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Intersection of the Multivesicular Body Pathway and Lipid Homeostasis in RNA Replication by a Positive-Strand RNA Virus▽

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Like many positive-strand RNA viruses, brome mosaic virus (BMV) RNA replication occurs in membrane-invaginated vesicular compartments. BMV RNA replication compartments show parallels with membrane-enveloped, budding retrovirus virions, whose release depends on the cellular multivesicular body (MVB) sorting pathway. BMV RNA replication compartments are not released from their parent membranes, but might depend on MVB functions for membrane invagination. Prior results show that BMV RNA replication is severely inhibited by deletion of the crucial MVB gene *DOA4* or *BRO1*. We report here that involvement of *DOA4* and *BRO1* in BMV RNA replication is not dependent on the MVB pathway's membrane-shaping functions but rather is due to their roles in recycling ubiquitin from MVB cargos. We show that deleting *DOA4* or *BRO1* inhibits the ubiquitination- and proteasome-dependent activation of homologous transcription factors Mga2p and Spt23p, which regulate many lipid metabolism genes, including the fatty acid desaturase gene *OLE1*, which is essential for BMV RNA replication. However, Mga2p processing and BMV RNA replication are restored by supplementing free ubiquitin, which is depleted in *doa4*Δ and *bro1*Δ cells. The results identify Mga2p and Spt23p processing and lipid regulation as sensitive targets of ubiquitin depletion and correctly predict multiple effects of modulating additional host genes *RFU1*, *UBP6*, and *UFD3*. Our results also show that BMV RNA replication depends on additional Mga2p-regulated genes likely involved in lipid metabolism beyond *OLE1*. Among other points, these findings show the potential for blocking viral RNA replication by modulating lipid synthesis at multiple levels.

Positive-strand RNA viruses replicate their RNA genomes in close association with proliferated and rearranged host intracellular membranes (17, 47, 62). Accordingly, lipid synthesis and the lipid composition of intracellular membranes play important roles in RNA replication by many positive-strand RNA viruses (12, 13, 16, 23, 32, 38–40, 64, 66, 72–74). Revealing the critical interactions between membrane rearrangement, lipid synthesis/composition, and viral RNA replication will further understanding of viral replication and could provide foundations for novel broad-spectrum antiviral strategies.

Brome mosaic virus (BMV) has been used as a model system to study gene expression, RNA replication, virus-host interaction, and evolution of positive-strand RNA viruses (70). BMV has a tripartite RNA genome and a single subgenomic mRNA, RNA4. Genomic RNA1 and RNA2 encode the two BMV RNA replication proteins, 1a and $2a^{pol}$, respectively. $2a^{pol}$ contains the viral RNA-dependent RNA polymerase domain. 1a, which contains RNA capping (3, 35) and NTPase/helicase domains (71), plays multiple, central roles in RNA replication complex assembly and function. For example, 1a directs itself, $2a^{pol}$, and viral RNA replication templates to perinuclear endoplasmic reticulum (ER) membranes that become the sites of viral RNA synthesis both in BMV's natural plant hosts and in cells of the yeast *Saccharomyces cerevisiae*, which also support BMV RNA replication, subgenomic mRNA transcription, and selective RNA encapsidation (15, 18, 29, 37, 57, 58, 63). Moreover, at these perinuclear ER membranes, 1a induces formation of ~60- to 75-nm spherular invaginations that serve as RNA replication compartments in yeast (63) and in plant hosts of BMV, such as *Nicotiana benthamiana* (X. Wang, A. Diaz, and P. Ahlquist, unpublished results). Many other positive-strand RNA viruses use similar vesicular membrane invaginations as RNA replication compartments (2, 17, 36).

BMV RNA replication is closely linked not only to formation of these vesicular RNA replication compartments (19, 42) but also to the lipid composition of the associated membranes (38–40). For example, even though normal numbers of replication compartments form, BMV RNA replication is inhibited by ≥20-fold when the activity of Δ9 fatty acid desaturase, which converts saturated to monounsaturated fatty acids (SFA and monoUFA, respectively), is reduced by mutations in yeast (39, 40) or by gene-specific RNA interference in *N. benthamiana* plants (X. Wang et al., unpublished data).

Previously, Kushner et al. (38) screened a yeast single-gene-deletion library and identified ~100 genes whose deletion inhibited or enhanced BMV RNA replication by >3-fold. Among the implicated genes, we focused on *DOA4* and *BRO1*, key components of the multivesicular body (MVB) pathway

