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The Chromatin Regulator DMAP1 Modulates Activity of the Nuclear Factor κ B (NF- κ B) Transcription Factor Relish in the *Drosophila* Innate Immune Response^{*[5]}

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Background: DMAP1 was identified as an interactant of the NF- κ B transcription factor Relish in a proteomic analysis.

Results: DMAP1 knockdown reduces activation of the IMD pathway response to immune challenge in *Drosophila*.

Conclusion: DMAP1 is new modulator of the IMD pathway acting in complex with chromatin remodeling factors.

Significance: We report a novel function for the evolutionarily conserved DMAP1 molecule in innate immunity.

The host defense of the model organism *Drosophila* is under the control of two major signaling cascades controlling transcription factors of the NF- κ B family, the Toll and the immune deficiency (IMD) pathways. The latter shares extensive similarities with the mammalian TNF-R pathway and was initially discovered for its role in anti-Gram-negative bacterial reactions. A previous interactome study from this laboratory reported that an unexpectedly large number of proteins are binding to the canonical components of the IMD pathway. Here, we focus on DNA methyltransferase-associated protein 1 (DMAP1), which this study identified as an interactant of Relish, a *Drosophila* transcription factor reminiscent of the mammalian p105 NF- κ B protein. We show that silencing of DMAP1 expression both in S2 cells and in flies results in a significant reduction of *Escherichia coli*-induced expression of antimicrobial peptides. Epistatic analysis indicates that DMAP1 acts in parallel or downstream of Relish. Co-immunoprecipitation experiments further

reveal that, in addition to Relish, DMAP1 also interacts with Akirin and the Brahma-associated protein 55 kDa (BAP55). Taken together, these results reveal that DMAP1 is a novel nuclear modulator of the IMD pathway, possibly acting at the level of chromatin remodeling.

Innate immunity comprises a heritable, multifaceted, and highly conserved defense system. Significant progress has been made during the past two decades in the understanding of the signaling pathways involved in innate immunity and of the receptors sensing infectious non-self (1–4). The well established genetic tools and the remarkable conservation of key aspects of the genetic regulation have established the fruit fly *Drosophila melanogaster* as a favorable model organism to decipher the principles of innate immune responses. Extensive studies have pointed to the role of two major innate immune pathways: (i) the Toll pathway (initially discovered for its role in early embryogenesis (5, 6)), which is predominantly activated by Gram-positive bacterial and fungal infections, and (ii) the immune deficiency (IMD)³ pathway, which is preferentially activated by Gram-negative bacterial infection (1, 7, 8). In *Drosophila*, three NF- κ B family members, namely Dorsal, DIF (Dorsal-related immunity factor), and Relish, play central roles in controlling the expression of hundreds of immune-responsive genes (9). Toll pathway activation occurs when microbial ligands, namely Gram-positive peptidoglycan and fungal β -glucan, interact with dedicated proteins in circulation, thus initiating proteolytic cascades, which culminate in the cleavage of the polypeptide Spaetzle (7, 8). Cleaved Spaetzle in turn interacts with the extracellular domain of the Toll receptor, triggering an intracellular signaling cascade, which sets free the cytosolic NF- κ B family members Dorsal and/or DIF from the inhibitory polypeptide Cactus, resulting in their nuclear translocation. In the case of the IMD pathway, Gram-negative bacterial peptidoglycan is sensed by a transmembrane receptor, which activates an intracellular signaling cascade leading to the proteolytic cleavage of Relish, and the concomitant nuclear translocation of its N-terminal Rel domain. Predominant among the immune response genes are the antimicrobial peptides. The relevance of these two pathways in the host defense of the flies is illustrated by the compromised survival of adults mutant for components of these pathways (10, 11). Recent evidence suggests that the IMD pathway, which is evocative of the human tumor necrosis factor receptor (TNF-R) signaling pathway, is more complex than initially anticipated. For one, it involves a range of post-translational modifications, positive and negative regulators, and tissue-specific modulators (e.g. gut) (12). Further, it is not only activated by Gram-negative

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³ The abbreviations used are: IMD, immune deficiency; BAP, Brahma-associated protein; DMAP1, DNA methyltransferase associated protein 1; DNMT, DNA methyltransferase; nt, nucleotides; QRT-PCR, quantitative RT-PCR; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); PGRP-LC, peptidoglycan recognition protein LC; FL, full-length; C-ter, C-terminal; N-ter, N-terminal.

bacteria, but can react in the absence of infection to endogenous ligands, namely DNA (13, 14).

