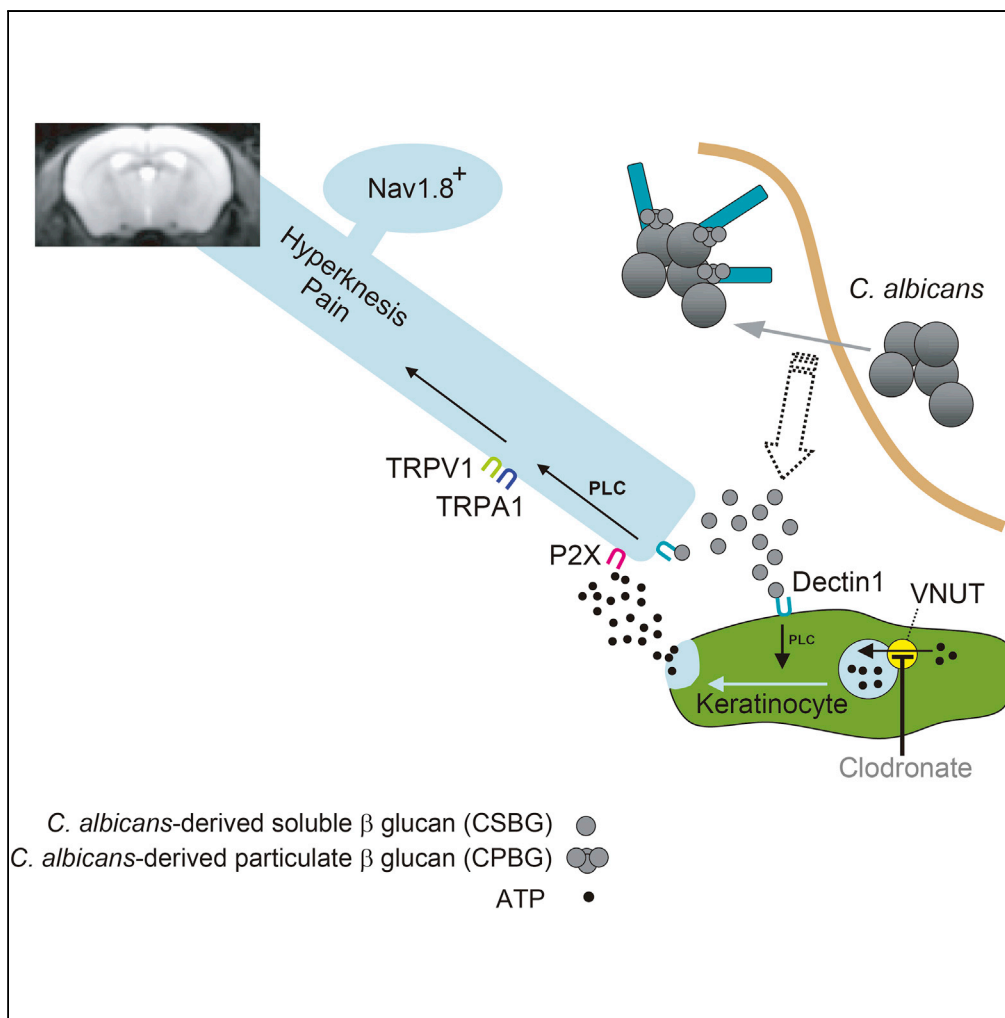


Article

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**HIGHLIGHTS**

$\beta$ -Glucan-induced pain is abolished in Dectin-1- and TRPV1/TRPA1-deficient mice

$\beta$ -Glucan stimulates nociceptors via Dectin-1-PLC axis, activating neurons

$\beta$ -Glucan-induced allodynia is dependent on extracellular ATP and VNUT

VNUT inhibitor clodronate can be used to treat  $\beta$ -glucan-induced allodynia

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## Article

# The ATP Transporter VNUT Mediates Induction of Dectin-1-Triggered *Candida* Nociception

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## SUMMARY

***Candida albicans* infection can cause skin, vulvar, or oral pain. Despite the obvious algescic activity of *C. albicans*, the molecular mechanisms of fungal nociception remain largely unknown. Here we show that the *C. albicans*-specific signaling pathway led to severe mechanical allodynia. We discovered that *C. albicans*-derived  $\beta$ -glucan stimulated nociceptors depending on Dectin-1, and two pathways in inflammatory pain. The major pathway operates via the Dectin-1-mediated ATP-P2X<sub>3</sub>/P2X<sub>2/3</sub> axis through intercellular relationships between keratinocytes and primary sensory neurons, which depends on the ATP transporter vesicular nucleotide transporter (VNUT). The other pathway operates via the Dectin-1-mediated PLC-TRPV1/TRPA1 axis in primary sensory neurons. Intriguingly, *C. albicans*-derived  $\beta$ -glucan has the ability to enhance histamine-independent pruritus, and VNUT inhibitor clodronate can be used to treat unpleasant feelings induced by  $\beta$ -glucan. Collectively, this is the first report to indicate that Dectin-1 and VNUT mediated innate sensory mechanisms that detect fungal infection.**

## INTRODUCTION

*Candida albicans* is an opportunistic fungus that thrives in the skin, mouth, vagina, and nipples. Immuno-deficiency within hosts and poorly controlled diabetes have increased the rates of invasive *C. albicans* infections, evoking skin or oral pain. Notably, approximately 75% of women experience vulvovaginal candidiasis at some point during their lifetime and 5% of women experience recurrent episodes of infection (Egan and Lipsky, 2000). It is well established that such mucosal invasion of *C. albicans* induces mechanical allodynia and itching (Farmer et al., 2011). For example, nipples are a common site of *C. albicans* infection, and breast candidiasis in a lactating mother is characterized by severe nipple pain (Amir et al., 2013). It has been well established that ion channels expressed in primary sensory neurons play a critical role in the sensing of pain (Woolf and Costigan, 1999). Transient receptor potential cation channel subfamily vanilloid member 1 (TRPV1) and transient receptor potential cation channel subfamily ankyrin member 1 (TRPA1) are vital ion channels that mediate nociceptive signaling (Julius, 2013). A recent report suggested that nociceptors directly sense Gram-positive bacterial components such as  $\alpha$ -hemolysin (Chiu et al., 2013). Another group reported that Gram-negative bacterial components, such as lipopolysaccharide, are sensed by TRPA1 (Meseguer et al., 2014). Thus, nociceptors may directly sense bacterial infection like innate immune cells.

From inside to outside, the *C. albicans* cell wall is composed of  $\beta$ -glucan and mannan (Gow et al., 2012). In response to invading fungi, innate immune cells recognize fungal surface mannan through Toll-like receptor (TLR) 4, leading to the production of cytokines via the activation of adaptor protein MyD88 and TRIF (Underhill and Lliev, 2014). A recent report suggested that mannan is also detected by TLR2, mannose receptor, Dectin-2, DC-SIGN, and Mincle (Lionakis and Netea, 2013). During the budding growth phase,  $\beta$ -glucan is exposed to the fungal surface and is sensed by Dectin-1 (Saijo et al., 2007). Ligand-stimulated Dectin-1 assembles a multimeric complex and induces signaling via the ITAM-like motif, leading to the activation of the CARD-9-Bcl-10-Malt-1 trimer (CBM trimer) and the NLRP3-ASC-ICE complex (NLRP3 inflammasome). Activation of the CBM trimer and the NLRP3 inflammasome is indispensable for the induction of

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Continued



nuclear factor (NF)- $\kappa$ B-dependent pro-inflammatory cytokine production and interleukin (IL)-1 $\beta$  maturation, respectively (Underhill and Lliev, 2014). Recently, the first fungal cytolytic peptide, named candidalysin, was discovered (Moyes et al., 2016). Because candidalysin is secreted from *C. albicans* and permeabilizes the epithelial membrane, it may contribute to the pathogenesis of fungal inflammation. Recently, our group discovered that *C. albicans* stimulates nociceptors via the  $\beta$ -glucan receptor Dectin-1 to induce Calcitonin gene-related peptide (CGRP). Notably, nociceptor-derived CGRP suppressed  $\beta$ -glucan-induced inflammation and osteoclast multinucleation via Jdp2-mediated NF- $\kappa$ B repression and inhibition of actin polymerization, respectively (Maruyama et al., 2017). Thus, nociceptors may modulate the fungal osteomyelitis, but mechanisms by which they sense and feel fungal invaders remains largely unknown.

In this study, we noticed that Dectin-1-deficient mice were unresponsive to fungal pain. *C. albicans*-derived soluble  $\beta$ -glucan (CSBG) induces robust pain via the Dectin-1-mediated ATP-P2X<sub>3</sub>/P2X<sub>2/3</sub> axis and Dectin-1-mediated phospholipase C (PLC)-TRPV1/TRPA1 axis. Furthermore, CSBG has the ability to enhance Mrgpr ligand chloroquine (CQ)-induced itch behaviors. Strikingly, inhibition of the ATP transporter vesicular nucleotide transporter (VNUT) by using clodronate abolished the unpleasant feelings induced by  $\beta$ -glucan.

## RESULTS

### *C. albicans*-Derived Soluble $\beta$ -Glucan (CSBG) Is a Critical Irritant Released from *C. albicans*

*C. albicans* injected into the hind paw of mice induced pain-related behaviors (Figure S1A). The pain sensation reportedly depends on the direct stimulation of primary sensory neurons by the fungus (Kashem et al., 2015). *C. albicans* was found to have already spread its hyphae, as observed in culture, when patients report a pain sensation in the early phase of invasive *Candida* infection (Figure 1A). The hyphae directly damage living cells; however, the molecular mechanisms inducing the pain sensation remain unclear. Recent report suggested that candidalysin, a fungal cytolytic peptide, is released from the hyphae and may evoke calcium influx into the cells (Moyes et al., 2016). Candidalysin induced slight mechanical allodynia; however, allodynia was also induced by the injection of Ece1 $\Delta/\Delta$  *C. albicans*, which cannot produce candidalysin, similar to wild-type *C. albicans* (Figures S1B–S1D). Furthermore, candidalysin did not induce intracellular calcium increases in the dorsal root ganglion (DRG) neurons isolated from mice (Figure S1E, observations of 43 cells by 3 trials). Therefore, we assumed that there are other molecules causing neural activation followed by uncomfortable sensations. To investigate these molecules, we focused on components of the fungal body and found that  $\beta$ -glucan was secreted from the fungus when cultured for 2 hr at 37°C (Figure 1B).  $\beta$ -Glucan is released as CSBG or *C. albicans*-derived particulate  $\beta$ -glucan (CPBG) in the infected regions (Figure S1F). CSBG might be a particularly important component for the pathological condition because when injected into the hind paw this  $\beta$ -glucan enhanced pro-inflammatory cytokine release, including tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-1 $\beta$ , and hind paw enlargement due to the infiltration of myeloid cells in the CSBG-injected area (Figures S1G–S1I). To explore whether the nature of  $\beta$ -glucan-induced pain *in vivo* is similar to TRPV1-mediated pain, fractional amplitude of low-frequency fluctuations (ALFF) analysis of resting-state brain fMRI (Zang et al., 2007; Zou et al., 2008) was performed to quantify the levels of CSBG or capsaicin-induced pain (Figures 1C–1G). The sensation of pain has been associated with activation of the primary somatosensory cortex (S1) and insula (Schweinhardt and Bushnell, 2010). Both CSBG and capsaicin injection significantly increased ALFF in the S1, and CSBG-evoked ALFF was 1.5-times more potent than that evoked by capsaicin (Figure 1F). A significant increase in insula ALFF was observed only in CSBG-treated mice (Figure 1F). Cross-correlation analysis revealed that differences in partial pairwise correlation coefficients between CSBG and capsaicin were significant between the anterior cingulate cortex (ACC) and hippocampus, between the ACC and S1, between the motor area and hippocampus, and between the thalamus and amygdala (Figure 1G). Because these areas are all involved in pain sensation (Bushnell et al., 2013), we conclude that *in vivo* CSBG nociception displays different characteristics in TRPV1-mediated pain.