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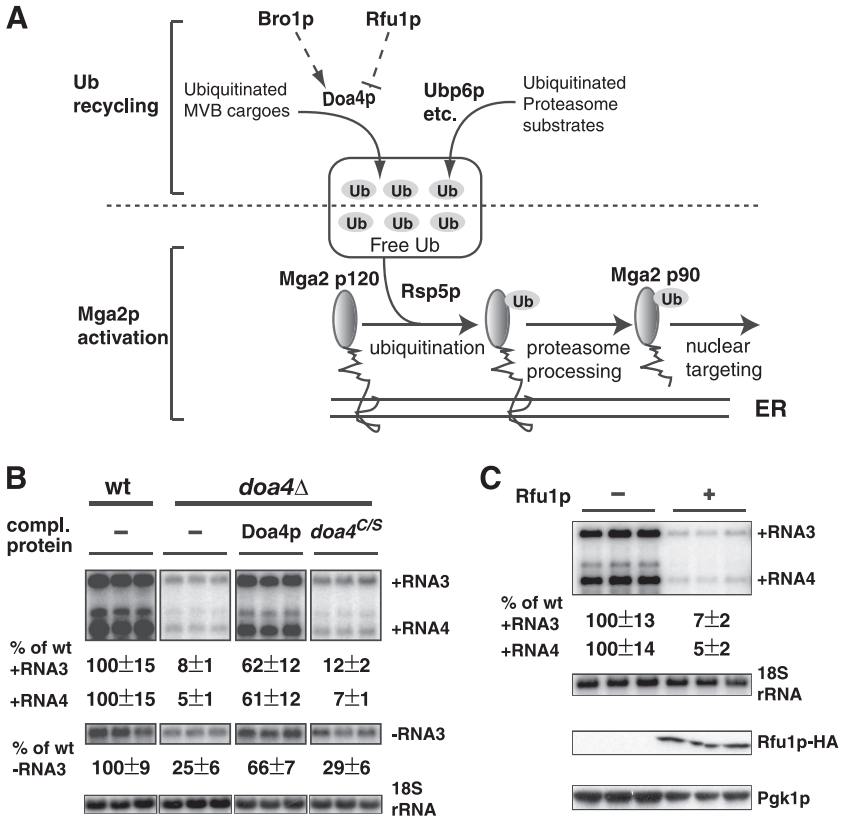

FIG. 1. Doa4p and its deubiquitinating activity are required for BMV RNA replication. (A) Schematic illustrating the connection between free ubiquitin (Ub) levels and activation of transcription activator Mga2p (and Spt23p). Ub is removed, and thus recycled, from ubiquitinated MVB cargoes via Doa4p or from polyubiquitinated proteasomal substrates via Ubp6p and other deubiquitinating enzymes. While Bro1p recruits Doa4p to MVB and promotes Doa4p's enzymatic activity, Rfu1p inhibits Doa4p activity. Mga2p (Mga2 p120) is ubiquitinated by ubiquitin ligase Rsp5p. The C-terminal membrane-spanning domain is degraded by proteasomes, and the N-terminal activation domain (Mga2 p90) is released from perinuclear ER membranes and subsequently targeted to the nucleus. See the text for more details. (B) The BMV RNA replication defect is complemented (comp.) by wt Doa4p but not by the *doa4*$^{C/S}$ mutant in *doa4*Δ cells. wt DOA4 and the *doa4*$^{C/S}$ mutant were expressed from their endogenous promoter by using centrameric plasmids. (C) BMV RNA replication is severely affected in yeast cells overexpressing *RFU1*. *RFU1* expression was driven by the *GAL1* promoter in a high-copy-number plasmid. For both panels B and C, total RNA was extracted from yeast cells expressing BMV components. BMV positive- and negative-strand RNAs were detected by Northern blotting using probes specific to BMV RNAs. 18S rRNA bands were detected with an 18S rRNA probe. The measured BMV RNA signals were normalized to that of 18S rRNA. The blot showing negative-strand RNA accumulation was exposed longer than that for positive-strand RNA for comparison. Total proteins were extracted from equal numbers of yeast cells and analyzed by SDS-PAGE and immunoblot analysis with monoclonal anti-HA or anti-Pgk1p antibodies. All experiments were done multiple times in triplicate or duplicate (Fig. 8B). Representative blots are shown for each figure.

through which mono- or oligoubiquitinated cargo proteins, such as signaling receptors, are targeted to vacuoles/lysosomes for degradation (27, 33, 53). These cargoes are sorted into intralumenal vesicles that invaginate away from the cytoplasm into the endosomal lumen and then pinch off to create MVBs. This MVB pathway is hijacked by retroviruses and other enveloped viruses to support their topologically similar virion budding (10, 11, 48). For such viruses, disrupting viral recruitment or function of MVB pathway components arrests the release of budding virions at a late stage (10, 11, 48).

Before cargoes are sorted into intralumenal MVB vesicles and degraded, ubiquitin (Ub) is obligatorily removed from these cargoes and recycled by *DOA4*-encoded deubiquitinating enzyme Doa4p (5, 6, 21, 33, 43, 69). Doa4p is also involved in removing poly-Ub from proteins targeted for degradation by proteasomes (50). Doa4p is recruited to MVB sites primarily by Bro1p (4, 44, 49, 59), which also activates Doa4p's deubiquitinase activity (Fig. 1A) (59). Bro1p is the yeast ortholog of mammalian *ALX1/AIP1*, through which the MVB pathway is recruited by late domains of HIV and other retroviruses to mediate budding (14, 46, 48). As the assembly of BMV RNA replication complexes shares multiple similarities with budding retroviral virions (63), we investigated whether dependence of BMV RNA replication on *DOA4* and *BRO1* involved the MVB pathway. We report here that inhibition of BMV RNA replication by deleting *DOA4* or *BRO1* is independent of the MVB pathway's membrane-shaping functions, but rather is due to downregulated expression of Δ9 fatty acid desaturase and other genes on which BMV RNA replication depends. We further show that upon deletion of *DOA4* or *BRO1*, a reduction in free Ub inhibits activation of specific transcription factors that induce expression of Δ9 fatty acid desaturase (Fig. 1A) and many other lipid metabolism genes. The results further demonstrate the crucial dependence of positive-strand RNA virus replication on a balanced spectrum of lipid synthesis and modification and the potential for

blocking viral replication by modulating such synthesis at multiple levels.