To further characterize IMD signaling, this laboratory previously undertook a proteomic approach to identify new interactants of its 11 canonical components. This study led to the identification of 369 interacting proteins (15). Of these, 96 were identified in complex with Relish, suggesting an essential regulation at the level of this NF- κ B family member. The functions of these interactants have not yet been investigated. Here, we report that one of these interactants, DNA methyl transferase-associated protein 1 (DMAP1), is a novel modulator of Relish activation in *Drosophila* cell culture and in flies.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A PCR fragment of DMAP1 ORF tagged with either V5 or Myc sequence was amplified from an EST clone (LD35228, nt 99–1400) and subcloned into EcoRI-XbaI sites of the metallothionein promoter-driven pMT-V5-HisA vector (Invitrogen). Other DMAP1 deletion constructs were generated in the same way based on the amino acid sequence position shown in Fig. 2A. pPAC-Relish Δ S29-S45 (16), pMT-Toll Δ LRR (17), pAC-PGRP-LC (18), and pAC-Akirin-V5 (18) constructs were described previously. The pMK33-BAP55-HA-FLAG construct was provided by J. M. Reichhart.⁴

Cell Culture and Transfection—*Drosophila* S2 cells were maintained at 23 °C in Schneider's medium (Biowest) supplemented with 10% FCS. A total of 6×10^5 cells were transfected in 24-well plates by calcium phosphate precipitation with 50 ng of the AttacinA-luciferase or *Drosomycin-luciferase* reporter vector (17), 10 ng of an Actin5C-*Renilla* luciferase transfection control vector, the expression vectors, and each dsRNA (2.0 μ g/well). After transfection, the cells were stimulated with heat-killed *Escherichia coli* for 24 h, and both firefly and *Renilla* luciferase activities on the cell lysate were measured using Dual-Luciferase assay kit (Promega).

Cell Staining—S2 cells were fixed 4 days after transfection with methanol/water/acetic acid (95:4:1, by volume) for 20 min. They were then permeabilized with cold methanol for 15 min, incubated overnight with anti-V5 monoclonal antibody (500-fold dilution in PBST/PBS containing 0.1% Triton X-100), washed four times with PBST, incubated with Alexa Fluor 488 anti-mouse IgG (500-fold dilution in PBST, Molecular Probes) for 2 h, and washed four times with PBST. VECTASHIELD mounting medium with DAPI was used to visualize nuclei (Vector Laboratory). Specimens were observed under Zeiss Axioskop 2 microscope.

Synthesis of dsRNAs—Templates for dsRNA preparation were PCR-derived fragments between two T7 promoter sequences. Fragments for each gene were as follows: GFP (nt 26–302, GenBankTM L29345), DMAP1 dsA and dsB region (nt 834–1138 and 1150–1419, National Center for Biotechnology Information (NCBI) NM_137659), IMD (nt 550–919, NCBI NM_133166), dMyD88 (nt 929–1240, NCBI NM_136635), and Akirin (nt 656–1051, NCBI NM_139856). dsRNAs were synthesized with the MEGAscript T7 transcription kit (Ambion).

Annealed dsRNAs were ethanol-precipitated and dissolved in distilled water for the transfection.