### CSBG Potently Caused Allodynia Depending on Keratinocyte-Derived ATP

CSBG injection induced inflammatory conditions in hind paws (Figures S1H and S1I). Interestingly, mechanical allodynia was strongly induced by CSBG and CPBG, although mannan also slightly induced allodynia (Figure 2A). The  $\beta$ -glucan- or mannan-induced allodynia recovered after 24 hr, whereas pro-inflammatory cytokine release still occurred (Figures 2A and 2B). Among the *C. albicans* components, CSBG is the strongest analgesic substance. However, the cytokine stimulatory capacity of CSBG is weak compared with other *C. albicans* components (Figure 2B). Moreover, the allodynia induced by *C. albicans* was not reduced in

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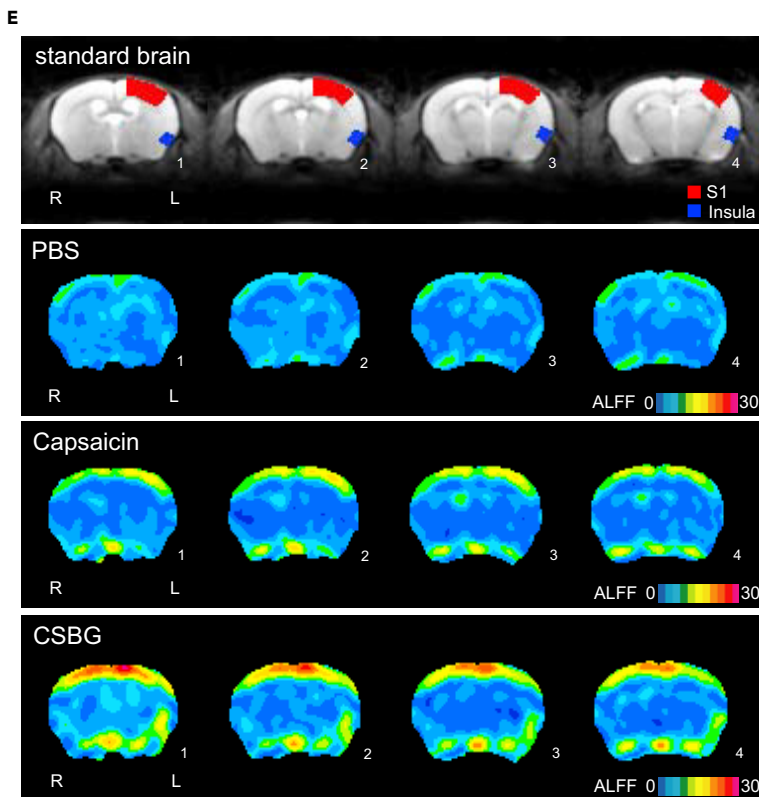
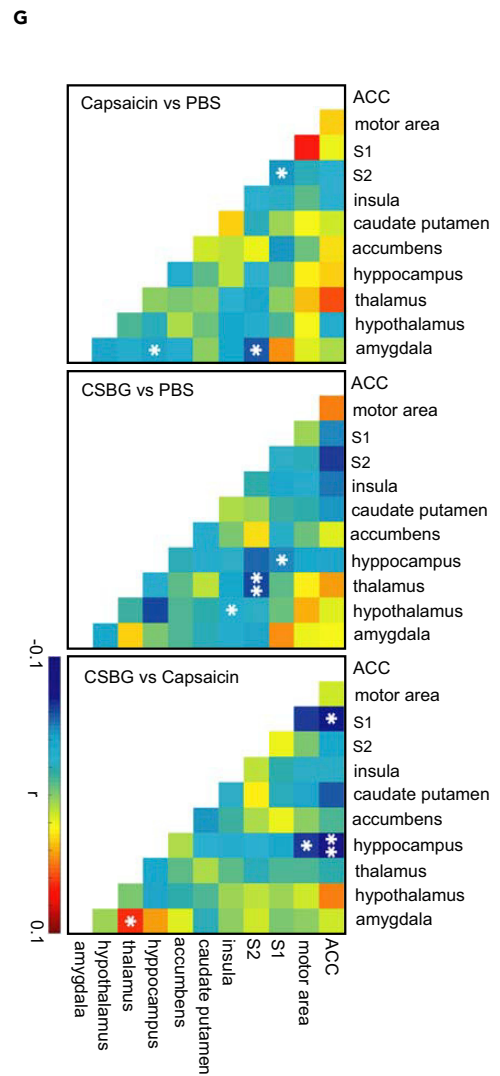
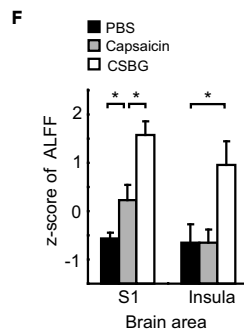
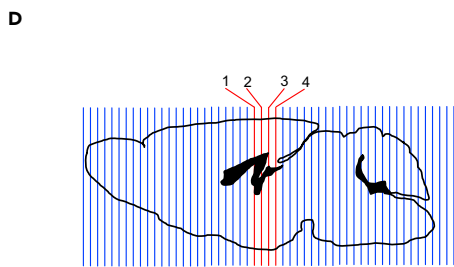
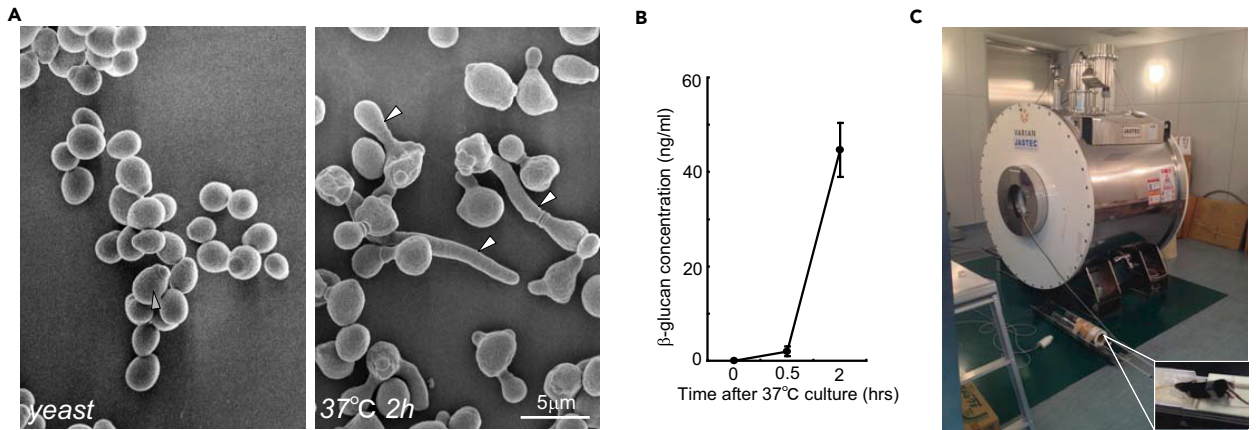
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**Figure 1. fMRI Analysis of *C. albicans*-Induced Pain Sensation**

(A and B) Morphology (A, white arrowheads: hyphae) and soluble  $\beta$ -glucan concentration (B,  $n = 4$ ) in yeast form *C. albicans* cultured at 37°C.

(C) Mouse MRI system. Mice were maintained under light anesthesia. phosphate buffered saline (PBS), capsaicin, or CSBG were injected into the right hind paw of mice, and fMRI images were recorded (30 min after injection,  $n = 6$ /group).

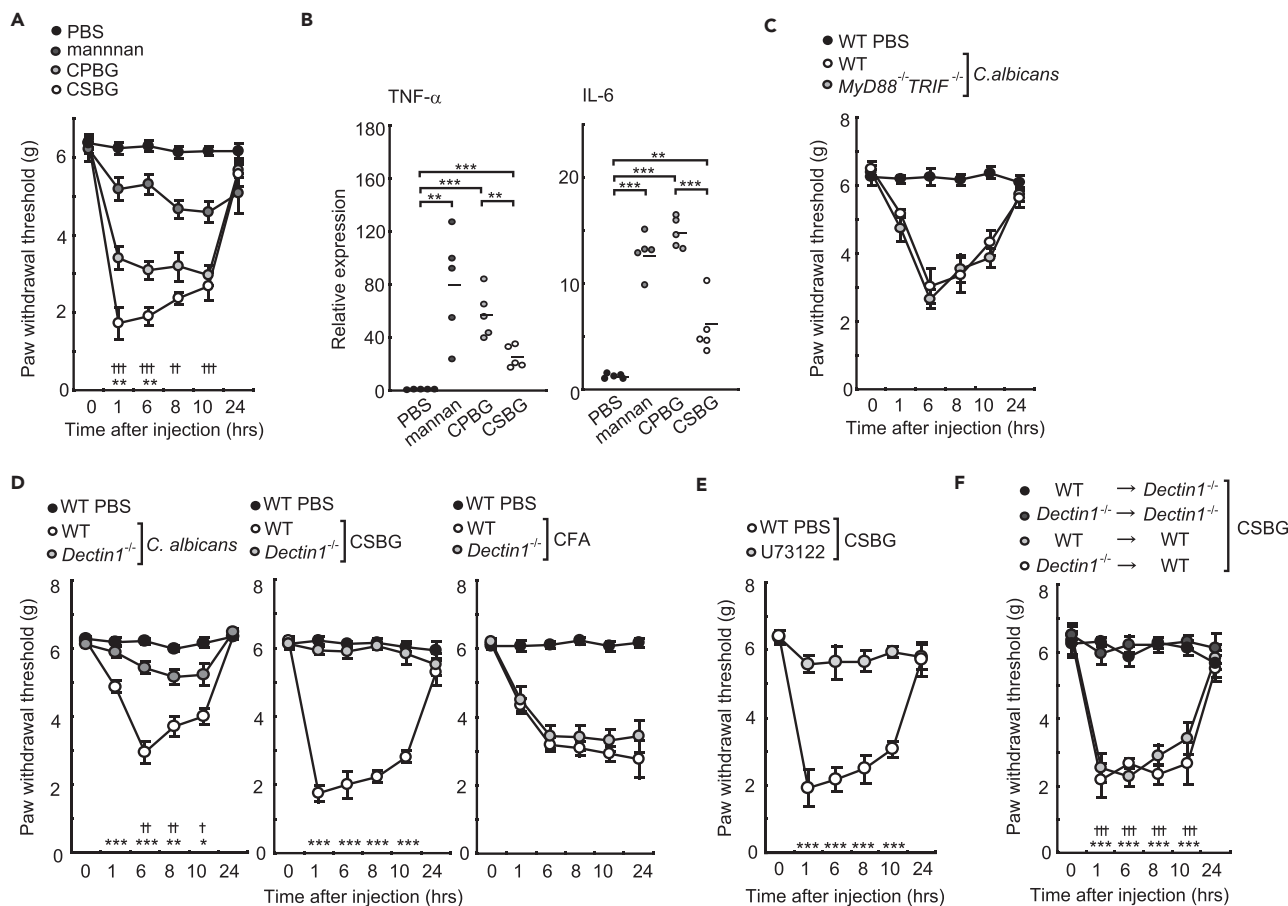
(D–G) A total of 55 sections of the brain were analyzed by MRI (D). Averaged ALFF maps of sections 1–4 in (D) are displayed (E). Group average z-scores of ALFF in the S1 and insula were calculated (F). Differences in partial pairwise correlation coefficients between the 2 groups are indicated and asterisks denote a significant difference (G). The difference maps of “capsaicin versus PBS,” “CSBG versus PBS,” or “CSBG versus capsaicin” show the difference in correlation between the 2 groups for the capsaicin-treated and the PBS-treated groups, the CSBG-treated and the PBS-treated groups, and the CSBG-treated and the capsaicin-treated groups, respectively. Blue color indicates that the former is greater than the latter. Red color indicates that the latter is greater than the former.

