and Mincle, we established a general role for Vav proteins in CLR signaling in myeloid antigen-presenting cells. However, our results that CLR-mediated MAPK activation and inflammasome signaling are not impaired in *Vav1/2/3* triple-deficient BMDCs reveal that Vav proteins only mediate specific CLR effector mechanisms. Recent studies have indicated the involvement of Vav1 and Vav3 in Dectin-1-mediated zymosan phagocytosis and reactive oxygen



**Figure 4. Vav Proteins Control CLR-Triggered NF- $\kappa$ B Activation**

(A–E) BMDCs from the indicated genotypes were stimulated with *C. albicans* hyphae (MOI 0.5) for various times or with LPS (100 ng/ml) for 45 min. (A) Syk and PLC $\gamma$ 2 phosphorylation was determined in cell lysates by immunoblot with anti-phospho-Syk and anti-phospho-PLC $\gamma$ 2 antibodies. (B) Activation of the MAP kinases Erk1/2, p38, and JNK was analyzed with anti-phospho-Erk1/2, anti-phospho-p38, and anti-phospho-JNK antibodies. (C) Cell lysates were analyzed by immunoblot with anti-phospho-PKC $\delta$  and anti-phospho-IKK $\alpha/\beta$  antibodies.  $\beta$ -actin served as a loading control. (D and E) Nuclear extracts from WT and Vav1/2/3<sup>-/-</sup> (D), or Card9<sup>-/-</sup> BMDCs (E) were analyzed by immunoblot with antibodies against the NF- $\kappa$ B subunits p65, c-Rel, and RelB; anti-lamin B antibodies indicate equal protein loading.

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species (ROS) production in neutrophils and the recruitment of Vav1 to phagocytic cups for *C. albicans* engulfment by macrophages (Li et al., 2011; Strijbis et al., 2013). Although these effector mechanisms are important during innate immune responses, they are not regulated by the Card9 pathway (Drewniak et al., 2013; Goodridge et al., 2009; Gross et al., 2006). However, the Card9 signaling pathway appears to be the central mammalian host defense mechanism against fungi, given that multiple genetic studies in humans have recently identified that several Card9 loss-of-function defects are causative for several fungal diseases, including various types of mucocutaneous candidiasis; superficial, extensive, and deep dermatophytosis with *Trichophyton* spp.; subcutaneous phaeohyphomycosis; invasive *Candida* infections of the digestive tract and central nervous system; *Candida* endophthalmitis and osteomyelitis; and disseminated *Exophiala* disease of the liver, brain, and lung (Grumach et al., 2015; Pérez de Diego et al., 2015).

We assessed enzymatic Malt1 paracaspase activity as a direct functional marker for Card9 complex activity in Vav-deficient mice. Given that this activity was severely impaired in Vav1/2/3 triple-deficient BMDCs, our results establish Vav molecules as regulators of the Card9/Bcl10/Malt1 complex. Consistent with the essential function of a Vav/Card9-mediated mechanism for CLR-induced NF- $\kappa$ B activation, the NF- $\kappa$ B response is blocked in Vav1/2/3-deficient mice in response to *C. albicans* infection or selective CLR triggering, and Vav1/2/3-deficient animals exhibit dramatically impaired proinflammatory cytokine production in response to *C. albicans* infection and CLR stimulation *in vitro* and *in vivo*. Therefore, we propose a mechanistic model in which Syk is engaged by CLRs upon fungal sensing, thereby triggering the phosphorylation and activation of Vav proteins, which subsequently activate the Card9 complex for NF- $\kappa$ B control and antifungal gene transcription.