Co-immunoprecipitation Assay—A total of 2×10^6 S2 cells were co-transfected by calcium phosphate precipitation with 5 μ g of each expression vector. After the transfection in the presence of 500 μ M CuSO₄ for 48 h, a total of $4–6 \times 10^6$ cells were lysed in cell extraction buffer (Invitrogen) with protease inhibitors (Roche Diagnostics), and cleared cell lysates were incubated with anti-V5-agarose beads (Sigma) overnight under constant rotation at 4 °C. The beads were washed and resuspended in 2 \times sample buffer. The aliquots were used for Western blot analysis using commercially available antibodies against FLAG (Abcam, Ab1162), V5 (Sigma v8137), Myc (Abcam, Ab9106), and Actin5C (clone 4, Millipore). 1% of the volume of cell lysate was used as input.

QRT-PCR Analysis—To measure expression of the endogenous *AttacinA*, *CecropinA1*, and *Drosocin* genes, the dsRNA soaking method using 96-well plates was used. Briefly, 20 μ g of either *dsGFP*, *dsIMD*, or *dsDMAP1* were prespotted per well, mixed with 45,000 cells in 30 μ l of serum-free medium for 1 h before the addition of serum-containing medium. After 7 days of dsRNA soaking, the cells were stimulated with heat-killed *E. coli* for 3 h. Total RNA extraction, cDNA synthesis, and quantitative PCR using primers against *AttacinA*, *Cecropin A1*, *Drosocin*, and *DMAP1* (forward 5'-TTA AGT TAA CGC GGC CCG TTT C-3' and reverse 5'-ATC CAT TTA TTT CCC CAT GCG G-3') were carried out using Cells-to-CT kit according to the manufacturer's instructions (Ambion). *Rp49* copy number was used for normalization.

Fly Strains and Genetics—Flies were grown on standard medium at 25 °C. To generate conditional DMAP1 knockdown adult flies, we used a GAL4-GAL80^{ts} system (19). UAS-DMAP1-RNAi (20) and UAS-GFP-RNAi (used as a negative control) were crossed with Actin-GAL4/CyO; Tub-GAL80^{ts} flies at 18 °C. Emerged adult flies were then transferred to 29 °C to activate the UAS-GAL4 system for 7–9 days. Microbial challenges were performed by pricking adult flies with a sharpened tungsten needle dipped into either concentrated *E. coli* (1106) or concentrated *Micrococcus luteus* (CIP A270) solution. Total RNA extraction with TRIzol (Invitrogen), iScript cDNA synthesis (Bio-Rad), and quantitative PCR using *Diptericin* (*Dpt*)- and *Drosomycin* (*Drs*)-specific primers were described previously (21).

RESULTS

DMAP1 Interacts with Relish—In a preliminary RNAi screen against a subset of Relish interactants, DMAP1 reproducibly scored as a positive regulator of the IMD pathway (15) (see below). We first validated in an independent experiment the interaction of DMAP1 and Relish. Co-immunoprecipitation assays in S2 cells transfected with vectors expressing a tagged version of DMAP1 and Relish Δ S29-S45, a constitutively active version of the protein with a short internal truncation (16), confirmed that the two proteins indeed interact. Both the full-length (FL) version of Relish and the N-terminal domain (Rel-68) were pulled down with DMAP1 (Fig. 1A).

DMAP1 Is a Positive Regulator of the IMD Pathway—We next examined the impact of DMAP1 function on activation

⁴J. M. Reichhart, unpublished data.