Error bars, SE; \* $p < 0.05$ ; \*\* $p < 0.01$  (two-sample, two-tailed t test).

*MyD88*- and *TRIF*-deficient mice (Figure 2C). Therefore, inflammatory responses through TLR pathways may not be involved in allodynia. However, Dectin-1 may be an important receptor for mechanical allodynia because *C. albicans*- or CSBG-induced allodynia was drastically reduced in Dectin-1-deficient mice, although Complete Freund's adjuvant (CFA)-induced allodynia was the same between wild-type and Dectin-1-deficient mice (Figure 2D). Notably, CSBG-induced allodynia was suppressed by the PLC inhibitor, U73122 (Figure 2E). Thus, downstream signaling of Dectin-1 activation is important for inducing allodynia.

It has been established that Dectin-1 is mainly expressed in immune cells. Therefore, we investigated the efficacy of the immune system, which may be involved in the pain sensation. Wild-type or Dectin-1-deficient bone marrow engrafted onto Dectin-1-deficient mice showed no mechanical allodynia in response to CSBG, whereas wild-type or Dectin-1-deficient bone marrow-engrafted wild-type mice showed similar allodynia in response to CSBG (Figure 2F). Furthermore, *Bcl-10*- or *Malt-1*-deficient strains showed mechanical allodynia similar to wild-type mice in response to CSBG, CPBG, or heat-killed (hk) *C. albicans* (Figure 3A). CSBG-induced mechanical allodynia in mice deficient in inflammasome component genes (Figure 3B), histamine receptor H1 (*Hrh1*)-deficient mice (Figure 3C), non-obese diabetic/severe combined immunodeficiency mice (lacking T and B cells, Figure 3C), clodronate liposome-treated mice (lacking macrophages, Figures 3D and 3E), Ly6G antibody-injected mice (lacking neutrophils, Figures 3F and 3G), ibuprofen-treated mice (Figure 3H), and TNF- $\alpha$ -antibody-injected mice (Figure 3I) was indistinguishable from that in wild-type mice. These results clearly indicated that the immune system is not involved in *C. albicans*-induced allodynia, and we hypothesized that a non-hematopoietic cell-derived factor contributes to *C. albicans*-induced allodynia. Among the non-immune cells, keratinocytes express Dectin-1 (Figure 4A), and it is reported that an intercellular signal passes from the keratinocytes to neurons via ATP (Mandadi et al., 2009). To test whether *C. albicans* induce ATP secretion from keratinocytes, we stimulated keratinocytes with mannan, CPBG, CSBG, and *C. albicans*. ATP was strongly released by CPBG, CSBG, and *C. albicans* in a Dectin-1-dependent manner, whereas ATP release from isolated DRG neurons was weaker than that from keratinocytes (Figures 4B, 4C, and S2A). To test whether ATP exocytosis is involved in Dectin-1-mediated allodynia, we focused on the VNUT. Keratinocytes isolated from VNUT-deficient mice showed dramatically impaired ATP release in response to CSBG and *C. albicans* (Figures 4C and S2B). VNUT is also reportedly expressed in DRG neurons (Nishida et al., 2014), where ATP release was also found to be reduced (Figure 4C). Thus, keratinocytes mainly produce ATP and cell-cell interactions with primary sensory neurons are important for protecting the infected area, although the neurons could be activated by autocrine stimulation. Strikingly, CSBG or hk *C. albicans*-induced mechanical allodynia was almost abolished in VNUT-deficient mice, although the expression levels of Dectin-1, TRPV1, and TRPA1 were similar between wild-type and VNUT-deficient mice (Figures 4D, 4E, and S2C). Collectively, our findings provide the first evidence that *C. albicans*-induced allodynia is dependent on Dectin-1-stimulated keratinocyte-derived ATP.

It has been reported that cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activation through P2X receptor in DRG neurons is involved in the pathogenesis of neuropathic pain (Tsuda et al., 2007). Therefore, we next investigated the effect of A317491, an inhibitor of P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors (Jarvis et al., 2002), on CSBG-induced allodynia. A317491 treatment significantly suppressed CSBG-induced mechanical allodynia (Figure 4F). Recently, it has been reported that ATP activates inflammasomes through P2X receptors (Gombault et al., 2013) and the activated inflammasomes may induce allodynia. Therefore, we investigated the effects of  $\alpha,\beta$ -methyleneadenosine 5'-triphosphate ( $\alpha\beta$ mATP) on allodynia in inflammasome-deficient mice, including *NLRP3*, *ASC*, and *ICE* mutant strains. However,  $\alpha\beta$ mATP-induced allodynia was not reduced in these deficient mice (Figure 4G). Thus, we conclude that the pathway to induce



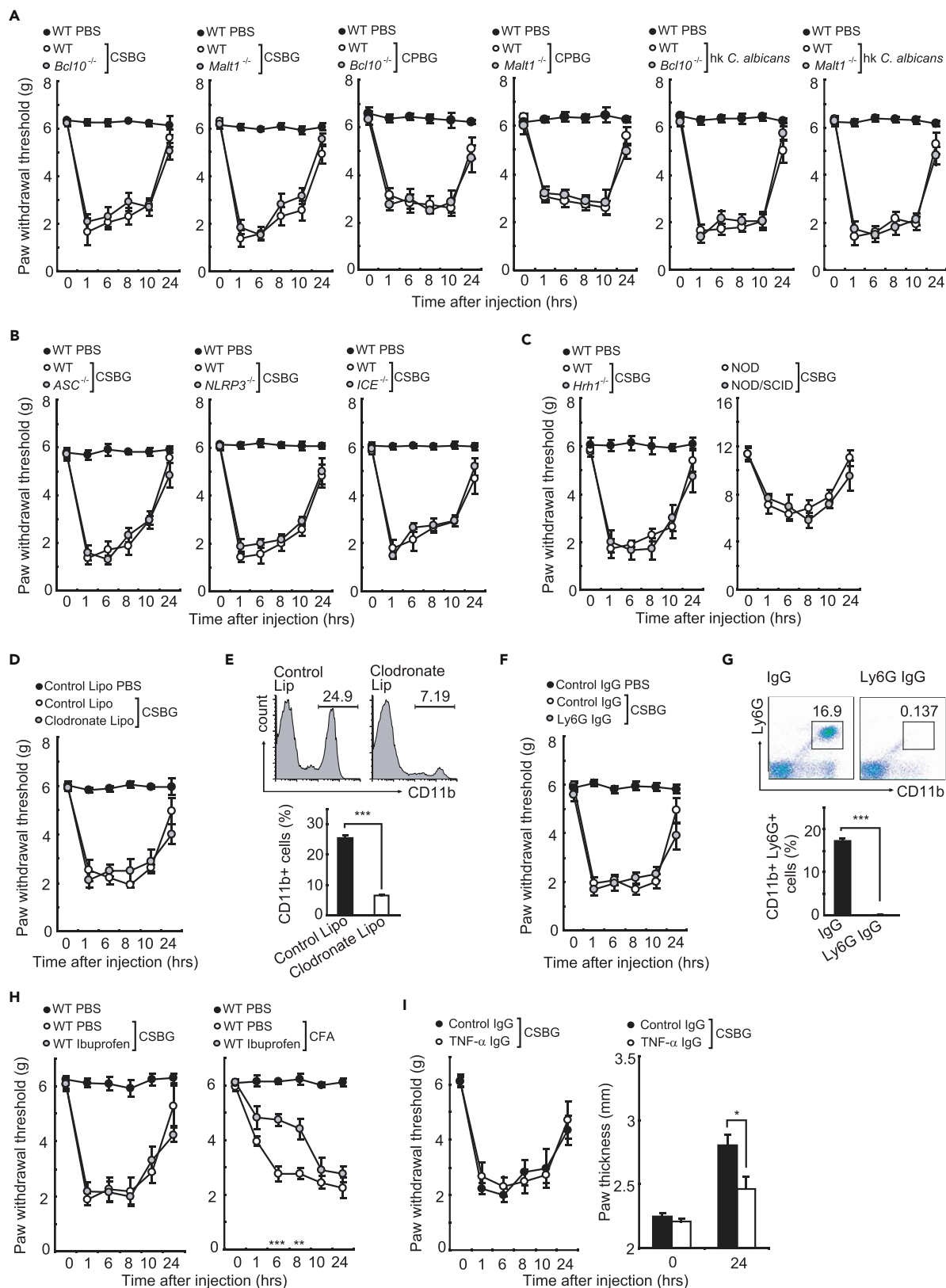
**Figure 2. β-Glucan-Induced Allodynia Is Dependent on Dectin-1**

(A) Mechanical allodynia in response to *C. albicans* components (n = 10/group; †, mannan versus phosphate buffered saline (PBS); \*CPBG versus CSBG). (B) Mannan, CPBG, or CSBG were injected into the hind paws of mice. After 24 hr, TNF-α and IL-6 expression levels in the hind paws were measured using qPCR (n = 5). (C) *C. albicans*-induced mechanical allodynia in *MyD88*<sup>-/-</sup>, *TRIF*<sup>-/-</sup>, and wild-type (WT) mice (all groups n = 7). (D) Mechanical allodynia with hind paw injection of *C. albicans*, CSBG, or CFA in *Dectin1*<sup>-/-</sup> and WT mice (n = 8/group; †, WT PBS versus WT; \*, WT versus *Dectin1*<sup>-/-</sup>). (E) CSBG-induced mechanical allodynia after pretreatment with U73122 (10 μM, 25 μL) (n = 6/group). (F) Mechanical allodynia with hind paw injection of CSBG in WT or *Dectin1*<sup>-/-</sup> mice irradiated and reconstituted with bone marrow from *Dectin1*<sup>-/-</sup> or WT mice (n = 6/group; †, WT → *Dectin1*<sup>-/-</sup> versus WT → WT; \**Dectin1*<sup>-/-</sup> → *Dectin1*<sup>-/-</sup> versus *Dectin1*<sup>-/-</sup> → WT). Error bars, SE; \*or † p < 0.05; \*\*or †† p < 0.01; \*\*\*or ††† p < 0.001.

allodynia by *C. albicans* is molecular signaling between Dectin-1 in keratinocytes and P2X<sub>3</sub> and P2X<sub>2/3</sub> in primary sensory neurons.

**Dectin-1-Mediated Activation of TRPV1 and TRPA1 was Critically Involved in CSBG-Induced Pain**

Our MRI results suggest that CSBG induced the acute pain sensation (Figure 1). Our previous work suggested that mRNA expression of Dectin-1 is detected in DRG (Maruyama et al., 2017). To check which DRG population expresses Dectin-1, we next analyzed mRNA expression of Dectin-1 in Nav1.8-positive DRG (Figures S3A and S3B). The *Nav1.8Cre* mice were coupled with *ROSA26-tdRFP* mice and Nav1.8-positive DRG neurons were separated by fluorescence-activated cell sorting (FACS) analysis depending on the red fluorescent protein (RFP) fluorescence level. Because the Dectin-1 expression level was higher in Nav1.8-positive neurons (Figures S3A and S3B), Dectin-1 appeared to be expressed in Nav1.8-positive primary DRG neurons (Figures S3C–S3E). We next investigated the acute irritant effect of CSBG in Dectin-1-deficient mice (Figure S3F). CSBG-induced pain-related behaviors (cheek wiping) were slightly



**Figure 3.  $\beta$ -Glucan-Induced Allodynia Is Independent of Immunity**

(A–C) CSBG or heat-killed *C. albicans*-induced mechanical allodynia in the indicated mice (A, n = 8; B, n = 10; C, n = 5). (D and E) Clodronate liposome or control liposome were injected into wild-type (WT) mice. After 24 hr, CSBG-induced allodynia was analyzed (D). Cells were prepared from spleens and analyzed by FACS analysis (E) (n = 8). (F and G) Anti-Ly6G antibody or isotype control antibody were injected into WT mice. After 24 hr, CSBG-induced allodynia was analyzed (F). Cells were prepared from spleens and analyzed by FACS (G) (n = 8). (H) Nonselective cyclooxygenase inhibitor, ibuprofen (200 mg/kg), or the vehicle control were orally administered 1 hr before CSBG or CFA injection into the hind paws. Ibuprofen significantly suppressed CFA-induced allodynia, but not CSBG-induced allodynia (n = 8). (I) Anti-TNF- $\alpha$  antibody or isotype control antibody was injected into WT mice. After 24 hr, CSBG-induced allodynia (left) and paw thickness (right) were quantified (n = 5). Error bars, SE; \*p < 0.05; \*\* p < 0.01; \*\*\*p < 0.001.

observed in wild-type mice but not in Dectin1-deficient mice. In contrast, pain-related behaviors were not reduced in Bcl-10- or Malt-1-deficient mice (Figure S3G). Thus, acute pain caused by *C. albicans* may depend on Dectin-1 and pro-inflammatory cytokines may be unnecessary for  $\beta$ -glucan-induced acute pain.