How Vav proteins are mechanistically coupled to Card9 control remains to be determined. We previously demonstrated that Card9 is phosphorylated by PKC $\delta$  at Thr231 to induce Card9 effector function. However, the activation of PKC $\delta$  is only partially impaired in Vav-deficient BMDCs, whereas IKK activation is almost completely defective. Similarly, in PKC $\delta$ -deficient cells, IKK phosphorylation is abolished, whereas phosphorylation of Vav1 is only slightly compromised, suggesting, together, that Vav and PKC $\delta$  operate at the same level upstream of Card9. Because Vav proteins can control cytoskeletal reorganization and serve as scaffolding platforms in other signaling systems (Acton et al., 2012; Bustelo, 2014; Spurrell et al., 2009; Tybulewicz, 2005), we speculate that Vav family members could bring Card9 into the vicinity of key upstream regulators such as PKC $\delta$  and potential, still unknown factors to allow Card9 activation. Moreover, because Vav1/2/3 do not only regulate CLR-induced Card9-NF- $\kappa$ B signaling but also control other cellular responses, such as phagocytosis and ROS production (Li et al., 2011; Strijbis et al., 2013), the precise molecular inter-

dependence of these mechanisms should be defined in the future.

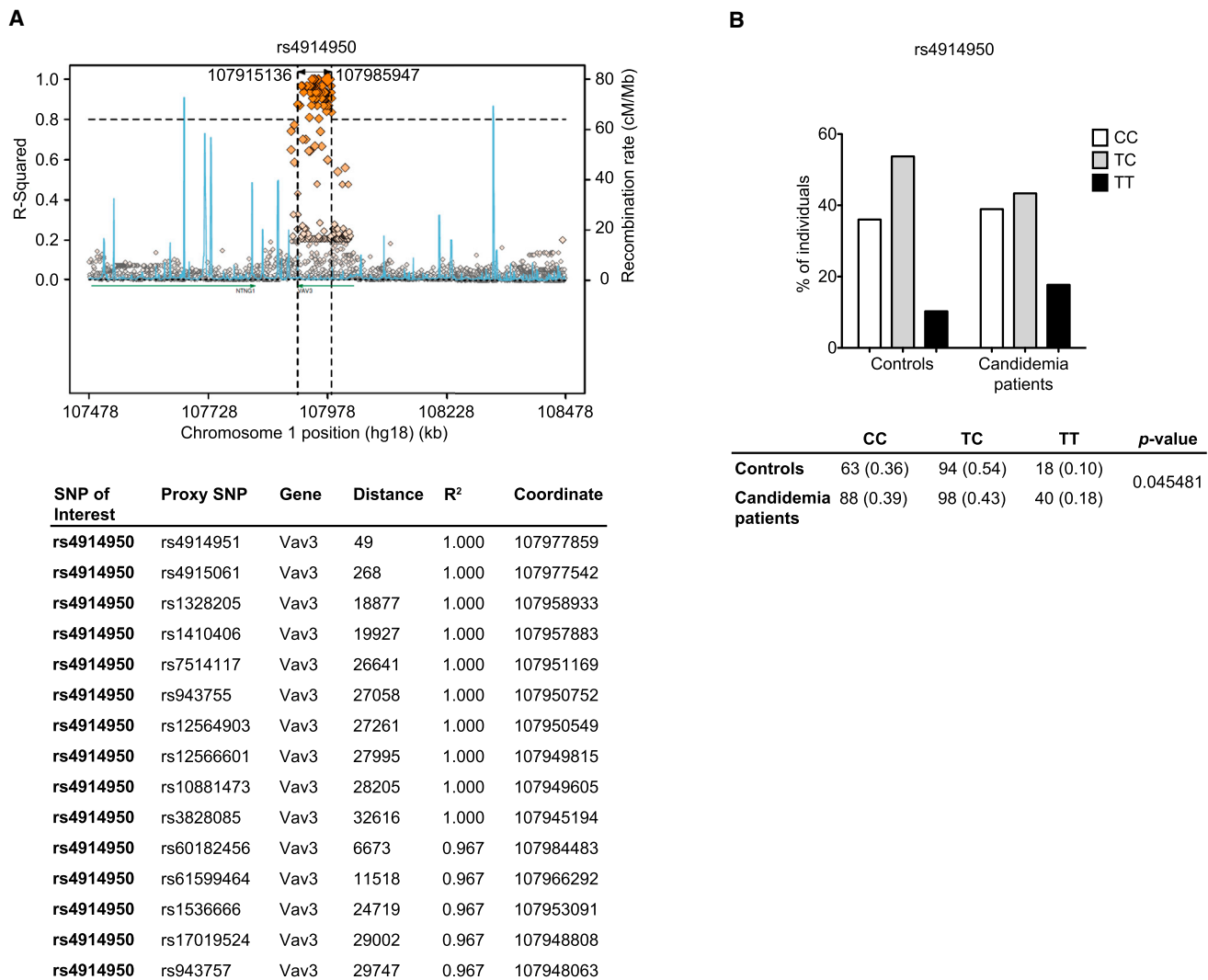
In accordance with our molecular model, similar to Card9 deficiency, a complete deficiency in all Vav isoforms results in a massive susceptibility to *C. albicans* infection. Likewise, a conditional deficiency of Syk only in dendritic cells also abolishes innate resistance to acute systemic *C. albicans* infection (Whitney et al., 2014), and Whitney et al. (2014) elegantly demonstrated that the early innate cytokine response is required to orchestrate innate anti-fungal functions, including neutrophil anti-fungal activity. Nevertheless, in our infection models presented here, it must be noted that the Vav mutant mice analyzed were germline Vav-deficient animals and that Vav proteins are not selectively expressed in Syk and Card9-containing innate immune cells, including dendritic cells, macrophages, and neutrophils, but are also expressed in lymphocytes, which signal through Card9-independent mechanisms (Gross et al., 2006; Hara et al., 2007). Still, the rapid death of Vav1/2/3 triple-deficient mice upon fungal infection indicates that an innate immune defect is indeed responsible for the observed phenotype. This hypothesis is also in line with the established roles of Vav1 and Vav3 in mediating phagocytosis and ROS production in macrophages and neutrophils upon fungal sensing (Li et al., 2011; Strijbis et al., 2013). Future studies with conditional Vav mutant mice will further dissect the individual roles of Vav signaling in dendritic cells, macrophages, and neutrophils as well as in adaptive immune subsets during complex fungal infection scenarios *in vivo*. In addition, *in vivo* results in individual Vav1-, 2-, and 3-deficient animals also revealed that, although there is functional redundancy among the three Vav family members in infection control, Vav3 is the single most important Vav family member for antifungal defense in mice. This finding is consistent with our results in patients who link a polymorphism in the VAV3 gene to susceptibility to *Candida* infection, suggesting that the function of Vav molecules is similar to Card9 function conserved between mice and men.