MATERIALS AND METHODS

Yeast strain and cell growth. Yeast strain BY4743 and its various single-gene-deletion derivatives were used in all experiments. Cultures were grown at 30°C in defined synthetic medium containing 2% galactose as a carbon source. Leucine, uracil, histidine, or combinations thereof were omitted to maintain plasmid selection. Cells were grown in galactose medium for 2 passages (36 to 48 h) and harvested when the optical density at 600 nm (OD_{600}) was between 0.4 and 1.0. To make medium supplemented with fatty acids, Tergitol NP-40 was added to a final concentration of 1% to solubilize the fatty acids. Equimolar amounts of palmitoleic acid (16:1) and oleic acid (18:1) were added to NP-40-containing medium to the specified concentrations (67).

Plasmids and plasmid construction. Expression of BMV 1a and $2a^{pol}$ was driven by the galactose-inducible and glucose-repressible *GAL1* promoter in most experiments. BMV 1a was expressed from plasmid pB1YT3 in all experiments that did not include expression of $2a^{pol}$ (71). To express both 1a and $2a^{pol}$ for assaying BMV RNA replication, plasmid pB12VG1 was used (38). *CUP1* promoter-driven BMV RNA3 was launched from plasmid pB3VG128-H in most experiments in medium lacking copper (38). To assay 1a-mediated RNA3 stability, we used plasmid pB3MS82, which expresses BMV RNA3 from the *GAL1* promoter (68).

Wild-type [wt] *DOA4* was expressed from plasmid pDOA4-8 (50) or pCR30 (59), using its endogenous promoter. The $doa4^{C/S}$ and $doa4^{AAFA}$ mutants were expressed from pCR64 and pCR15-U, respectively (59). pCR15-U was made by subcloning the $doa4^{AAFA}$ mutant sequence from pCR15 (59) to pRS416, a low-copy-number centromeric plasmid. Ubiquitin was expressed from pYEP96-U, which expresses the ubiquitin gene from the *CUP1* promoter (22). In all experiments, however, no additional copper was added to the medium. The pYEP96-U plasmid was made by subcloning the cassette containing the copper promoter, Ub open reading frame (ORF), and *CYC1* terminator from pYEP96 (22) into pRS426, a high-copy-number 2μ plasmid. Myc-tagged Mga2p and Spt23p were expressed from 2μ plasmids YEpLac181-^{myc}MGA2 and pRS426-$^{myc}SPT23^{HA}$, respectively (55). pRS426-$^{myc}SPT23^{HA}$ was made by subcloning the $^{myc}SPT23^{HA}$ fragment from pRS416-$^{myc}SPT23^{HA}$ into pRS426. A construct overexpressing His_6- and hemagglutinin (HA)-tagged *RFU1* was purchased from Open Biosystem, and the *RFU1* ORF was inserted into pBG1805, a 2μ plasmid under the control of the *GAL1* promoter. The *bro1-2* mutant, which has an early stop codon at position 820 amino acids (aa), was expressed from pSS41 and pGO369 for low and high expression levels, respectively (59). The *bro1-C* mutant (Bro1 aa 692 to 844) was expressed from pJEN6, a centromeric plasmid. Expression of both *bro1-2* and *bro1-C* was driven by the endogenous promoter.

Cell fractionation assays. Yeast spheroplasts were prepared from 5 OD_{600} units of cells and were lysed in lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM dithiothreitol, and 1:200 dilution of yeast protease inhibitor mix [Sigma]). Half of the lysate was retained as the total extracted protein. The other half of the lysate was centrifuged at 4°C for 5 min at 20,000 × *g*. The supernatant was removed and retained, and the pellet was resuspended in lysis buffer. Equal volumes of the total (T), supernatant (S), and pellet (P) fractions were used for SDS-PAGE and Western blotting.

Western blotting. Yeast cells were grown to an OD_{600} of 0.4 to 1.0, and 2 OD_{600} units of cells was harvested. Proteins were extracted as described previously (40), and equal volumes of extracted total proteins were used for electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. Expression of target proteins was detected with the following antibodies and dilutions: rabbit anti-BMV 1a at 1:10,000, rabbit anti-Bro1p at 1:5,000 (a gift from Gregory Odorizzi, University of Colorado), mouse anti-BMV $2a^{pol}$ at 1:4,000, mouse anti-Pgk1p (A6457; Molecular Probes) at 1:10,000, mouse anti-Dpm1p (A6429; Molecular Probes) at 1:1,000, mouse anti-Myc (OP10; Calbiochem) at 1:2,000, mouse anti-Ub (P4D1; Santa Cruz Biotechnology) at 1:2,000, and mouse anti-HA (32-6700; Invitrogen) at 1:2,000, using horseradish peroxidase (HRP)-conjugated secondary antibodies and Supersignal West Femto substrate (Thermo Scientific).

RESULTS

Doa4p deubiquitinase activity is required for BMV RNA replication. A previous genomewide yeast deletion mutant screen (38) revealed that BMV RNA replication depends on *DOA4*. In that screen, a BMV RNA3 derivative expressing *Renilla* luciferase (RLuc) was used as a viral RNA replication reporter. To eliminate the possibility that the BMV replication defect in cells with the complete *DOA4* open reading frame deleted (*doa4*Δ) was specific to the Rluc reporter, we compared the accumulation of BMV RNA3 replication products in *doa4*Δ and wild-type (wt) cells expressing BMV 1a, $2a^{pol}$, and template RNA3 (Fig. 1B). Viral RNA replication and subgenomic mRNA synthesis were assayed by strand-specific Northern blotting and normalized to 18S rRNA levels. In *doa4*Δ cells, positive-strand RNA3 accumulated to only 10 to 20% and RNA4 to 5 to 10% of wt replication levels (Fig. 1B), which agreed well with the previous Rluc-based measurements (38). Interestingly, negative-strand RNA3 in *doa4*Δ cells was detected at 20 to 30% of the wt level (Fig. 1B), indicating a more moderate reduction in this earlier replication step.