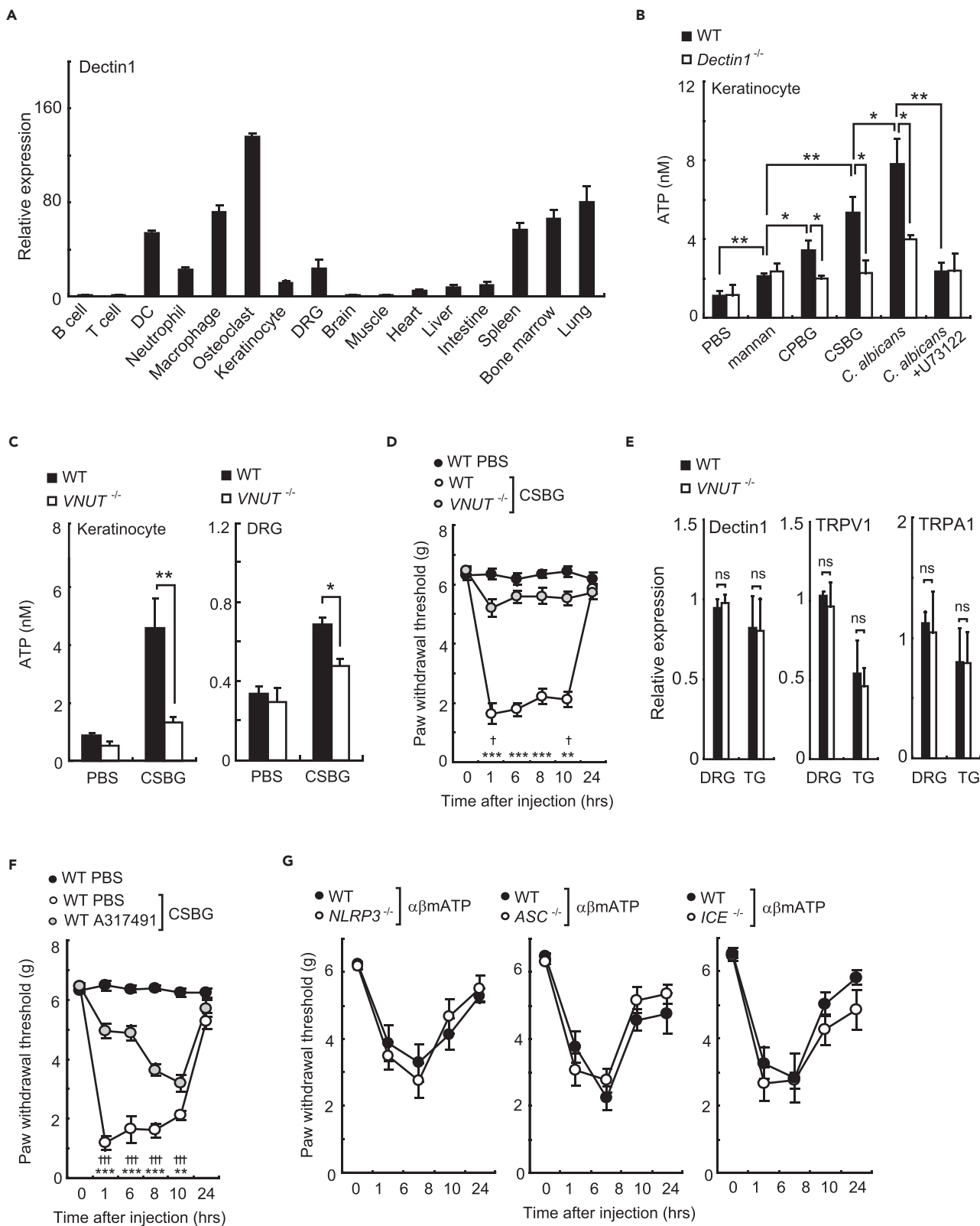
Although an extract from hk *C. albicans* did not induce potent calcium increases (data not shown), we analyzed CSBG response in DRG neurons from wild-type and Dectin-1-deficient mice. Calcium imaging suggests the functional expression of Dectin-1 in isolated DRG neurons (Figures S3H and S3I). Unexpectedly, 5 of 136 neurons in wild-type mice showed rapid increases in intracellular calcium concentrations that occurred within 1 min after CSBG application (Figure S3G). No rapid calcium increases were detected in Dectin-1-deficient cells (total 64 cells by 6 trials) and vehicle (total 30 cells by 3 trials). We also discovered that DRG neurons express spleen tyrosine kinase (Syk), a critical factor of Dectin-1 signaling, and that CSBG can induce phosphorylation of PLC $\gamma$ 2 in DRG neurons depending on Dectin-1 (Figures S3C and S3J–S3L). Notably, PLC inhibitor U73122 significantly reduced  $\beta$ -glucan-induced pain (Figure 2E). It is thought that TRP channel activation could also be accelerated by the PLA<sub>2</sub>-PKC axis (Nishizuka, 1992) and TRPV1 and TRPA1 activations downstream of GPCR depend on PLC $\beta$  activation, and these pathways are crucial in inflammatory conditions induced by factors such as bradykinin (McMahon and Wood, 2006). However, our novel finding suggests the importance of phosphorylated PLC $\gamma$ 2 in DRG neurons on inflammatory conditions induced by  $\beta$ -glucan. These results indicate that some DRG neurons could be directly involved in CSBG detection. However, acute pain-related behaviors were induced by hk *C. albicans* injection (Figure S4). Furthermore, the behaviors were reduced in TRPV1- and/or TRPA1-deficient mice and inhibited by U73122 treatment. Thus, the relationships between keratinocytes and DRG neurons could be important in *in vivo* situation, although *in vivo* ATP releases could not be analyzed.

Next, we investigated whether TRPV1 and TRPA1 were involved in CSBG-induced allodynia; TRPV1- or TRPA1-deficient mice showed incomplete reduction of mechanical allodynia, which was completely inhibited in TRPV1/TRPA1 double-deficient mice (Figures 5A–5C). Strikingly, hk *C. albicans*-induced acute pain-related behaviors were also inhibited in mice deficient in TRPV1 and/or TRPA1 (Figure S4). Thus, these results indicate that the Dectin-1-mediated activation of TRPV1 and TRPA1 in primary sensory neurons is an important signaling pathway in  $\beta$ -glucan-induced pain sensation in *Candida* infection.

**Clodronate Inhibits CSBG-Mediated Enhancement of Histamine-Independent Itch Sensation and CSBG-Induced Mechanical Allodynia**

In a clinical situation, *Candida* infection causes an itch sensation. To clarify the pruritogenic effects of *C. albicans* *in vivo*, we investigated whether *C. albicans* triggers acute pain-related behaviors (wiping) with or without itching using a cheek injection model (Figure 6A). *C. albicans* was observed to cause wiping behaviors, and this response was significantly enhanced by heat killing. The *C. albicans* culture supernatant also caused wiping behaviors, and this effect was not changed by heating of the supernatant. Scratching behaviors were never observed, indicating that the acute phase of infection with *C. albicans* may cause pain rather than itching. Therefore, we hypothesized that  $\beta$ -glucan enhances the itch sensation induced by the pruritogen CQ, because TRPA1 is reportedly involved in cell signaling of the itch sensation, whereas TRPV1 is not required in this histamine-independent itch pathway (Wilson et al., 2011). Interestingly, CSBG and CPBG significantly enhanced CQ-induced itch-related behaviors and these behaviors were inhibited in Dectin-1-deficient mice (Figures 6B and 6C). Recently, it has been reported that clodronate is a strong inhibitor of VNUT (Kato et al., 2017). A low concentration of clodronate impaired vesicular ATP release from cells. To check the prophylactic potential of clodronate to  $\beta$ -glucan-mediated mechanical allodynia and enhancement of histamine-independent itch sensation, we intravenously or subcutaneously injected





**Figure 4.  $\beta$ -Glucan-Induced Allodynia Is Dependent on ATP**

(A) Expressions of Dectin-1 in various cells and tissues (n = 3).

(B) ATP levels in the culture supernatant of keratinocytes stimulated for 3 hr by mannan (100  $\mu$ g/mL), CSBG (100  $\mu$ g/mL), CPBG (100  $\mu$ g/mL), *C. albicans*, or *C. albicans* plus PLC inhibitor U73122 (10  $\mu$ M) (n = 4).

(C) ATP levels in the culture supernatant of keratinocytes or DRG from *VNUT*<sup>-/-</sup> and WT mice stimulated by CSBG (100  $\mu$ g/mL) for 3 hr (n = 4).

(D) CSBG-induced mechanical allodynia in *VNUT*<sup>-/-</sup> and WT mice (n = 8–18; *VNUT*<sup>-/-</sup> CSBG; †, WT phosphate buffered saline (PBS) versus WT; \* WT versus *VNUT*<sup>-/-</sup>).

(E) Dectin-1, TRPV1, and TRPA1 expression levels in the DRG from *VNUT*<sup>-/-</sup> mice and control mice (n = 3).

(F) A317491 (10  $\mu$ M, 25  $\mu$ L) or PBS were injected into the hind paws of WT mice. After 30 min, CSBG was injected into the hind paws and mechanical allodynia was measured (n = 6/group; †, WT PBS versus WT; \*, WT versus WT A317491).

(G) Mechanical allodynia in  $\alpha$  $\beta$ mATP-treated *NLRP3*<sup>-/-</sup>, *ASC*<sup>-/-</sup>, *ICE*<sup>-/-</sup>, and WT mice (n = 6/group).

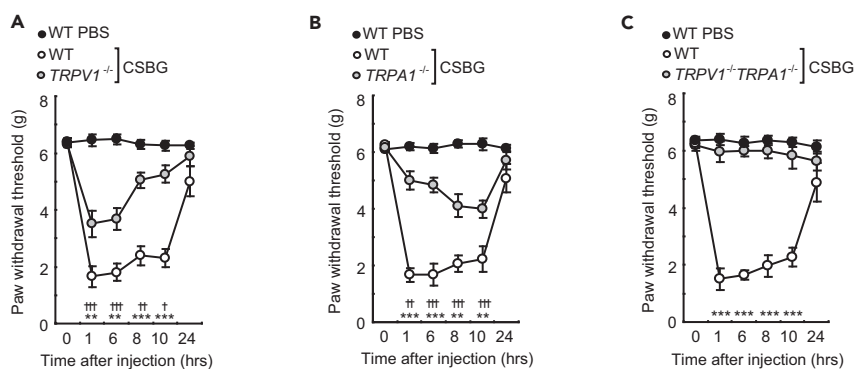
Error bars, SE; \*or † p < 0.05; \*\*p < 0.01; \*\*\*or ††† p < 0.001.

clodronate into mice 60 min before the ligand injection. To our surprise, clodronate pretreatment significantly inhibited  $\beta$ -glucan-induced mechanical allodynia and  $\beta$ -glucan-mediated enhancement of CQ-induced itch behavior (Figures 6D and 6E). Strikingly, CSBG-plus-CQ-induced itch-related behaviors were dramatically impaired in *VNUT*-deficient mice (Figure 6F). Collectively, these findings clearly indicate that Dectin-1 and *VNUT* are crucial components to express neural functions involved in both pain and itch sensations evoked by  $\beta$ -glucan and clodronate can be used to treat unpleasant feelings induced by fungal infection (Figure 6G).