In addition to fungal components, CLRs also recognize structures present on mycobacteria (Ishikawa et al., 2009; Lobato-Pascual et al., 2013; Miyake et al., 2013; Schoenen et al., 2010), viruses such as dengue virus (Chen et al., 2008), and parasites such as *Schistosoma mansoni* (Ritter et al., 2010), as well as endogenous ligands under sterile inflammatory conditions (Roth and Ruland, 2013). We therefore believe that our findings have implications beyond antifungal immunity. Consistent with this hypothesis, Vav1/2/3<sup>-/-</sup> mice were almost completely impaired in systemic inflammatory responses upon innate immune stimulation with the mycobacterial pathogen-associated molecular patterns (PAMP) analog TDM, implying that, like Card9 (Dorhoi et al., 2010), Vav proteins might also be critical for innate antimycobacterial immune responses. In addition, accumulating evidence indicates important roles for CLR/Card9 signaling in inflammatory disorders. Several independent studies have shown an

(F and G) BMDCs from mice of the indicated genotypes were pretreated with the proteasome inhibitor MG132 for 30 min and then stimulated with curdlan (0.5 mg/ml) (F) or *C. albicans* hyphae (MOI 1) (G) for 2 or 4 hr. Cell lysates were analyzed by immunoblot using antibodies against RelB and  $\beta$ -actin.