The BMV RNA replication defect in *doa4*Δ cells was largely complemented by expressing wt Doa4p (Fig. 1B). The nearly 70% complementation observed is close to the maximal possible restoration given the inevitable loss of the *DOA4*-expressing plasmid from some cells due to general inefficiencies in plasmid segregation during mitosis (24). To determine if Doa4p deubiquitinase activity was required for BMV RNA replication, we used a Doa4p $doa4^{C/S}$ mutant, which has a Cys_{781}→Ser substitution that suppresses Doa4p's deubiquitinating activity (51) and its ability to support cargo protein sorting through the MVB pathway (59). To avoid possible nonphysiological effects from overexpression of $doa4^{C/S}$ (51), as from a high-copy-number plasmid, this mutant and wt *DOA4* were independently expressed from the endogenous *DOA4* promoter on low-copy-number centromeric plasmids. The $doa4^{C/S}$ mutant protein is stable in yeast (51), but in contrast to the strong complementation by wt *DOA4*, expression of $doa4^{C/S}$ only slightly increased the low levels of BMV RNA replication in *doa4*Δ cells (Fig. 1B). Thus, Doa4p's deubiquitinase activity is essential to support BMV RNA replication.

RFU1 (*r*egulator of *f*ree *u*biquitin chains *1*) was recently identified as an inhibitor of Doa4p that helps maintain cellular Ub homeostasis (Fig. 1A) (34). Rfu1p interacts with Doa4p directly and inhibits Doa4p's enzymatic activity *in vitro*. Overexpression of *RFU1* led to phenotypes similar to those of the *doa4*Δ mutant, including depleted free Ub levels and heat stress sensitivity (34; also see the next section). Therefore, we overexpressed HA-tagged *RFU1* (Rfu1p-HA) (Fig. 1C) under the control of the strong GAL1 promoter in wt cells and checked its effect on BMV RNA replication. Overexpressing *RFU1* inhibited BMV RNA replication by 13-fold (Fig. 1C), independently confirming that Doa4p's deubiquitinating activity is indeed required to support BMV RNA replication.

BMV RNA replication is restored in *doa4*Δ cells by added Ub. In addition to defects in MVB cargo protein sorting, deletion of *DOA4* leads to depletion of free Ub, defective protein proteolysis in proteasomes and vacuoles/lysosomes, hypersensitivity to stress and amino acid analogs, and a strong sporulation defect (69). The Ub deficiency causes the remaining listed defects since supplementation with Ub largely restores the majority of affected functions (69). As expected, Western blotting confirmed an equivalent decrease in free Ub in the *doa4*Δ cells used here (Fig. 2A).

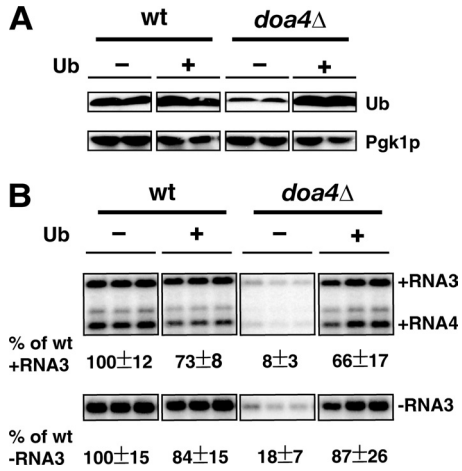

FIG. 2. Supplemented ubiquitin complements BMV RNA replication defect in *doa4*Δ cells. (A) Reduced levels of free Ub are complemented by supplemented Ub. Total protein extraction and Western blotting were done as in Fig. 1 with monoclonal anti-Ub or anti-Pgk1p antibodies. (B) Supplemented Ub restores BMV RNA replication to close to wt levels. Ub was expressed from plasmid pYEP96-U under the control of the copper promoter and grown in medium lacking copper. RNA extraction and Northern blotting were done as in Fig. 1.