**DISCUSSION**

Here we showed that CSBG is a potent irritant secreted by *C. albicans*. A previous report suggested that MyD88 signaling mediates Dectin-1 ligand zymosan-induced allodynia accompanied by the production of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  (Guerrero et al., 2012). Contrary to this report, our *in vivo* analysis revealed that innate immunity has limited effects on CSBG-induced allodynia. Discrepancies between our findings and this previous report may be explained by the difference in ligand composition (zymosan is composed of mannan plus  $\beta$ -glucan). Significantly, no allodynia was detected when CSBG was injected into the hind paw of *VNUT*-deficient mice, which cannot secrete ATP in response to Dectin-1 stimulation. A recent report suggested that clodronate inhibits *VNUT* at a half maximal inhibitory concentration of 15.6 nM without affecting other vesicular transporters, acting as an allosteric modulator through competition with Cl<sup>-</sup> (Kato et al., 2017). Our *in vivo* behavioral assay clearly indicated that low-dose clodronate treatment can be used to abolish the unpleasant feelings induced by  $\beta$ -glucan. Intriguingly, systemic injection of large-dose clodronate liposome to deplete macrophages had no impact on  $\beta$ -glucan-induced allodynia. Such discrepancy may be explained by the severe cytotoxicity of high-concentration clodronate liposome. Notably, inflammasome components were dispensable for  $\alpha$  $\beta$ mATP-induced allodynia, suggesting that ATP signaling in nociception does not use the inflammasome cascade. Recently, it has been reported that nociceptors directly sense *Staphylococcus aureus* cytolytic components such as  $\alpha$ -hemolysin (Chiu et al., 2013). In contrast to *S. aureus*, *C. albicans*-derived newly discovered cytolytic peptide candidalysin did not contribute to enhance the intracellular calcium concentrations in DRG. In contrast, infection with candidalysin-deficient *C. albicans* revealed a slight impairment in allodynia compared with wild-type *C. albicans*. Thus, candidalysin may induce cytotoxicity in keratinocytes, leading to an increase in extracellular ATP, which weakly contributes to allodynia, and to our knowledge, this is the first study to provide evidence that extracellular ATP evokes fungal allodynia. A recent study in mice revealed that repeated vaginal *C. albicans* infections cause mechanical allodynia accompanied by mucosal hyperinnervation with nociceptors (Farmer et al., 2011). This study also reported that vaginal mechanical allodynia can persist long after the resolution of active *C. albicans* infection. Vulvar pain associated with previous infection (vulvodynia) affects large numbers of women of childbearing age (Farmer et al., 2011). Because the most promising treatment of severe vulvodynia is surgical excision of the vulval tissue (Goldstein and Burrows, 2008), ATP- or *VNUT*-targeted therapy such as clodronate treatment may be a promising drug repositioning.

In this study, we also showed that CSBG activates Nav1.8-positive DRG neurons via Dectin-1 to evoke acute pain. A previous *in vitro* study suggested that hk *C. albicans* induces a calcium influx in TRPV1-positive nociceptors (Kashem et al., 2015). Contrary to this study, cross-correlation analysis of resting-state fMRI clearly indicated that *in vivo* CSBG nociception displays different characteristics in TRPV1-mediated pain. In myeloid cells, Dectin-1 signaling is mainly activated by CPBG, which triggers phagocytosis, leading to the clustering of Dectin-1 receptors in synapse-like structures, from which the regulatory tyrosine phosphatases CD45 and CD148 are excluded (Goodridge et al., 2011). Our results indicated that the algescic activity



**Figure 5. TRPV1 and TRPA1 Were Indispensable for  $\beta$ -Glucan-Induced Allodynia**

(A–C) Mechanical allodynia following hind paw injection with CSBG in (A)  $TRPV1^{-/-}$ , (B)  $TRPA1^{-/-}$ , and (C)  $TRPV1^{-/-}TRPA1^{-/-}$  mice ( $n = 6$ – $8$ /group; †, WT phosphate buffered saline (PBS) versus WT; \*, WT versus mutant) ( $n = 10$ /group). Error bars, SE; †  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* or †††  $p < 0.001$ .

of CPBG was weaker than that of CSBG. Because DRG neurons do not express CD45 or CD148 and are not considered to be phagocytic, Dectin-1 signaling in DRG neurons may behave differently compared with that in myeloid cells. Our behavioral analysis further suggested that *C. albicans*-induced acute pain is dependent on the Dectin-1-mediated activation of the PLC-TRPV1/TRPA1 axis. In this study, we also showed the synergic effect of Dectin-1 signaling on CQ-mediated itch behaviors. Dectin-1 signaling may activate TRPA1, which could be the mechanism that causes the vulvovaginal candidiasis-induced itch.

Collectively, *C. albicans* stimulates Nav1.8-positive nociceptors via the Dectin-1 to induce acute pain. *C. albicans*-derived  $\beta$ -glucan induces allodynia, which depends on the ATP transporter VNUT, but not on the immune system. This suggests that fungal allodynia induction requires extracellular ATP. Our previous study revealed that hind paw  $\beta$ -glucan injection after nociceptor ablation or in TRPV1/TRPA1 deficiency showed dramatically increased osteoinflammation accompanied by impaired CGRP production (Maruyama et al., 2017). Notably, CGRP inhibited  $\beta$ -glucan-induced cytokine production and bone resorption by osteoclasts. These previous discoveries and the findings of this study clearly indicate that the signaling pathway from Dectin-1 to TRP channels is a novel molecular mechanism of pain generation and that CGRP production accelerated by sensory nervous excitation is critical for the resolution of fungal inflammation.

### Limitations of the Study

It would be intriguing to analyze keratinocyte- or DRG-specific Dectin-1- (or VNUT-) deficient mice. To address such an important issue as future direction is important.

### METHODS

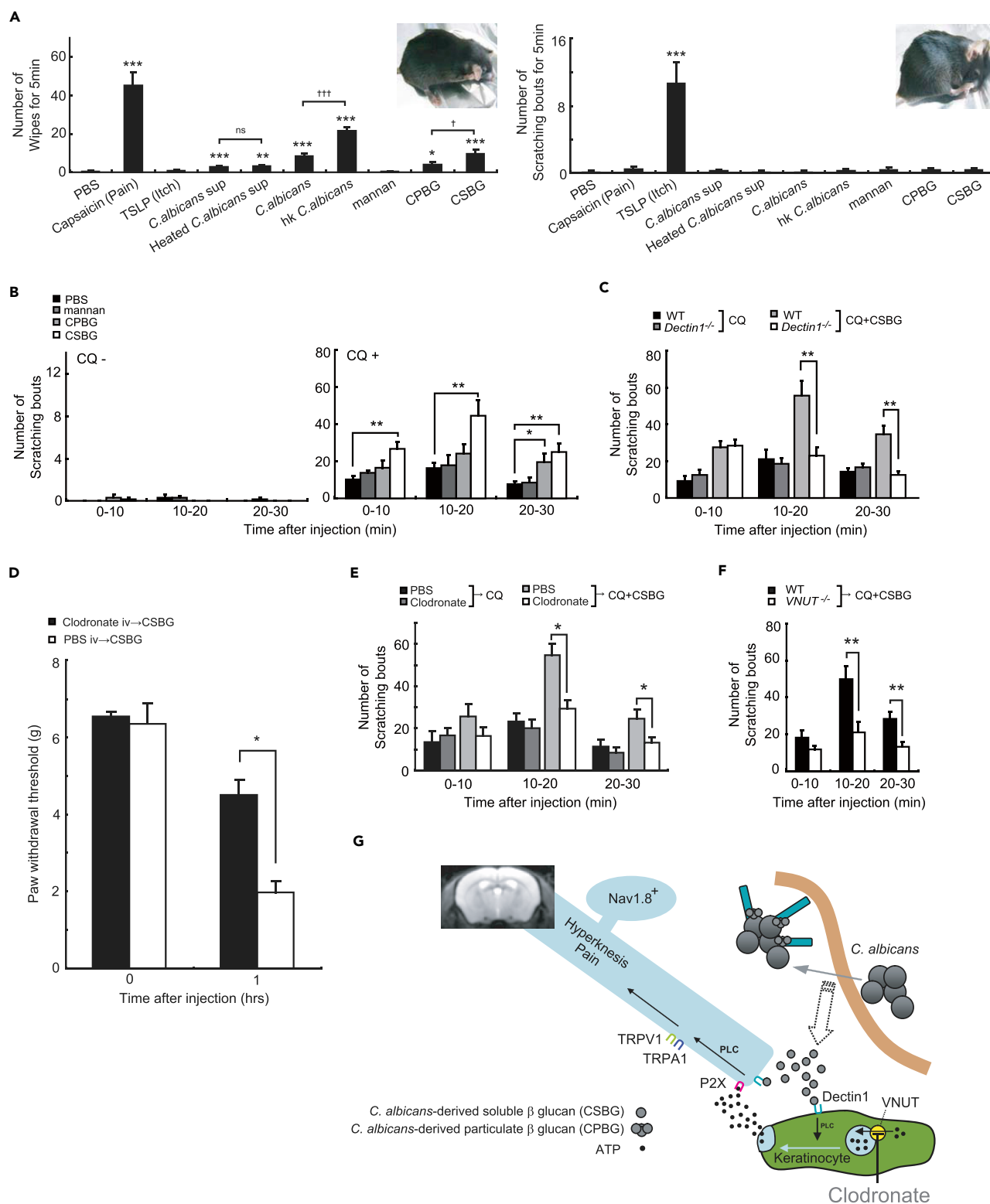
All methods can be found in the accompanying [Transparent Methods supplemental file](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and four figures and can be found with this article online at <https://doi.org/10.1016/j.isci.2018.08.007>.

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**Figure 6. β-glucan-Induced Allodynia and Enhancement of Histamine-Independent Itch were Neutralized by VNUT Inhibitor Clodronate**

(A) Wiping and scratching behaviors after injection of mice with *C. albicans* or its components (15 μg, n = 10 for each injection group).

(B) Mannan, CPBG, or CSBG were injected into the cheek with or without chloroquine (CQ), and scratching behaviors were observed (n = 7 for each injection group).

**Figure 6. Continued**

(C) CQ was injected into the cheek of *Dectin1*<sup>-/-</sup> or WT mice with or without CSBG, and scratching behaviors were observed (n = 7 for each injection group).  
 (D) CSBG-induced mechanical allodynia after preinjection with clodronate (10mg/kg intravenous [IV] injection, 60 min before CSBG injection into footpad. n = 6/group; \*, phosphate buffered saline (PBS) IV → CSBG versus clodronate → CSBG).  
 (E) PBS or clodronate (300μg/25ul) was injected into the cheek. After 60 min, CQ was injected into the cheek with or without CSBG, and scratching behaviors were observed (n = 7 for each injection group).  
 (F) CQ plus CSBG was injected into the cheek of *VNUT*<sup>-/-</sup> or WT mice, and scratching behaviors were observed (n = 7 for each injection group).  
 (G) Model for the novel innate sensory mechanisms of *Candida* infection. *C. albicans*-derived β-glucan directly stimulates Nav1.8-positive pain nerve via Dectin-1 to induce acute pain. *C. albicans*-derived β-glucan also induces allodynia and hyperknesis (abnormal pruriceptive state in which there is a normally pruritic stimulus). Notably, β-glucan-induced allodynia was not dependent on the immune system, but instead on keratinocyte-derived ATP.  
 Error bars, SE; \*or † p < 0.05; \*\*p < 0.01; \*\*\* or ††† p < 0.001.