(H) WT and Vav1/2/3<sup>-/-</sup> BMDCs were stimulated with curdlan (0.2 mg/ml) for 3 hr. Shown is the GSEA enrichment score of NF- $\kappa$ B target genes differentially expressed in curdlan-stimulated wild-type cells compared with Vav1/2/3-deficient BMDCs.

Data are representative of at least three independent experiments. See also Figure S1.



**Figure 5. A VAV3 Polymorphism Is Associated with Increased Susceptibility to Candidemia**

(A) Polymorphisms associated with candidemia were investigated in a group of 227 patients with *Candida*-positive blood cultures and compared with 176 patients from the same clinical wards without infection. SNPs associated with the VAV3 locus revealed a significant association with candidemia, with the significant SNPs distributed across the LD region of the gene. The VAV3 gene region on chromosome 1 is shown. Using the SNAP server, the SNP of interest (rs4914950) (biggest orange diamond) was plotted with all annotated polymorphisms in this gene (other diamonds). R<sup>2</sup> and recombination rate are indicated on the left and right y axis, respectively. Diamonds on the same horizontal line as the SNP of interest (biggest orange diamond) are in complete linkage disequilibrium (R<sup>2</sup> = 1) with this SNP and have the same disease association values. Diamonds below this line have a lower LD value with the SNP of interest and, therefore, differ in the association with the disease. The table below shows the top 15 polymorphisms that are linked to rs4914950.

(B) Genotype frequencies for the rs4914950 SNP that showed the strongest association with candidemia. The table below shows the total numbers and percentages (in parentheses) of genotypes in *Candida*-infected (candidemia patients) and non-infected (controls) individuals. Statistical comparisons of genotype frequencies between the two groups were made using  $\chi^2$  test.

association between Card9 polymorphisms and ulcerative colitis and Crohn's disease (Roth and Ruland, 2013), which are supported by a recent study in mice demonstrating a protective role for Card9 in intestinal inflammation (Sokol et al., 2013). Deep resequencing of inflammatory bowel disease genome-wide association study (GWAS) loci confirmed an association between Card9 and ulcerative colitis and Crohn's disease (Beaudoin et al., 2013; Hong et al., 2016). Moreover, additional genome-wide association studies in humans have identified significant associations between CARD9 and VAV3 gene loci and immunoglobulin A (IgA)

nephropathy (Kiryluk et al., 2014). In conjunction with the results presented here, these data suggest that the described Vav/Card9-dependent signaling mechanism could also play a role in sterile inflammatory diseases.

#### EXPERIMENTAL PROCEDURES

##### Mice

*Vav1*<sup>-/-</sup> (Turner et al., 1997), *Vav2*<sup>-/-</sup> (Doody et al., 2001), *Vav3*<sup>-/-</sup> (Sauzeau et al., 2006), *Card9*<sup>-/-</sup> (Gross et al., 2006), *Prkd*<sup>-/-</sup> (Leitges et al., 2001),

*Malt1*<sup>-/-</sup> (Ruland et al., 2003), and *Malt1*<sup>PM/-</sup> (Gewies et al., 2014) mice were described previously. Vav double-deficient (*Vav2/3*<sup>-/-</sup>) and triple-deficient (*Vav1/2/3*<sup>-/-</sup>) mice were generated by intercrossings of Vav single knockout animals and were provided by X.R.B. and V.L.T.. All animal work was conducted in accordance with German federal animal protection laws and approved by the Institutional Animal Care and Use Committee at the Technical University of Munich. Animals were used at 8–16 weeks of age.