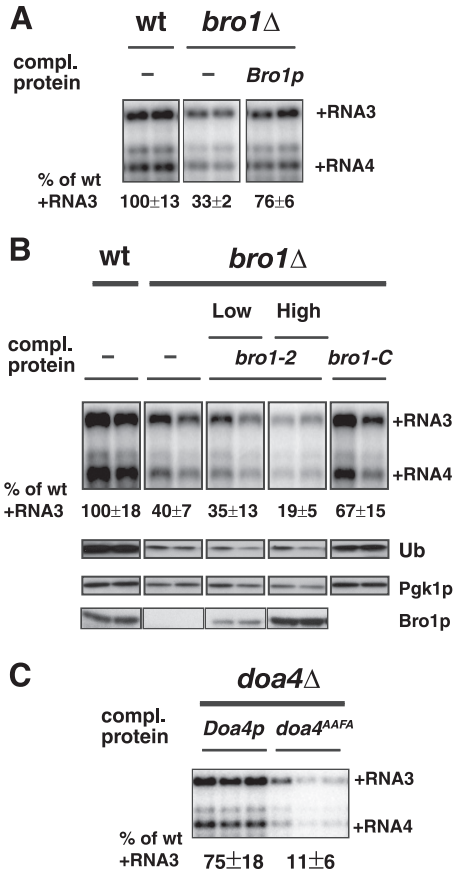

FIG. 3. Inhibited BMV RNA replication in *bro1*Δ cells correlates with a lack of Doa4p's enzymatic activity and a decrease in free Ub levels. (A) Inhibited BMV RNA replication is restored by supplementation with wt Bro1p in *bro1*Δ cells. (B) BMV RNA replication is related to the levels of free Ub in *bro1*Δ cells. The *bro1-2* mutant was expressed from either a low- or high-copy-number plasmid, and mutant *bro1-C* was expressed from a low-copy-number plasmid in *bro1*Δ cells. Note that the anti-Bro1p antiserum did not detect *bro1-C*. comp., complementing. (C) The functional interaction between the C termini of Doa4p and Bro1p is required for BMV RNA replication. The $doa4^{AAFA}$ mutant, which does not interact with Bro1p or support cargo sorting and deubiquitination, did not restore BMV RNA replication in *doa4*Δ cells. Accumulation of BMV RNAs and Ub, Bro1p, and Pgk1p levels were detected as in Fig. 1.

To determine whether this free Ub deficiency played a role in inhibiting BMV RNA replication in *doa4*Δ cells, Ub was expressed from plasmid pYEP96-U, which provides wt Ub levels (Fig. 2A) (69). In *doa4*Δ cells with supplemented Ub, all forms of BMV RNA, including positive- and negative-strand RNA3 and positive-strand RNA4, accumulated to levels similar to those in wt yeast cells harboring pYEP96-U (Fig. 2B). Similar to the maximum complementation achieved by expression of wt Doa4p (Fig. 1B), supplementation with Ub restored BMV RNA replication to ~66% of that in wt cells, suggesting that a Ub-related pathway or pathways may be responsible for most or all inhibition of BMV RNA replication in *doa4*Δ cells.

BMV RNA replication deficiency in *bro1*Δ cells is linked to Doa4p activity and decreased free Ub. Like *DOA4*, *BRO1* was implicated in BMV RNA replication in a prior yeast deletion library screen (38). We found in repeated experiments that replication of wt BMV RNA3 was inhibited in *bro1*Δ cells by 2.5- to 4-fold (Fig. 3A), confirming the *BRO1* dependence of BMV replication and showing that this effect was independent of the RLuc reporter used in the original primary screening.

Bro1p has multiple functions in the MVB pathway, including activating Doa4p's deubiquitinase function and contributing to Doa4p recruitment to the MVB pathway (4, 59). Bro1p contributes to Doa4p recruitment by an interaction between their N termini, while Bro1p's crucial stimulation of Doa4p's deubiqutinase activity is mediated by interaction of the Bro1p C terminus with the Doa4p C-terminal catalytic domain (4, 44, 49, 59). Disruption of this C-terminal Bro1-Doa4p interaction drastically inhibits Doa4p's deubiquitinase and MVB cargo-sorting functions (49, 59).

To test the possible relationship between *BRO1*'s effects on BMV RNA replication and *DOA4*, we performed complementation tests with the wt *BRO1* allele and selected *BRO1* mutants. The *bro1-2* mutant has a Q_{820}→Stop substitution that deletes the C-terminal 25 amino acids of Bro1p, blocks Doa4p deubiquitinase activation, and produces multiple phenotypes paralleling those of *doa4*Δ cells (59). As expected, expressing wt *BRO1* in *bro1*Δ cells complemented the defect in BMV RNA replication (Fig. 3A). Unlike wt Bro1p, expression of *bro1-2* from a low-copy-number plasmid had little or no significant effect on RNA3 replication, while expression of *bro1-2* from a high-copy-number plasmid inhibited the already low BMV RNA replication a further 2-fold (Fig. 3B). The negative effects of overexpression of *bro1-2*, whose N terminus interacts with and recruits Doa4p without activation (59), might result from suppression of the low basal activity of Doa4p or from nonproductive sequestration of Doa4p from other roles on the proteasome and other sites (50).

To further assess the importance of Bro1p-mediated Doa4p deubiquitinase activation for BMV RNA replication, we also tested the converse approach of disrupting this activation by

mutating *DOA4* in the presence of wt *BRO1*. The *DOA4* allele used was *doa4*^{AAFA}, which substitutes AAFA for a C-proximal YPFL motif (aa 826 to 829) essential for the Doa4p-Bro1p interaction that stimulates Doa4p's enzymatic activity (59). The *doa4*^{AAFA} mutant stably accumulates in cells, and like green fluorescent protein (GFP)-tagged wt Doa4p, GFP-tagged *doa4*^{AAFA} properly localizes to MVBs (59). Unlike wt *DOA4*, however, expressing *doa4*^{AAFA} in *doa4*Δ cells did not complement the defect in BMV RNA replication (Fig. 3C).

Next we expressed in *bro1*Δ cells a C-terminal fragment of Bro1p comprising aa 692 to 844, which lacks the N-terminal Bro1p sequence that facilitates Doa4p recruitment to MVB sites but retains all sequences needed to bind and activate Doa4p's C-terminal deubiquitinase domain (59). This C-terminal Bro1p fragment complemented BMV RNA replication nearly as efficiently as wt Bro1p (Fig. 3A and B).