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**AUTHOR CONTRIBUTIONS**

K.M. initiated, designed, and conducted the entire project. Y.T. and Yu Yamoni performed Ca<sup>2+</sup> imaging. T.Y., Y.M., Yoshichika Yoshioka, H.N., and A.S. contributed to MRI analysis. E.S., T.K., Y.K., B.R.S., H.K., M.M.M., H.T., and N.T. designed and conducted the immunological experiments. K.I. and N.O. provided *C. albicans*-derived β-glucan and contributed to the experimental design. Y.I. provided Dectin-1-deficient mice and conducted animal experiments. Yoshinori Moriyama and M.N. provided *VNUT*-deficient mice and contributed to the experimental design. Y.T. and M.T. provided TRPV1- and TRPA1-deficient mice and designed the physiological experiments. S.A. supervised the research. The manuscript was written by K.M., Y.T., and M.T.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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ISCI, Volume 6

## Supplemental Information

### The ATP Transporter VNUT Mediates

### Induction of Dectin-1-Triggered

### *Candida* Nociception

Kenta Maruyama, Yasunori Takayama, Erika Sugisawa, Yu Yamanoi, Takashi Yokawa, Takeshi Kondo, Ken-ichi Ishibashi, Bikash Ranjan Sahoo, Naoki Takemura, Yuki Mori, Hisashi Kanemaru, Yutaro Kumagai, Mikaël M. Martino, Yoshichika Yoshioka, Hisao Nishijo, Hiroki Tanaka, Atsushi Sasaki, Naohito Ohno, Yoichiro Iwakura, Yoshinori Moriyama, Masatoshi Nomura, Shizuo Akira, and Makoto Tominaga

## **Supplemental Information**

### **The ATP transporter VNUT mediates induction of Dectin-1-triggered *Candida* nociception**

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*Kenta Maruyama and Yasunori Takayama contributed equally to this work.*



Figure S1

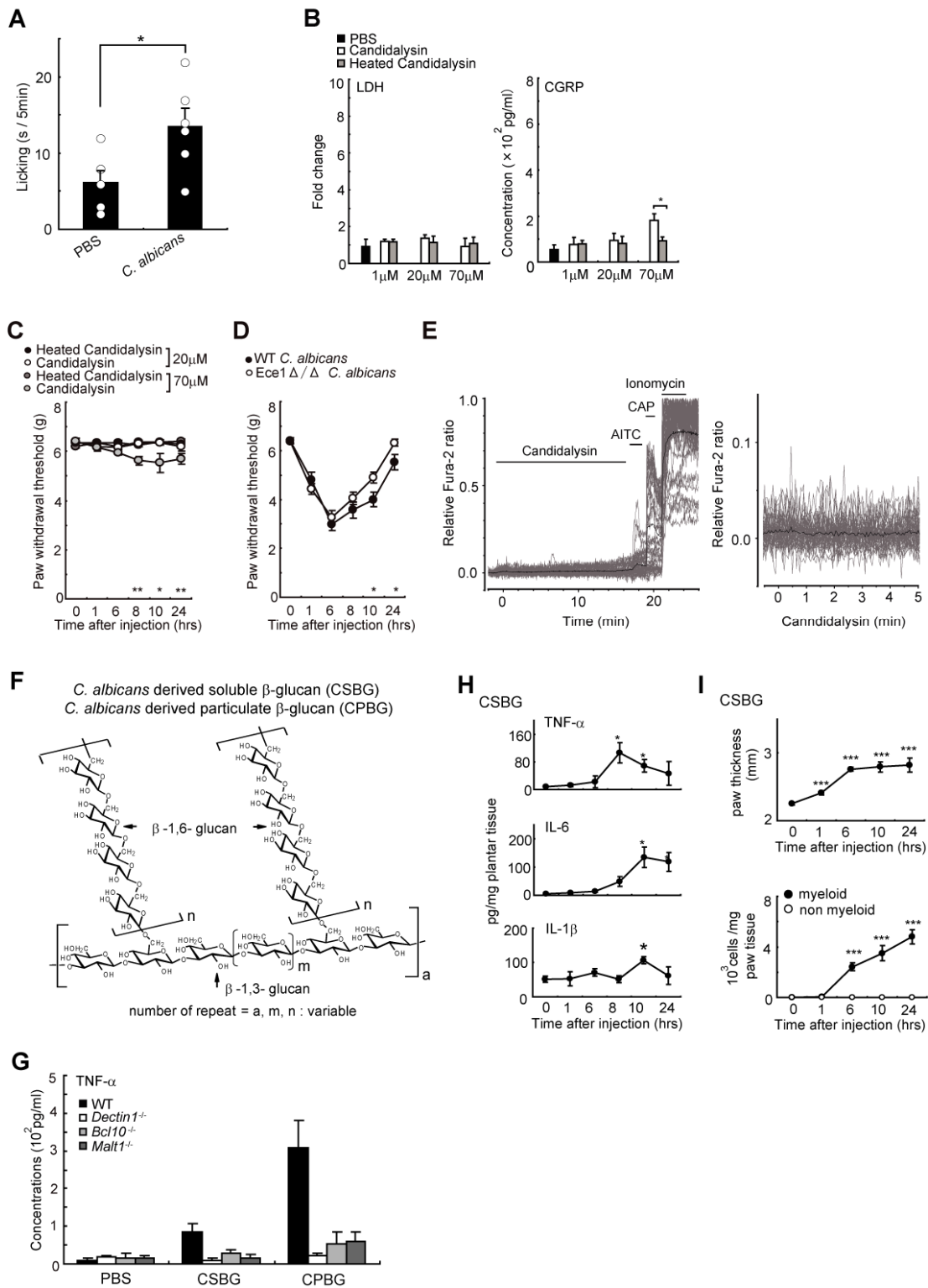
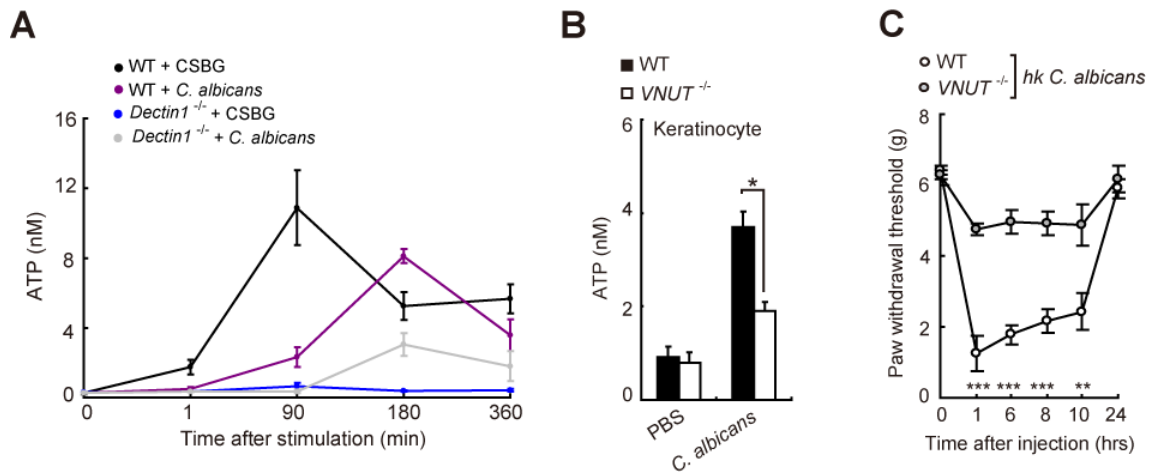


Figure S1. *C. albicans* and its components induce pain-related behaviors and inflammation.

Related to Fig. 1, Figure 2 and Figure 3. (A) *C. albicans*-induced pain-related licking behaviors (n=6).

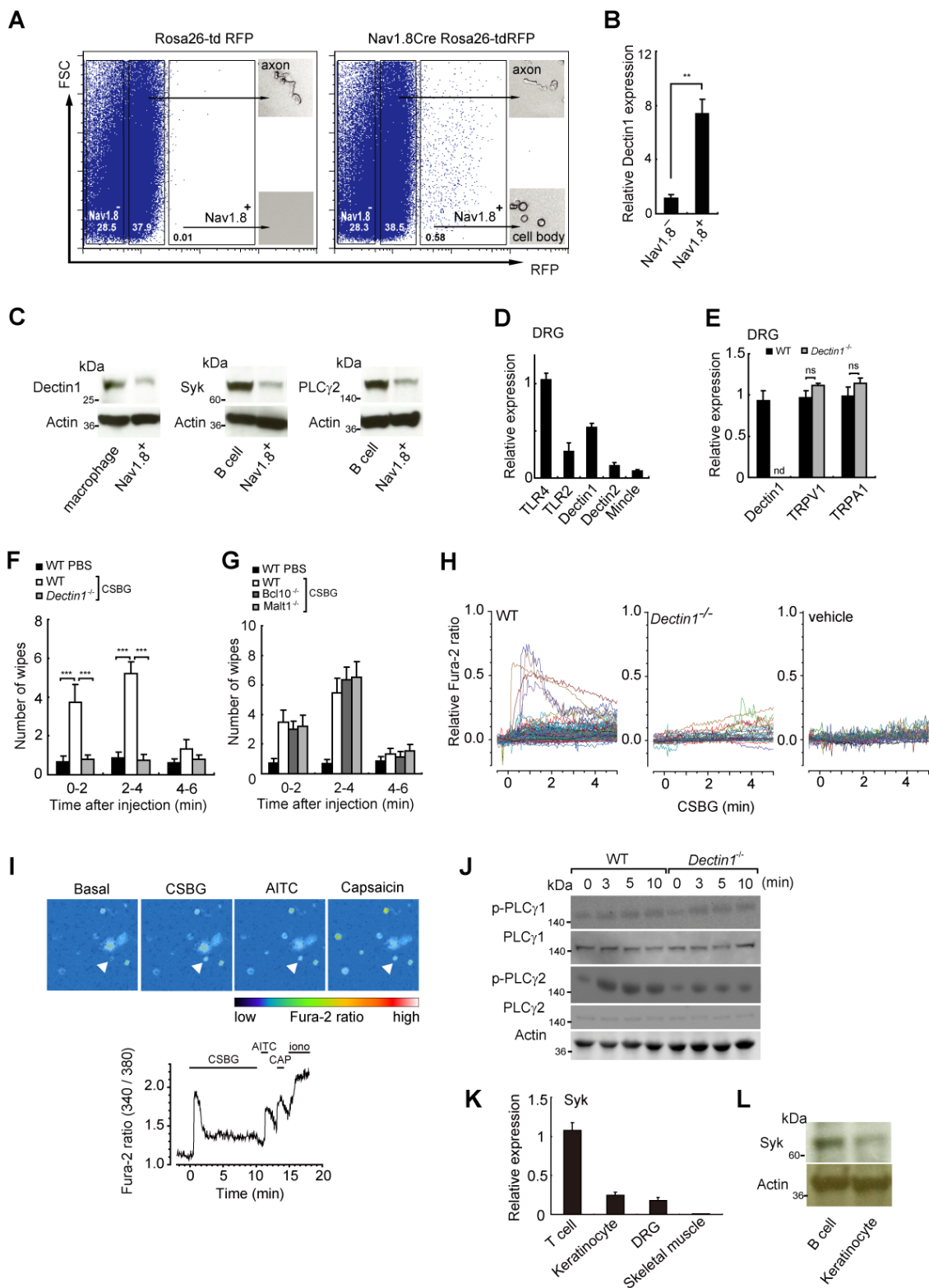
(B) LDH release (left) or CGRP production (right) from DRG neurons treated with 70  $\mu$ M candidalysin for 24 h (n=3). (C) Candidalysin and heated candidalysin (20  $\mu$ M or 70  $\mu$ M) were injected into the hind paws of WT mice and mechanical allodynia was analyzed (n=10; \*, heated candidalysin 70  $\mu$ M vs. candidalysin 70  $\mu$ M). (D) WT *C. albicans* or *Ece1 $\Delta$ / $\Delta$  C. albicans* were injected into the hind paws of WT mice and mechanical allodynia (n=10) was analyzed. (E) Relative Fura-2 ratio with candidalysin (left), and expanded traces (right). Black lines, average value of relative Fura-2 ratio; gray lines, response of each neuron (n=43). (F) Structure of CPBG and CSBG. *C. albicans*-derived  $\beta$ -glucan is made up of long branched 1,6- $\beta$ -glucan and 1,3- $\beta$ -glucan segments. (G, H) TNF- $\alpha$  production by CSBG or CPBG stimulated macrophages (G) and various cytokine levels in CSBG-injected hind paw tissues measured by ELISA (n=8). (I) Paw thickness and myeloid or lymphoid cell numbers in CSBG-injected hind paw tissues were measured (n=8). Error bars, S.E; \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