#### Cell Culture and Stimulation

BMDCs were derived from bone marrow as described previously (Gross et al., 2006) and stimulated with curdlan (Wako Pure Chemicals Industries), LPS (ultrapure, from *Escherichia coli* strain K12, InvivoGen), Pam3CSK4 (InvivoGen), Dectin-2 monoclonal antibody (AbD Serotec), Rat IgG2a isotype control (R&D Systems), TDB (Avanti Polar Lipids), or *C. albicans* strain SC5314 as indicated. For tyrosine kinase or proteasome inhibition, the Syk inhibitor R406 (Rigel), the Src kinase inhibitor PP2 or its inactive analog PP3 (both from Calbiochem), or MG132 (Sigma) were used. Cell culture reagents were purchased from Invitrogen, and fetal calf serum (FCS) was purchased from HyClone. Mouse recombinant granulocyte-macrophage colony-stimulating factor was purchased from PeproTech.

#### Cytokine Measurement

Cytokine levels were measured by ELISA (BD Biosciences and eBioscience) or by cytometric bead array (CBA; BD Biosciences) according to the manufacturer's instructions.

#### Immunoprecipitation and Immunoblot Analysis

Cell lysates or cell supernatants were subjected to standard immunoblot analysis techniques as described previously (Strasser et al., 2012). Cytosolic and nuclear extracts were prepared as described previously (Ferch et al., 2007). For immunoprecipitation experiments, BMDCs were left unstimulated or stimulated with *C. albicans* as indicated and lysed in an NP-40-containing buffer. Lysates were pre-cleared by incubation with protein G Sepharose (GE Healthcare 4 Fast Flow) and then incubated with anti-phospho-tyrosine antibodies. Immune complexes were precipitated with protein G Sepharose and subjected to protein immunoblotting as indicated. Quantification of immunoblots was performed by densitometry using ImageJ software. Densitometry data were expressed relative to levels of appropriate loading controls and normalized to specific treatment conditions.

#### Statistical Analysis

Data were analyzed and graphed using Excel (Microsoft Office) and Prism (GraphPad). For comparison between two groups, two-tailed Student's *t* test was used. Analysis across more than two groups on a single dataset was performed using one-way ANOVA and post hoc Tukey-Kramer test. Survival data were analyzed using the log-rank test. Genotype frequencies between controls and candidemia patients were compared using the  $\chi^2$  test. The level of significance was defined as \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001.

#### ACCESSION NUMBERS

The accession number for the gene expression profiling raw data reported in this paper is NCBI GEO: GSE83736.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.11.018>.

#### AUTHOR CONTRIBUTIONS

S.R. and J.R. designed the study. S.R., H.B., M.J., K.N., and P.A.K. performed experiments. S.R., H.B., M.J., K.N., P.A.K., M.N., and J.R. analyzed the results. M.J., V.K., M.J., and M.N. conducted patient sample collection and clinical data analyses. A.Y. and B.H. performed bioinformatic analyses. S.R., M.J.,

and A.Y. generated the figures. C.P.C., L.V., M.M.M., V.L.T., and X.R.B. provided critical reagents. S.R. and J.R. wrote the paper.