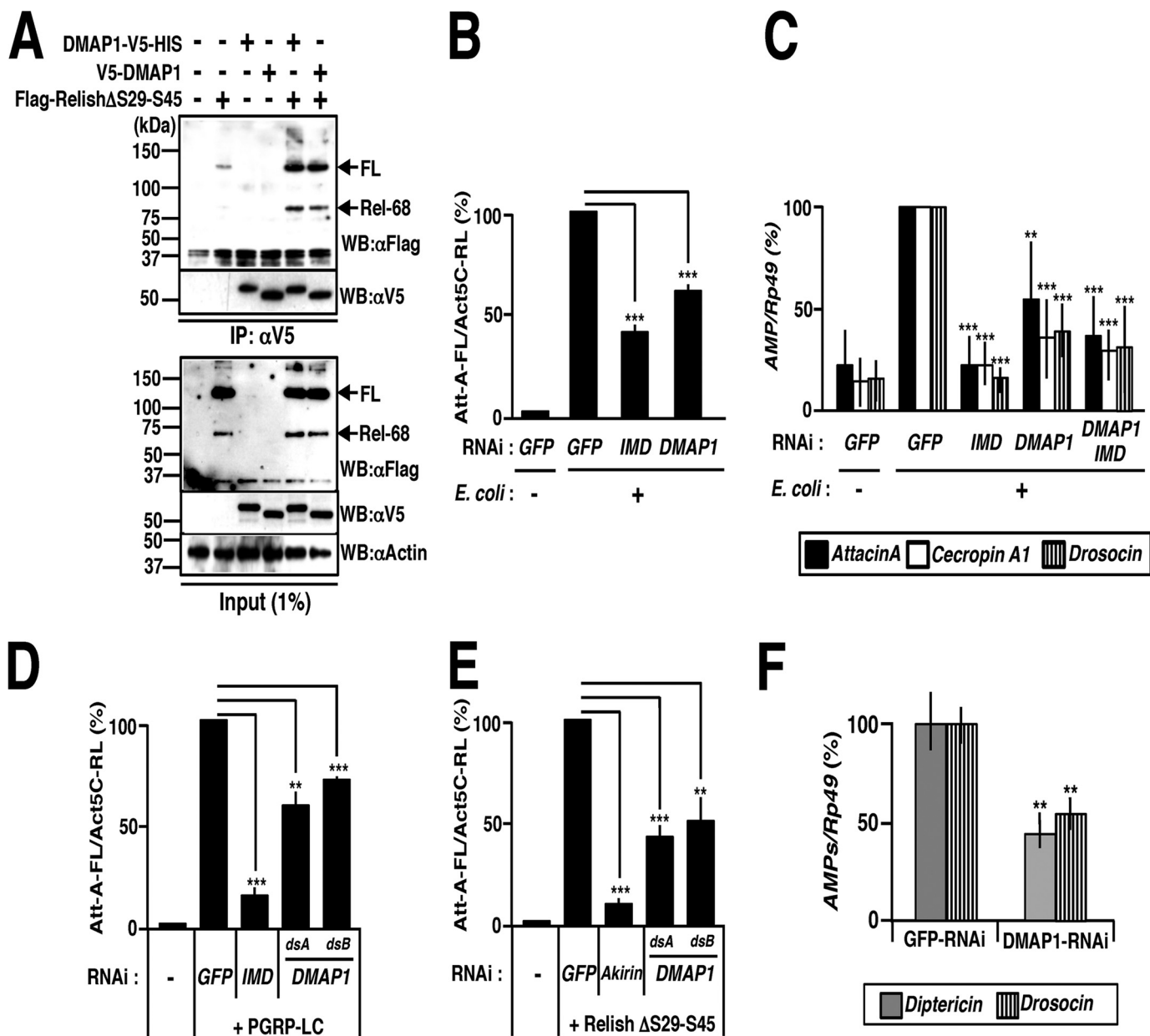


FIGURE 1. Functional characterization of DMAP1 in the IMD pathway. A, S2 cells were co-transfected with plasmids expressing either N terminus or C terminus V5-tagged DMAP1 and FLAG-Relish Δ S29-S45. Cell lysates were co-immunoprecipitated (IP) and immunoblotted (WB) with the indicated antibodies. Empty vector transfection (–) and single overexpression of either construct were used as controls. 1% of the volume of cell lysate was used as input. B, S2 cells were transfected with the indicated dsRNA together with the AttacinA-luciferase (Att-A-FL) and the transfection control Actin5C-Renilla luciferase (Act5C-RL). After stimulation with heat-killed *E. coli*, the ratio of Att-A-FL/Act5C-RL was measured. C, expression of endogenous *AttacinA*, *Cecropin A1*, and *Drosocin* genes was monitored by QRT-PCR after knocking down expression of the indicated genes and stimulation with heat-killed *E. coli*. D and E, PGRP-LC (D) or Relish Δ S29-S45 (E) expression vectors were co-transfected with dsRNAs targeting two independent regions of DMAP1 (dsA and dsB) and the Att-A-FL and Act5C-RL reporters. The value for control dsGFP-treated cells was set at 100%. F, conditional DMAP1 knockdown adult flies were pricked with *E. coli*, and the expression of *Diptericin* and *Drosocin* at 6 h after infection was monitored by QRT-PCR. GFP-RNAi flies were used as control. – indicates non-infected. For panels B–F, the data represent the mean and S.D. of at least two independent experiments, and the difference between control GFP and each target RNAi is statistically significant (Student's *t* test: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