Thus, the results with all three mutants were consistent with Bro1p activation of Doa4p deubiquitinase function being the major if not sole contribution of Bro1p to BMV RNA replication. This conclusion was further underscored by Western blots showing that, as in *doa4*Δ cells (Fig. 2B), free Ub levels in *bro1*Δ cells were greatly reduced relative to those in wt cells (Fig. 3B) (4). Moreover, *bro1-2* expression, which did not restore BMV RNA replication, did not restore free Ub levels, whereas *bro1-C* expression stimulated BMV RNA replication and free Ub levels in parallel (Fig. 3B). Since multiple, independent findings implied that deletion of Bro1p affected BMV RNA replication indirectly, through the loss of its stimulating effect on Doa4p deubiquitinase, we next concentrated on how loss of Doa4p function inhibited BMV RNA replication.

Doa4p is not required for early steps of BMV RNA replication. Loss of Doa4p or its activity increases the half-life of many cellular proteins (50, 51). Therefore, we checked whether deletion of *DOA4* affects the accumulation of BMV replication proteins 1a and 2a^{pol}. Western blotting showed that 1a and 2a^{pol} levels were unaffected in *doa4*Δ cells (Fig. 4A), indicating that the BMV RNA replication defect in *doa4*Δ cells is not due to altered accumulation or balance of the viral replication proteins.

BMV 1a recruits viral RNA replication templates into a membrane-associated, RNase-resistant state with a likely physical location inside the viral spherules (63). This is reflected by a dramatic 8- to 20-fold increase in viral RNA template accumulation depending on the amount of 1a protein expressed (28, 63). BMV genomic RNA3 accumulation in the presence of 1a increased 15-fold in *doa4*Δ cells, very similar to the 13-fold increase in wt cells (Fig. 4B), indicating that recruitment of viral RNA replication templates was not affected by deletion of *DOA4*.

BMV 1a also stimulates 2a^{pol} accumulation and recruits 2a^{pol} to ER membranes through an interaction between the 1a C terminus and 2a^{pol} N terminus (15, 31). We examined the distribution of 2a^{pol} in *doa4*Δ cells without or with 1a by cell fractionation and Western blotting (Fig. 4C). In *doa4*Δ cells without 1a, 2a^{pol} was mainly detected in the membrane-depleted supernatant, similar to soluble cytoplasmic protein Pgk1p and opposite from ER marker Dpm1p, which accumulates primarily in the membrane-enriched pellet fraction (Fig. 4C). As in wt cells, 2a^{pol} accumulation increased several-fold and shifted to the membrane-enriched pellet upon 1a expression in *doa4*Δ cells, indicating that recruitment of 2a^{pol} to membranes by 1a was not affected in *doa4*Δ cells (Fig. 4C).

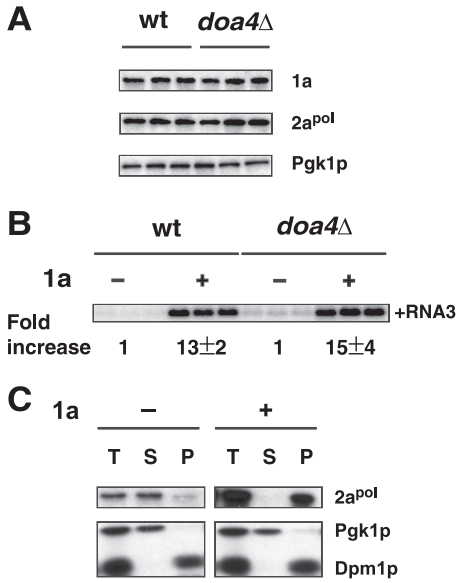

FIG. 4. *DOA4* is not required for early steps of BMV RNA replication. (A) BMV 1a and 2a^{pol} accumulate to equal levels in wt and *doa4*Δ cells. Accumulation of BMV 1a or 2a^{pol} protein was analyzed as in Fig. 1 with anti-1a or anti-2a^{pol} antibodies. (B) BMV RNA3 is stabilized by 1a in *doa4*Δ cells. BMV RNA3 only or RNA3 and 1a were expressed in wt and *doa4*Δ cells. Accumulation of RNA3 was detected by using a BMV-specific probe as in Fig. 1. (C) BMV 2a^{pol} is recruited to the membrane fraction by 1a in *doa4*Δ cells. Yeast cells expressing 2a^{pol} only or coexpressing 2a^{pol} and 1a were spheroplasted and lysed to yield a total protein fraction (T). The lysate was centrifuged to obtain membrane-depleted supernatant (S) and membrane-enriched pellet (P) fractions. Equal volumes of total, supernatant, and pellet fractions were subjected to SDS-PAGE, and Pgk1p, Dpm1p, and BMV 2a^{pol} were detected by using antibodies against each protein. Note that Pgk1p is a soluble cytoplasmic protein, while Dpm1p is an ER membrane protein.

Collectively, the above data indicate that in *doa4*Δ cells BMV RNA replication was inhibited within the short interval after 1a-mediated recruitment of RNA templates and 2a^{pol} to the ER membrane, but before negative-strand RNA synthesis.