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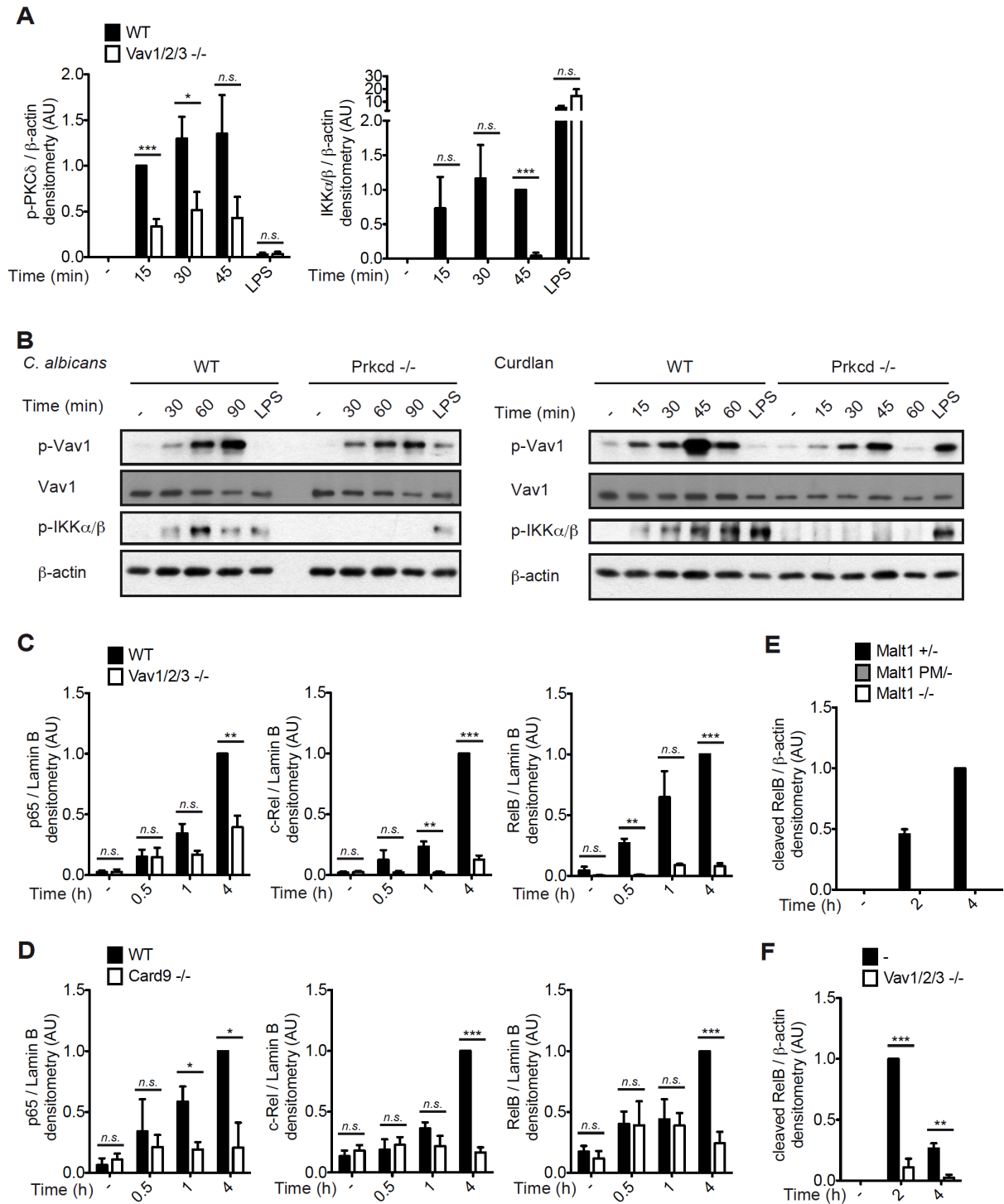
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## **Supplemental Information**

### **Vav Proteins Are Key Regulators of Card9 Signaling for Innate Antifungal Immunity**

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**Figure S 1**



**Figure S 1. Vav proteins control CLR-triggered NF- $\kappa$ B activation, Related to Figure 4.** (A, C, D) BMDCs from the indicated genotypes were stimulated with *C. albicans* hyphae (MOI 0.5) for various times or with LPS (100 ng/ml) for 45 minutes. (A) Cell lysates were analyzed by immunoblot with anti-phospho-PKC $\delta$  and anti-phospho-IKK $\alpha/\beta$  antibodies. Densitometrical quantification of phospho-PKC $\delta$  (left) and phospho-IKK $\alpha/\beta$  (right) relative to  $\beta$ -actin, normalized to WT BMDCs treated with *C. albicans* for 15 minutes (left) or 45 minutes (right). (B) BMDCs from WT or *Prkcd*<sup>-/-</sup> mice were stimulated with *C. albicans* hyphae (MOI 0.5) (left) or curdlan (0.5 mg/ml) (right) for various times, or with LPS (100 ng/ml) for 30 minutes. Cell lysates were