of the IMD pathway in tissue-cultured cells. For this, S2 cells were treated with dsRNA against DMAP1, and the activation of the IMD pathway was monitored using the AttacinA-luciferase reporter, a conventional readout of the IMD pathway activation (17). We first confirmed that DMAP1 expression was indeed silenced in cells treated with dsDMAP1 as compared with control dsGFP (supplemental Fig. S1A). DMAP1 knockdown resulted in ~40% reduction of the AttacinA-luciferase activity upon stimulation with heat-killed *E. coli* (Fig. 1B). To exclude

the possibility that this phenotype results from the artificial luciferase system, expression of endogenous *AttacinA*, *CecropinA1*, and *Drosocin* genes, three classical standard markers for the activation of the IMD pathway in S2 cells, was also measured. The inducibility of *AttacinA*, *CecropinA1*, and *Drosocin* expression was significantly impaired in DMAP1 knockdown experiments (Fig. 1C). This reduced inducibility did not result from cell viability or cell proliferation defects as neither the activity of the transfection efficiency control

Actin5C-*Renilla* luciferase reporter nor the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay, which determines the number of viable cells based on mitochondrial dehydrogenase activity, was largely affected in our experimental condition (data not shown). Silencing of both IMD and DMAP1 did not show synergistic effect (Fig. 1C), further indicating that DMAP1 and IMD act in the same pathway.

To further explore the involvement of DMAP1 in other innate immunity signaling pathways, we monitored the expression of readout markers of the Toll (*Drosomycin*) or JAK-STAT (*Turandot M* (*TotM*)) pathways and noted that neither of them were reduced upon DMAP1 knockdown. Rather, expression of both *Drosomycin* and *TotM* was increased when DMAP1 was silenced (supplemental Fig. S2, A and C). Taken together, these results indicate that DMAP1 functionally contributes to the activation of the IMD pathway by *E. coli* stimulation.

DMAP1 Acts Either in Parallel or Downstream of Relish—To further characterize the role of DMAP1 in the IMD pathway and determine its position within the IMD pathway, we performed epistatic experiments. For this, we overexpressed the genes *PGRP-LC*, encoding the IMD pathway receptor, or *Relish* Δ S29-S45 (Fig. 1, D and E) in the presence or absence of *dsDMAP1*, and monitored the AttacinA-luciferase activity. As previously reported, *PGRP-LC* overexpression results in an IMD-dependent activation of the AttacinA-luciferase reporter (18). Similar results were obtained when cells were transfected with dsRNAs targeting different, non-overlapping regions of DMAP1, although the effect was not as strong as in the case of IMD knockdown (Fig. 1D). We next performed a similar experiment in *Relish* Δ S29-S45-expressing cells. When DMAP1 was silenced in cells, the activity of the AttacinA-luciferase reporter was also significantly reduced, albeit not as strongly as in the knockdown of the highly conserved Relish regulator Akirin (18) (Fig. 1E). This result indicates that DMAP1 acts either in parallel or downstream of Relish, which is in agreement with the physical interaction between the two proteins (Fig. 1A).