Activation of transcription factor Mga2p is inhibited in *doa4*Δ cells. Pathway and literature analysis revealed that the regulated expression of Δ9 fatty acid desaturase, which is required for BMV RNA replication in yeast (39, 40) and plants (X. Wang et al., unpublished data), was a candidate pathway connecting *DOA4* function and Ub depletion to BMV RNA replication (Fig. 1A). Fatty acid desaturase and other lipid synthesis genes are regulated by conserved processes that retain key features from yeast to *Drosophila* to humans, including the proteolytic release and activation of membrane-anchored, cytosolic transcription factors that then translocate into the nucleus to activate transcription of lipid metabolism genes (1, 20, 56). Specifically, in yeast, Δ9 fatty acid desaturase gene *OLE1* and other lipid synthesis genes are regulated by the homologous, functionally related transcription activators Mga2p and Spt23p (75). Mga2p and Spt23p precursor forms (p120s) are sequestered in the ER membrane through C-terminal membrane-spanning domains (Fig. 1A). Upon changes

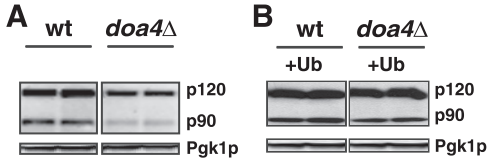

FIG. 5. Activation of transcription factor Mga2p is inhibited in *doa4*Δ cells. N-terminally Myc-tagged Mga2p was expressed in wt and *doa4*Δ cells alone in panel A or along with Ub and BMV 1a in panel B. Accumulation of Mga2p p120 and p90 was detected with an anti-Myc monoclonal antibody by Western blotting.

in lipid composition or environmental stresses, Mga2p and Spt23p are mono- or oligoubiquitinated by ubiquitin ligase Rsp5p, the C-terminal membrane anchors are recognized and degraded by the proteasome, and their N-terminal transcription activation domains (p90s) are released and translocated into the nucleus to induce transcription of *OLE1* and other genes linked to lipid metabolism (Fig. 1A) (7, 8, 25, 26, 54–56, 65). Loss of Doa4p function might inhibit Mga2p and Spt23p activation and thus *OLE1* expression because consequent reduction of free Ub levels may inhibit Mga2p and Spt23p ubiquitination. Alternatively, or in addition, since a subset of Doa4p is associated with proteasomes (50), deletion of *DOA4* might alter proteasome function and lead to inefficient Mga2p and Spt23p processing. Moreover, as shown above, loss of *DOA4* function mirrors the effects of reduced Ole1p activity in inhibiting BMV RNA replication at a point between RNA replication complex assembly and negative-strand RNA synthesis (40).

To compare levels of accumulation of the transcriptionally inactive precursor p120 and processed, active p90 forms of Mga2p and Spt23p in wt and *doa4*Δ cells, we expressed N-terminally Myc-tagged Mga2p and Spt23p, allowing both p120 and p90 to be detected using an anti-Myc antibody (26, 55). For standard growth conditions and the yeast BY4743 strain used here, Mga2p produced a much stronger Western blot signal and so was used for further analysis (Fig. 5). While the levels of accumulated BMV 1a, $2a^{pol}$, and host protein Pgk1p (Fig. 4A and 5) were similar in both wt and *doa4*Δ cells, accumulation of both the p120 and p90 forms of Mga2p decreased in *doa4*Δ cells. However, the decrease in accumulation of the transcriptionally active p90 form was more dramatic (Fig. 5A), consistent with a decrease in Mga2p processing. The reason for the reduction in p120 levels in *doa4*Δ cells is currently unclear, but this reduction was minor (22 to 28%) compared to the 80 to 90% inhibition of BMV RNA replication. To determine if limited free Ub caused the reduced activation of Mga2p in *doa4*Δ cells, Ub was supplemented from plasmid pYEP96-U to wt levels (Fig. 2A). This increased accumulation of Mga2p p90 close to wt levels (Fig. 5B), showing that the reduced in free Ub in *doa4*Δ cells is the major cause of inhibited Mga2p activation.

Inhibition of BMV RNA replication in *doa4*Δ cells is linked to inhibition of *OLE1* expression. Since Mga2p and Spt23p regulate *OLE1* transcription, we analyzed *OLE1* mRNA levels. In *doa4*Δ cells, *OLE1* mRNA accumulation was typically reduced 3-fold or more below wt levels (Fig. 6A). We similarly observed a 3-fold reduction of *OLE1* transcript levels in cells overexpressing *RFU1* (data not shown). This is consistent with

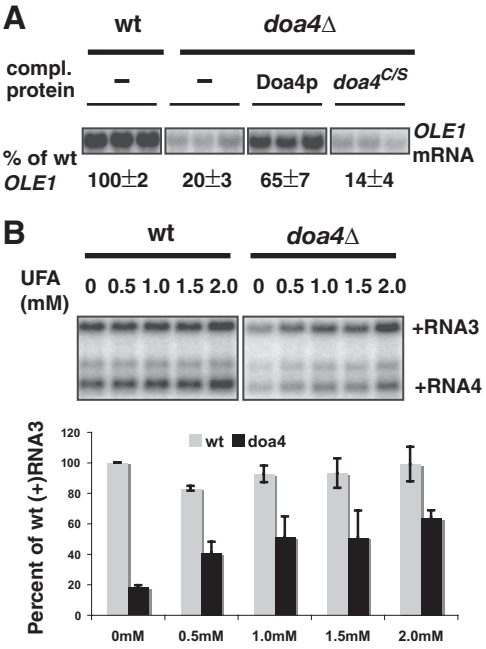

FIG. 6. Inhibited BMV RNA replication in *doa4*Δ cells is linked to inhibition of OLE1 expression. (A) Decreased accumulation of *OLE1* mRNA correlates with defective BMV RNA replication in *doa4*Δ cells. *OLE1* mRNA was detected by Northern blotting with an *OLE1*-specific probe. The RNA signals were normalized to that of 18S rRNA. (B) BMV RNA replication was measured after addition of increasing concentrations of UFA (an equimolar mixture of palmitoleic and oleic acids) to the yeast growth medium. Accumulation of BMV RNA was detected as in Fig. 1. Values represent the means of four independent repeats.