analyzed by immunoblot with anti-phospho-Vav1 (Tyr174), anti-Vav1, and anti-phospho-IKK $\alpha$ / $\beta$  antibodies.  $\beta$ -actin serves as a loading control. Representative data of two independent experiments are shown. (C, D) Nuclear extracts were analyzed by immunoblot with antibodies against the NF- $\kappa$ B subunits p65, c-Rel and RelB. Quantification of nuclear p65, c-Rel and RelB by densitometry, relative to Lamin B, normalized to WT BMDCs treated with *C. albicans* hyphae for 4 hours. (E, F) BMDCs from mice of the indicated genotypes were pretreated with the proteasome inhibitor MG132 for 30 minutes, and then stimulated with curdlan (0.5 mg/ml) (E) or *C. albicans* hyphae (MOI 1) (F) for 2 or 4 hours. Cell lysates were analyzed by immunoblot using antibodies against RelB and  $\beta$ -actin. Densitometrical quantification of cleaved RelB relative to  $\beta$ -actin, normalized to WT BMDCs treated with curdlan for 4 hours (E), or with *C. albicans* for 2 hours (F). (A, C-F) Data of at least three independent experiments are shown as means ( $\pm$ s.e.m.). \* $p$  < 0.05, \*\* $p$  < 0.01 and \*\*\* $p$  < 0.001, Student's *t*-test. Not significant (*n.s.*). Arbitrary units (AU).



## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***C. albicans* infections.** Mice were infected with  $1 \times 10^5$  colony-forming units (c.f.u) of *C. albicans* (strain SC5314) as described (Gross et al., 2006) and monitored daily for health and survival according to institutional guidelines. To determine fungal burden, organ homogenates were plated in dilutions on CHROMagar (BD Biosciences). For histological analyses, kidney sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) according to standard protocols. Six hours after infection, serum was collected to analyze cytokine levels by cytometric bead array (CBA; BD Biosciences).

**Administration of TDM.** Squalane-in-water emulsions containing TDM (Trehalose 6,6'-dimycolate from *Mycobacterium bovis*, Sigma) were prepared by ultrasonic treatment as previously described (Yarkoni and Rapp, 1978). The final concentrations of emulsion components were as follows: 1.5 or 0 mg/ml (vehicle control) TDM, 9% squalane (Sigma), 1% Tween 80 (Sigma), and 90% saline. The TDM emulsions or the squalane-tween-saline vehicle controls were administered to groups of animals via tail vein injection (0.1 ml per mouse). After 24 hours, serum was collected to determine cytokine levels by CBA (BD Biosciences).

**Antibodies.** Primary antibodies anti-Caspase-1, p10 (sc-514), anti-c-Rel (sc-71), anti-lamin B (sc-6217), anti-phospho-Vav (Tyr 174-R, sc-16408-R), and anti-Vav (sc-132) were from Santa Cruz, and anti-NF- $\kappa$ B p65 (4764), anti-phospho-Erk1/2, (Thr202/Tyr204, 9101), anti-phospho-IKK $\alpha/\beta$  (Ser176/180, 2697), anti-phospho-JNK (Thr183/Tyr185, 9251), anti-phospho-p38 (Thr180/Tyr182, 4511), anti-phospho-PLC $\gamma$ 2 (Tyr759, 3874), anti-phospho-Syk (Tyr525/526, 2711), anti-phospho-PKC $\delta$  (Tyr311, 2055), anti-phospho-tyrosine (9411), and anti-RelB (4922) were purchased from Cell Signaling. Anti- $\beta$ -actin was purchased from Sigma.