DMAP1 Modulates Antimicrobial Peptide Expression in Vivo—We next analyzed whether DMAP1 plays a role in antimicrobial peptide expression *in vivo*. Because there are no null mutants available, we used a UAS-DMAP1-RNAi line from Vienna *Drosophila* RNAi Center (VDRC) (20). We first crossed this DMAP1-RNAi line with ubiquitous GAL4 drivers such as *daughterless-GAL4* or *Actin5C-GAL4*. All the progeny from these crosses exhibited larval-pupal lethality, suggesting that DMAP1 is required for development (data not shown). We then tried the tissue-specific *Collagen-GAL4* driver, where GAL4 is expressed in immune-responsive organs such as fatbody and hemocytes. In this case, a small number of escaper flies emerged, but they were fragile and susceptible to stress such as injury (data not shown). We therefore used a conditional DMAP1 knockdown strategy at the adult stage using the thermo-sensitive *Actin-GAL4*; *Tub-GAL80^{ts}* flies (19). Briefly, after the crossing and development at 18 °C, newly emerged adult flies were shifted to 29 °C to inactivate the Gal80 system and then to induce the UAS-GAL4 system. A ~60% reduction in DMAP1 RNA expression was observed in 7–9-day-old flies (supplemental Fig. S1B).

We observed a weak but statistically significant reduction of the infection-induced expression of *Diptericin* as well as *Drosomycin*, another commonly used *in vivo* marker gene for activation of the IMD pathway (Fig. 1F). When flies were stimulated with the Gram-positive bacteria *M. luteus*, expression of the Toll pathway marker gene *Drosomycin* was not reduced in DMAP1-silenced flies. Of note, as observed in tissue-cultured cells, DMAP1 silencing resulted in increased expression of *Drosomycin* (supplemental Fig. S2B). The significance of this up-regulation is discussed below. Taken together, these results indicate that *in vivo* DMAP1 participates in the activation of the IMD pathway in adults, and further suggest that it also plays an important role during development.

DMAP1 Interacts with Akirin and BAP55—The above results raise the question of the mode of action of DMAP1, a poorly characterized protein containing a highly conserved SANT (SWI-SDA2-NcoR-TFIIB) domain, involved in histone tail binding, and a coiled coil domain, responsible for protein oligomerization (Fig. 2A). Because Relish resides both in the cytosol and nucleus, we first wanted to clarify where DMAP1 localizes in *Drosophila* cells. Although we reproducibly could detect cytosolic staining in ~20% of the transfected cells, the tagged full-length DMAP1-V5 protein localizes mainly to the nucleus (Fig. 2B). A similar staining pattern was observed when the truncated version of the protein Δ C-ter was expressed (Fig. 2B). By contrast, the Δ N-ter truncated DMAP1 protein was mainly cytosolic (Fig. 2B), suggesting that the SANT domain is partially responsible for the nuclear localization. We also noticed that both the Δ N-ter and the Δ C-ter proteins co-immunoprecipitated with Relish, suggesting that both regions are sufficient for Relish interaction (Fig. 2C). It was previously shown that the nuclear protein Akirin acts in the IMD pathway at the level or downstream of Relish (18). We therefore explored whether DMAP1 interacts with Akirin. As shown in Fig. 2D, DMAP1 co-immunoprecipitated with Akirin. To further characterize the role of DMAP1, we looked for putative interactants in protein databases. Interestingly, both DPiM (22) and String 9.05 (23) databases pointed to the Brahma complex protein BAP55 as an interactant for DMAP1. Co-immunoprecipitation experiments in transfected S2 cells indeed revealed that DMAP1 physically interacts with BAP55 (Fig. 2E).

DISCUSSION

The p105-like NF- κ B family member Relish is a key player in the IMD signaling pathway in *Drosophila*, regulating tens of genes in response to infection. Regulation of Relish activity is a critical aspect of the immune response as abnormally high or sustained activation of the IMD pathway can be detrimental to the host (24, 25). We previously described a proteomic analysis to identify novel partners of Relish (15). Here, we report that one of these novel interactants, DMAP1, modulates activation of the IMD pathway in tissue culture cells and *in vivo*. We further show that in addition to Relish, DMAP1 also interacts with Akirin and BAP55. Our results unravel a novel function for DMAP1 in innate immunity, and also establish a connection between this molecule and the Brahma chromatin remodeling complex.