Figure S2



**Figure S2. VNUT is critical for *C. albicans*-induced pain.** Related to Figure 4. (A) ATP levels in the culture supernatant of keratinocytes stimulated by CSBG (100  $\mu$ g/ml) or *C. albicans* (n=4). (B) ATP levels in the culture supernatant of keratinocytes from *VNUT*<sup>-/-</sup> and WT mice stimulated by *C. albicans* for 2 h (n=4). (C) *hk C. albicans*-induced mechanical allodynia in *VNUT*<sup>-/-</sup> and WT mice (n=7). Error bars, S.E; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

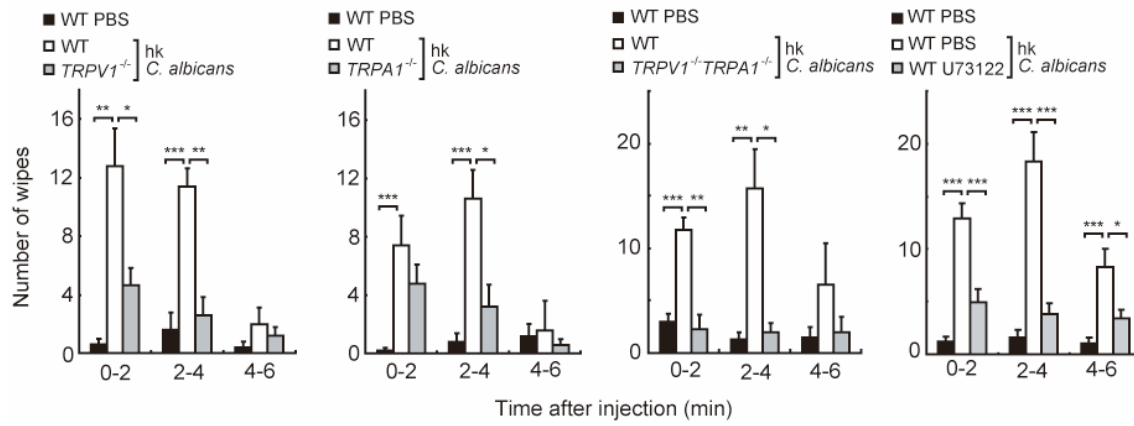
Figure S3



**Figure S3. Direct activation of nociceptor by Dectin-1.** Related to Figure 1 and Figure 5. (A) DRG from *Rosa26-tdRFP* and *Nav1.8Cre Rosa26-tdRFP* mice was dissociated and analyzed using

FACS Aria. (B) Nav1.8<sup>+</sup> neurons (RFP<sup>+</sup>) and Nav1.8<sup>-</sup> neurons (RFP<sup>-</sup>) were sorted from the DRG of *Nav1.8Cre Rosa26-tdRFP* mice. Dectin-1 and CGRP expression levels in both populations were measured by qPCR (n=3). (C) Nav1.8<sup>+</sup> neurons (RFP<sup>+</sup> Nav1.8<sup>+</sup> area cells in A) were sorted from the DRG of *Nav1.8Cre Rosa26-tdRFP* mice. Dectin1, Syk and PLC $\gamma$ 2 expression levels were analyzed by western blotting. (D) DRG fungal receptor expression (n=3). (E) DRG Dectin-1 and TRP channel expression (n=3). (F) Wiping behaviors after cheek injection of CSBG into *Dectin1<sup>-/-</sup>* and WT mice (n=7–15). (G) Wiping behaviors after cheek injection of CSBG into *Bcl10<sup>-/-</sup>*, *Malt1<sup>-/-</sup>*, and WT mice (n=7–15). (H) Intracellular calcium levels in hk *C. albicans*-treated DRG neurons. The Fura-2 ratio is relative to the peak ionomycin response. (I) All traces of relative Fura-2 ratio with 100  $\mu$ g/mL CSBG or the vehicle control in DRG neurons. (J) Phosphorylated PLC $\gamma$ 1 and PLC $\gamma$ 2 levels in DRG neurons from *Dectin1<sup>-/-</sup>* or WT mice stimulated by CSBG for the indicated times. (K) Expression of Syk in indicated cells (n=3). (L) Syk expression in keratinocyte was analyzed by western blotting. Error bars, S.E.; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

Figure S4



**Figure S4. *C. albicans*-induced nociception is mediated by the PLC-TRPV1/TRPA1 axis.** Related to Figure 1 and Figure 5. Wiping behaviors after cheek injection with hk *C. albicans* (into *TRPV1*<sup>-/-</sup> mice, *TRPA1*<sup>-/-</sup> mice, *TRPV1*<sup>-/-</sup> *TRPA1*<sup>-/-</sup> mice, PLC inhibitor [U73122]-treated WT mice, and WT mice [n=5–10]). Error bars, S.E.; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; ns, not significant.

## Transparent Methods

### *Mice*

For infection or behavioral experiments, 8- to 13-week-old female mice were used. *MyD88<sup>-/-</sup> TRIF<sup>-/-</sup>* mice (Adachi et al., 1998; Yamamoto et al., 2003), *Hrh1<sup>-/-</sup>* mice (Inoue et al., 1996), *TRPVI<sup>-/-</sup>* mice (Caterina et al., 2000), *TRPA1<sup>-/-</sup>* mice (original strain was backcrossed for more than eight generations with C57BL/6) (Bautista et al., 2006), and *VNUT<sup>-/-</sup>* mice (Sakamoto et al., 2014) were generated and control C57BL/6 mice were purchased from SLC or Japan CLEA. NOD/ShiJic-scidJcl (NOD SCID) mice (Koyanagi et al., 1997) and NOD wild-type mice were purchased from Japan CLER. *Dectin1<sup>-/-</sup>* mice (Saijo et al., 2007), *NLRP3<sup>-/-</sup>* mice (Mariathasan et al., 2006), *ASC<sup>-/-</sup>* mice (Mariathasan et al., 2004), and *ICE<sup>-/-</sup>* mice (Gu et al., 1997) were generated as previously described and were compared with WT littermate controls. *Bcl-10<sup>-/-</sup>* mice and *Malt-1<sup>-/-</sup>* mice were a gift from S. Gerondakis, S. Morris, and V.M. Dixit, respectively (Ruefli-Brasse et al., 2003; Xue et al., 2003) and were compared with WT littermate controls (in Fig 2K, control WT mice were purchased from Oriental Bio Service). *Rosa26-tdRFP* mice were generated as described previously (Luche et al., 2007). *Nav1.8-Cre* mice (B6;129-Scn10atm(cre)Jnw/H, stock id EM:04582) were purchased from the European Mouse Mutant Archive (EMMA). Animal experiments complied with the institutional animal care and use guidelines of Osaka University (animal 14013) and the National Institutes of Natural Sciences (16A074).

### *Pathogens and their components*

*C. albicans* strain THK519, designated *C. albicans*, was obtained from a patient admitted to Tohoku University Hospital. *C. albicans*-derived soluble  $\beta$ -glucan (CSBG) and *C. albicans*-derived particulate  $\beta$ -glucan (CPBG) were prepared from *C. albicans* strain NRBC1385 (same as ATCC18804, ATCC20308, CBS562, JCM1542, and NRRL Y-12983), purchased from the NITE Biological Resource Center (Chiba, Japan) as previously described (Ishibashi et al., 2002). To generate *C. albicans*-derived  $\beta$ -glucan, acetone-dried *C. albicans* NRBC1385 was suspended in 0.1 M NaOH with NaClO (final concentration, 1%) for 1 day at 4°C. After the reaction was completed, the reaction

mixture was centrifuged and the particulate fraction was collected and dried by ethanol and acetone. The dried particulate fraction was suspended in saline and sonicated for 30 s. After centrifugation, the supernatant was removed and the particulate fraction was designated CPBG (CPBG is insoluble to saline). To prepare CSBG, CPBG suspended in DMSO was ultrasonically disrupted and the resulting supernatant was designated as CSBG. To generate the vehicle control of CSBG, saline suspended CPBG was centrifuged and the resulting supernatant was designated as "vehicle". To evaluate the purity of CSBG, elemental analysis and <sup>13</sup>C-NMR spectrum analysis was performed. Assignment of <sup>13</sup>C-NMR Chemical shifts clearly indicates that our CSBG contains only β-1,3/β-1,6-glucan peaks (data not shown). *C. albicans* ECE1 homozygous knockout mutant strain (M2057, designated Ece1Δ/Δ *C. albicans*) and the wild-type control strain (M1477, designated WT *C. albicans*) were provided by Dr. B. Hube (Moyes et al., 2016). Yeast from fungal strains was cultured on potato dextrose agar plates (KOHJIN BIO, Saitama, Japan) at 30°C. After a 30-h incubation, cells were harvested, suspended in PBS, and counted using a hemocytometer and trypan blue staining. *C. albicans*-derived purified mannan, designated mannan, was obtained from the National Institute for Biological Standards and Control (NIBSC code 76/515, non WHO reference material). *C. albicans*-derived peptide Ece1-III K 62-92K (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK), designated here as candidalysin, was synthesized by GenScript (Lot No:16017867190002/PE2773, Purity 98.4%). *C. albicans* was heat killed at 56°C for 30 min. To generate hyphae from fungi, yeast from *C. albicans* was cultured in RPMI (Wako, Osaka, Japan) supplemented with 10% FCS for 2 h at 37°C. The β-glucan concentration in the fungal culture supernatant was quantified using Fungitec G test MKII Nissui (Nissui, Tokyo, Japan). To prepare *C. albicans* culture supernatant, *C. albicans* was cultured in RPMI supplemented with 10% FCS for 30 h at 37°C and centrifuged to collect the supernatant. Additional information on the fungal and bacterial strains, and the cells and reagents used, is provided in the Supplemental Information.

### ***In vivo injection of pathogens and their components***

Injection into the hind paws or cheeks was performed using a 30-gauge needle (Becton Dickinson).