**Real-time quantitative PCR.** Total RNA was isolated and transcribed using standard methods. The specific primer pairs were as follows: IL-1 $\beta$ , 5'-TGTAATGAAAGACGGCACACC-3' and 5'-TCTTCTTTGGGTATTGCTTGG-3';  $\beta$ -actin, 5'-AGACCTCTATGCCAACACAG-3' and 5'-TCGTACTCCTGCTTGCTGAT-3'. The qPCR Core kit for SYBR Green I (Eurogentec) and a LightCycler 480 Real-Time PCR System were used as indicated by the manufacturers. IL-1 $\beta$  mRNA expression was calculated as the ratio of the real-time PCR signal for IL-1 $\beta$  mRNA to that of the  $\beta$ -actin mRNA.

**RNA-seq library preparation and data analysis.** Total RNA was purified using TRIzol Reagent (Invitrogen) and the RNeasy Mini kit (QIAGEN) with on-column DNase treatment. Purified RNA was submitted to the Genomics & Proteomics Core Facility at the DKFZ (Heidelberg, Germany) where it was subjected to library preparation using the Illumina TruSeq RNA sample preparation kit v2. Libraries were pooled (three samples per lane) and sequenced on an Illumina HiSeq 2000 (50–base pair single-end reads). The raw data quality of all libraries was analyzed using FastQC (v. 0.11.1). Quality trimming and adapter removal was performed using the FASTX-Toolkit (v. 0.0.14). The fastq files were then mapped to the mouse genome downloaded from Ensembl (genome build NCBI37) using TopHat2 (Kim et al., 2013) (v. 2.4.2a). Mapped reads were processed through featureCount (Liao et al., 2014) (v. 1.4.6-p4) at the gene level to account for the number of reads per gene in all samples. This was followed by differential expression analysis using the DESeq2 (Love et al., 2014) (v. 1.8.2) package of R (v. 3.2.0). Count data were normalized using the size factor to estimate the effective library size (Anders and Huber, 2010). After calculating gene dispersion across all samples, a pair-wise comparison of two different conditions resulted in a list of differentially expressed genes for the stimulated wild-type vs. knockout samples. Genes with an adjusted p-value  $\leq 0.1$  were defined as differentially expressed and were used for downstream analyses. The p-values were adjusted for multiple testing to reduce the false discovery rate (FDR). Gene set enrichment analysis (GSEA) for NF- $\kappa$ B was performed as previously described (Subramanian et al., 2005). The NF- $\kappa$ B target gene set was taken from earlier studies (Compagno et al., 2009). GSEA was performed on a pre-ranked gene list based on their normalized mean read counts from the DESeq2 analysis. This list was then compared with the list of 120 NF- $\kappa$ B target genes to compute the GSEA enrichment scores. The algorithm implements weighted scores and was run with 1000 permutation tests.

**Candidemia patients, control volunteer cohorts, and genotyping.** In this study, we included 227 unrelated adult candidemia patients of European genetic background. Patients were enrolled after confirmation of at least one positive blood culture for a *Candida* species. The control cohort of 176 volunteers of European descent consisted of non-*Candida*-infected matched patients from the same clinical departments as the patient cohort. Controls were recruited consecutively from the same hospital wards as infected patients during the study period, with a similar balance of medical, surgical, and oncology patients. Review boards of the involved medical centers approved the study, and patients were enrolled after giving written informed consent (described in detail in (Jaeger et al., 2015)). Patients and control volunteers were genotyped on the Illumina ImmunoChip SNP array platform, which contains approximately 200,000 SNPs focused on genomic regions known to be involved in immune-mediated diseases (Trynka et al., 2011). After the application of quality control filters, we investigated

whether SNPs in the *VAV* genes influence susceptibility to candidemia in the case-control association. The publicly available SNAP server was used to plot the SNP of interest (rs4914950)(Johnson et al., 2008; Saxena et al., 2007).

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