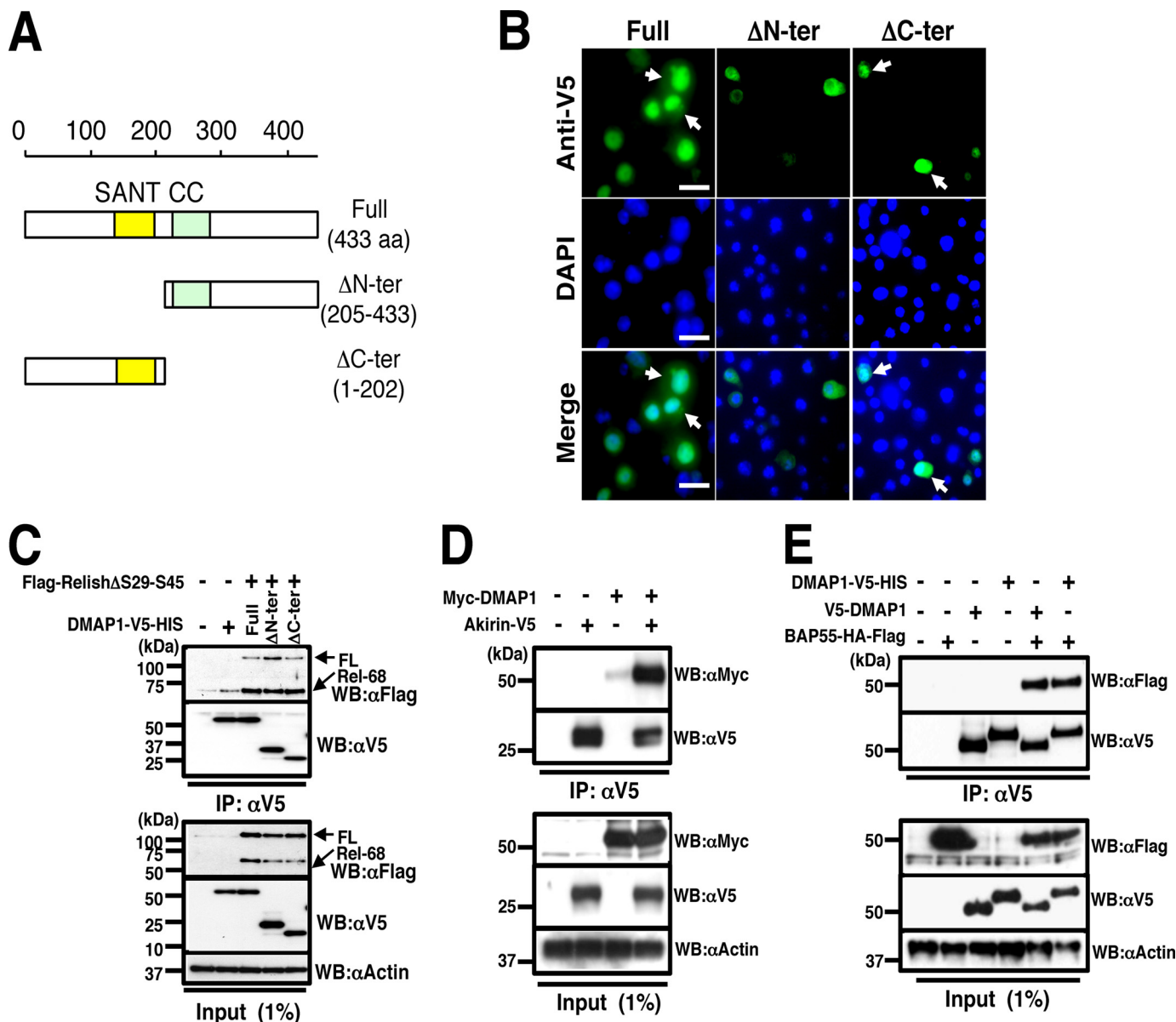


FIGURE 2. The nuclear protein DMAP1 interacts with the chromatin remodeling factors Akirin and BAP55. *A*, schematic presentation of DMAP1 full-length, ΔN-ter, and ΔC-ter constructs. *aa*, amino acids. *B*, S2 cells were transfected with each plasmid expressing V5-tagged DMAP1, stained with anti-V5 antibody, and revealed by Alexa Fluor 488 secondary antibody (green). DAPI (blue) was used to stain nucleus. *Bar* indicates 10 μm. *Arrows* indicate the cell stained both in the nucleus and in the cytosol. *C–E*, S2 cells were co-transfected with vectors expressing tagged DMAP1 (Myc or V5) and FLAG-RelishΔS29-S45 (*C*), Akirin-V5 (*D*), or BAP55-HA-FLAG (*E*). Cell extracts were immunoprecipitated (*IP*) and immunoblotted (*WB*) with the indicated antibodies. Empty vector transfection (–) and single overexpression of either construct were used as controls. 1% of the volume of cell lysate was used as input.

DMAP1 was initially identified in humans through a yeast two-hybrid screen as a protein interacting with the DNA methyl transferase 1, DNMT1 (hence the name DMAP1, which stands for DNA methyl transferase-associated protein 1) and was shown to function as a transcriptional corepressor through association with histone deacetylase 2 (HDAC2) (26). Subsequently, DMAP1 was biochemically identified as a component of the Tip60-p400 histone acetyltransferase complex (27, 28). Consistent with the loss-of-function phenotype for other members of the Tip60-p400 complex, such as Tip60 (29) and Trrap (30), DMAP1 KO mice exhibit early embryonic lethality even before the eight-cell embryo or blastocyst stage (31). It was recently shown that DMAP1 is required for Tip60 function (32). DMAP1 contains a highly conserved SANT domain, a his-

tone tail binding module (33), and may act as scaffold for the Tip60-p400 histone acetyltransferase complex (32). The *Drosophila* genome does not encode an ortholog of DNMT1, and no consistent pattern of DNA methylation could be detected in flies (34). Hence, the role of DMAP1 may be connected to Tip60-p400 nucleosome remodeling rather than to DNA methylation. Interestingly, our interactome analysis also identified other SANT domain-containing chromatin remodeling factors interacting with components of the IMD pathway such as Tip48/Reptin, Tip49/Pontin, and p400/Domino (15). It will therefore be interesting to test their involvement in antimicrobial host defenses.