For fungal infection, 25  $\mu$ l of the live or hk yeast form of the fungi in PBS was injected subcutaneously into the hind paws ( $3 \times 10^5$  c.f.u.) or cheeks ( $3 \times 10^7$  c.f.u.) and behavioral analysis was performed. For dead bacterial administration, 25  $\mu$ l of hk *S. aureus* ( $3 \times 10^8$  c.f.u.) in PBS was injected subcutaneously into the cheeks of mice. CSBG, CPBG, or mannan were subcutaneously injected into the hind paws or cheeks (15  $\mu$ g per 25  $\mu$ l PBS into hind paws and 150  $\mu$ g per 25  $\mu$ l PBS into cheeks). Capsaicin (40  $\mu$ g per 25  $\mu$ l PBS, M2028, Sigma-Aldrich, USA), TSLP (3  $\mu$ g per 25  $\mu$ l PBS, MAB555, R&D, USA), and chloroquine (200  $\mu$ g per 25  $\mu$ l PBS, C6628, Sigma-Aldrich, USA) were subcutaneously injected into the cheeks of mice. To test the algescic effect of ATP,  $\alpha, \beta$ -methylene ATP (100 nM, 25 ml, 5.05419.0001, Calbiochem, USA) was subcutaneously injected into the hind paws. After injection, mice were monitored and assayed for pain and/or itch behaviors and tissue inflammation.

### ***Behavioral analysis***

Mechanical sensitivity was measured using polypropylene tips attached to Von Fray apparatus (electronic Von Frey anesthesiometer, IITC Inc., Woodland Hills, CA, USA), as described previously (Zylka et al., 2008). Briefly, mice were acclimated to the testing room, equipment, and experimenter for 12–36 h before behavioral testing. Mice were tested in a resting state. The experimenter was blinded to the genotype during testing, and was trained to apply the polypropylene tip to the central hind paw with a gradual increase in pressure. A mirror placed below the grid provided guidance for the tip to the hind paw. To test the wiping and scratching behaviors, mice were placed in separate box (15  $\times$  15  $\times$  20 cm) and observed for 30 min, as previously described (Shimada and LaMotte, 2008).

### ***Scanning electron microscopic analysis***

Scanning electron microscopic analysis of *C. albicans* was performed, and samples were fixed with 3% formaldehyde in 0.1 M phosphate buffer (pH 7.4), and post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide in the same buffer. Next, samples were treated with 1% tannic acid solution, washed with water, and then fixed with 0.5% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M phosphate buffer (pH 7.4). Samples were then dehydrated in graded ethanol

concentrations ending with 100% ethanol and dried using a critical point drier. Samples were coated with osmium tetroxide vapor using a glow discharging device and observed using an S-4800 FE-scanning electron microscope (Hitachi High-Technologies Corp., Japan) at 1.5 kV and a 2 mm working distance.

### ***MRI analysis***

Structural T1-weighted, resting-state fMRI data were collected using a Varian 4.7T scanner (Unity INOVA, Varian Associates, Palo Alto, CA, USA) with a JASTEC Horizontal Magnet 4.7T (JMTB-4.7/310/SS, Japan Superconductor Technology, Kobe, Japan) with a 72-mm volume coil and a two-channel phased array mouse brain coil (Rapid Biomedical, Rimpar, Germany). Before each structural T1-weighted, resting-state fMRI scanning session, an exogenous contrast agent, USPIO (Molday ION Carboxyl, CL30Q02-7, Funakoshi, Japan) was injected into the caudal vein (12–15 mg/kg) to optimize the localization of fMRI signals (Leite and Mandeville, 2006). After placing the animal in a cradle, mice were maintained under light anesthesia with 1.5% isoflurane and then, CSBG, capsaicin or the PBS control were subcutaneously injected into the right hind paw (15 µg per 25 µl PBS). Anatomic images were obtained using a gradient-echo technique (GE3D; data matrix,  $128 \times 128 \times 64$ ; FOV, 2.0 cm<sup>3</sup>; TR/TE, 0.02/0.008 s; flip angle, 10°). From these images, nine 1-mm thick planes with no gaps were selected for functional imaging. In all scans, T2\*-weighted functional MR images were obtained using a gradient-echo imaging technique (data matrix,  $32 \times 32$ ; FOV, 2.5 cm<sup>2</sup>; TR/TE, 0.01/0.006 s; flip angle, 8°; slice thickness, 1 mm; scan time, 0.32 s/slice  $\times$  9 slices + 0.12 s delay=3.0 s). After data collection, normalization to standard space and smoothing was performed. Registration of the fMRI space data to standard space was carried out using bioimagesuite35 (<http://bioimagesuite.yale.edu>). After manual registration, an affine transformation with 12 degrees of freedom was performed. The standard space was chosen to be an anatomical scan from a rat of size  $128 \times 128 \times 55$  mm<sup>3</sup> with a voxel size of  $0.156 \times 0.156 \times 0.31$  mm<sup>3</sup>. Finally, 3D smoothing with a 0.8 mm size filter was carried out. To visualize the pain status in the brain, ALFF was calculated using the Resting-State fMRI Data Analysis Toolkit V1.8 REST (<http://restfmri.net/forum/index.php>). For each

voxel, a discrete Fourier transform was performed on the resting-state time series. The ALFF was computed by measuring the average square-root of the total power spectrum from 0.01–0.1 Hz on a voxel-by-voxel basis. Cross-correlation region-of-interest analysis and second level analysis were performed by UF2C (version 6.2), a plug-in of spm8 (version 6313), to make individual or group cross-correlation maps. A pairwise correlation was calculated between each pair of regions-of-interest, using our own MATLAB software. To compare the differences in the correlation values among the three groups, UF2C was also used to carry out second-level analysis. Seeds of the cross-correlation were supplied as a coordinates list. The shape of the seeds was a cube with four voxels per edge.

### ***Generation of bone marrow chimeric mice***

Donor bone marrow cells from *Dectin-1*<sup>-/-</sup> and age-matched control wild-type mice were collected and  $1 \times 10^7$  cells were intravenously injected into lethally-irradiated 4-week-old recipient mice. Mice were analyzed at 7 weeks after bone marrow transplantation.

### ***PCR and protein analysis***

RNA was extracted using TRIzol (Invitrogen Life Science Technologies). Reverse transcription was performed using ReverTra Ace (Toyobo Co. Ltd.). The quantity of mRNA was normalized to 18S rRNA using the TaqMan ribosomal control reagent kit (Applied Biosystems). Western blotting was performed as previously described (Maruyama et al., 2012). Immunohistochemistry of DRG and trigeminal ganglion (TG) is described in Supplemental Information.

### ***DRG neuron isolation***

The DRGs were separated from L1–L6 in mice after perfusion with 10 mL of ice-cold artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 5 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM MgSO<sub>4</sub>, 2.4 mM CaCl<sub>2</sub>, 10 mM glucose, 24 mM NaHCO<sub>3</sub>, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 1 h on ice). The tissues were incubated with 725 µg collagenase type IX (lot# SLBG3258V and SLBG3259V, Sigma-Aldrich) in 250 µL of Earle's balanced salt solution (Sigma-Aldrich) at 37°C for 25 min. Next, DRG

neurons were mechanically separated by 10–20 cycles of pipetting using a small diameter Pasteur pipette. The neurons were centrifuged three times at  $300 \times g$  for 5 min at 4°C. Then, supernatants were discarded and fresh PBS or aCSF was added to wash out the collagenase. The isolated neurons were placed on 12-mm-diameter coverslips (Matsunami, Japan) with 40  $\mu$ L of aCSF and were maintained at room temperature in a 95% O<sub>2</sub> and 5% CO<sub>2</sub> humidified chamber.

### ***Cells and reagents***

B cells and T cells were prepared from splenocytes using anti-B220 and anti-Thy-1.2 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. Splenic dendritic cells were prepared using anti-CD11c magnetic beads (Miltenyi Biotec). Splenic CD11b<sup>+</sup> macrophages were sorted by FACS Aria (BD Biosciences, Bedford, MA, USA). Antibodies for FACS analysis were purchased from BD Biosciences, and cells were stained with the indicated antibodies. Data were acquired using FACSCalibur (BD Biosciences), and analyzed by FlowJo software (Ashland, OR, USA). RFP-positive DRG neurons from *Nav1.8Cre Rosa26-tdRFP* mice were sorted using FACS Aria. Primary keratinocytes were obtained from newborn wild-type mice as previously described (Sokabe et al., 2010). Neutrophils were harvested from the peritoneal cavity of mice 2 h after intra-peritoneal injection of thioglycollate. Macrophage colony stimulating factor (M-CSF)-derived macrophages and conventional dendritic cells were generated by bone marrow cell cultures with M-CSF and GM-CSF, respectively. Keratinocytes ( $2 \times 10^5$ ) or DRGs were cultured with various stimuli, and ATP levels in the supernatant were quantified using an ATP Colorimetric/Fluorimetric Assay Kit (#K354-100, BioVision, USA). Cell death was quantified using an LDH Cytotoxicity Colorimetric Assay Kit (#K311-400, Bio Vision). Western blotting was performed as previously described (Maruyama et al., 2012). Proteins from DRG, Nav1.8 positive neuron and keratinocyte were detected using anti-Dectin1 (MAB1859, R&D Systems), anti-Syk (#2712, Cell Signaling), anti-PLC $\gamma$ 1 (EPR5358, ab109501), anti-PLC $\gamma$ 2 (#3872, Cell Signaling), anti-p-PLC $\gamma$ 1 (2821S, Cell Signaling), anti-p-PLC $\gamma$ 2 (3874S, Cell Signaling) and anti-actin (C-11, Santa Cruz Biotechnology).

### ***Calcium imaging***

Isolated DRG neurons were cultured at 37°C for 1 h in Earle's balanced salt solution (Sigma-Aldrich) with Fura-2 AM (Molecular Probes). Fura-2 fluorescence of isolated mouse DRG neurons was measured in a standard bath solution: 140 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose, 10 mM HEPES, pH 7.40 with NaOH, excited at wavelengths of 340 and 380 nm, and emission was monitored at 510 nm with a CCD camera (CoolSnap ES; Roper Scientific/Photometrics) or a sCMOS camera (Zyla DG-152XC1E-F1; ANDOR Technology). Data were acquired and analyzed by IPlab (Scanalytics), iQ (ANDOR), and ImageJ software. Ionomycin (5 μM) was applied to confirm cell viability.

### ***In vivo injection of antibodies and chemicals***

To deplete Ly6G-positive neutrophils, anti-Ly6G antibody (250 μg/head, RB6-8C5, eBioscience, USA) or isotype control antibody were intraperitoneally injected 24 h before CSBG injection into the hind paws of mice. To deplete T cells, anti-CD3 antibody (200 μg/head, 17A2, eBioscience) or isotype control antibody were intraperitoneally injected 12 h before CSBG injection into the hind paws of mice. To neutralize TNF-α, anti-TNF-α antibody (200 μg/head, TN3-19.12, BioLegend, USA) or isotype control antibody were intraperitoneally injected 12 h before CSBG injection into the hind paws of mice. To deplete macrophages, chlodronate liposome (200 μl/head, HYGIEIA Bioscience, Osaka, Japan) or control liposome were intraperitoneally injected 24 h before CSBG injection into the hind paws of mice. To inhibit cyclooxygenase pathway, nonselective cyclooxygenase inhibitor ibuprofen (200 mg/kg) or the vehicle control were orally administered using disposable animal feeding needles (FUCHIGAMI, cat no. 6202) 1 h before CSBG or CFA injection into the hind paws of mice. For PLC or ATP receptor inhibition, U73122 (10 μM, 25 μl, U6756, Sigma-Aldrich) or A317491 (10 μM, 25 μl, A2979, Sigma-Aldrich) were injected subcutaneously into both hind paws 30 min before ligand injection, respectively. For VNUT inhibition, Disodium Clodronate Tetrahydrate (D4160, TOKYO CHEMICAL INDUSTRY) was injected intravenously or subcutaneously 1h before ligand injection. For paw thickness evaluation, a digital micrometer (CD67-S PM, Mitutoyo, Kanagawa, Japan) was

used.

***Statistical analysis***

The difference in mean values between two groups was compared using Student's *t*-test, with  $p < 0.05$  considered to be significant.

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