Our results raise the question of the mechanism of DMAP1 function in the regulation of antimicrobial peptide gene expres-

sion. Our laboratory previously reported that the evolutionarily conserved nuclear protein Akirin modulates the activation of Relish in *Drosophila* by an as yet unknown mechanism (18). We show here that DMAP1 interacts with both Relish and Akirin, suggesting that Akirin and DMAP1 act together to regulate Relish activity. DMAP1 also interacts with BAP55, hinting that the Brahma complex participates in Relish regulation. The connection between DMAP1 and BAP55 may explain the increased activation of Toll and JAK-STAT pathways observed upon silencing of DMAP1 as the Brahma complex was previously identified as a negative regulator of these pathways in genome-wide RNAi screens (35, 36). Interestingly, the activity of another transcription factor, Twist, was recently shown to be regulated by both Akirin and the Brahma complex (37). Although acting in different contexts (e.g. immunity for Relish and development for Twist), both transcription factors regulate a large panel of genes in various tissues and require a tight control for gene expression. Several Twist-regulated genes in *Drosophila* embryo are not affected by silencing of Akirin (37) and, in mice, deficiency of Akirin2 affects some but not all NF- κ B-regulated genes (18). This indicates that Akirin is only required for expression of a subset of Twist or NF- κ B-regulated genes, suggesting that it may serve as a context-dependent modulator or selector of expression of downstream genes. We propose that DMAP1 is part of this modulator/selector complex.

In conclusion, we have identified a novel function for the SANT domain protein DMAP1 in innate immune signaling. The picture emerging from our data is that a chromatin remodeling process involving Akirin, DMAP1, and the Tip60 and Brahma complexes participates in the regulation of Relish activity in response to infection.

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REPORT: DMAP1 Modulates IMD Pathway Activity in *Drosophila*

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