earlier work showing that overexpression of *RFU1* inhibited Doa4p activity and decreased Ub levels (34). Plasmid-based expression of wt *DOA4* in *doa4*Δ cells restored accumulation of *OLE1* mRNA (Fig. 6A) in parallel to BMV RNA replication (Fig. 1B). Moreover, accumulation of *OLE1* mRNA was not increased in *doa4*Δ cells expressing the enzymatically inactive $doa4^{C/S}$ mutant (Fig. 6A), demonstrating a clear correlation between Doa4p enzymatic activity, *OLE1* mRNA accumulation, and BMV RNA replication.

To test if decreased BMV RNA replication was directly related to the downregulation of *OLE1* activity in *doa4*Δ cells, we provided increasing amounts of the unsaturated fatty acid (UFA) products of *OLE1* (an equimolar mixture of palmitoleic acid and oleic acid) in the growth medium. Adding up to 2 mM UFA did not affect the growth of wt and *doa4*Δ strains (data not shown). Supplemented UFA did not enhance BMV RNA replication in wt cells, but restored BMV RNA replication in a dose-dependent manner in *doa4*Δ cells (Fig. 6B). Accumulation of positive-strand viral RNA3 increased more than 3-fold at the highest UFA concentration, to about 65% of wt RNA replication levels. Levels of RNA4, which was more strongly inhibited than RNA3 in *doa4*Δ cells (Fig. 1B), saw a correspondingly greater 4-fold increase upon UFA addition, from ~10% to 40% compared to wt levels (Fig. 6B). However, we never observed full complementation of BMV RNA replication with addition of UFA, as can be achieved in the *ole1w* and *ole1*Δ mutants (40). This implied that decreased *OLE1* expres-

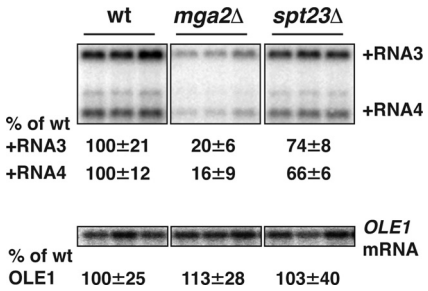

FIG. 7. BMV RNA replication is inhibited in *mga2*Δ mutant cells. BMV components were expressed in wt, *mga2*Δ, and *spt23*Δ cells. Accumulation of BMV RNAs and *OLE1* mRNA was assessed as in Fig. 1 with BMV RNA- or *OLE1*-specific probes.

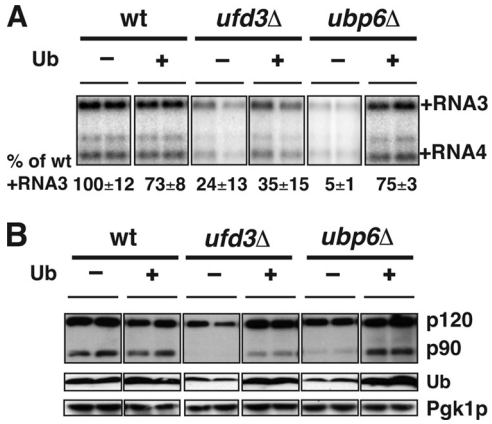

FIG. 8. Inhibited Mga2p activation and BMV RNA replication can be restored or partially restored in *ubp6*Δ and *ufd3*Δ cells by supplementation with Ub. (A) BMV RNA replication and (B) Mga2p activation were assessed in wt, *ufd3*Δ, and *ubp6*Δ cells with or without the addition of Ub. BMV 1a was expressed in all cells in panel B. Accumulation of BMV RNAs and activation of Mga2p were analyzed as in Fig. 1 and 5, respectively.

sion only partially accounts for the inhibition of BMV RNA replication in *doa4*Δ cells, as is addressed in the next section.

Other Mga2p-regulated genes are involved in BMV RNA replication. Besides *OLE1*, whose expression has been extensively studied, Mga2p and Spt23p collectively regulate at least 30 genes involved in lipid metabolism, including those synthesizing fatty acids (*FAS1* and *ACC1*), sterols (*ERG1*, *-3*, *-5*, and *-26*), and sphingolipids (*SUR1* and *-4*) (7). However, Mga2p and Spt23p regulate overlapping but not exactly matching sets of genes. While *OLE1* expression is induced by either Mga2p or Spt23p, other lipid metabolism genes, such as *SUR4* and *ERG5*, are preferentially induced by Mga2p (7). To test if other Spt23p- and/or Mga2p-regulated genes besides *OLE1* might be partially responsible for inhibiting BMV RNA replication in *doa4*Δ cells, we tested BMV RNA replication in *mga2*Δ and *spt23*Δ cells. In both cell types, *OLE1* transcripts accumulated to wt levels (Fig. 7), which was expected since either Mga2p or Spt23p is sufficient to activate *OLE1* expression (75).

Despite unaltered levels of *OLE1* mRNA, deletion of *MGA2* inhibited BMV RNA replication by more than 5-fold, compared to a mild decrease (25%) upon deletion of *SPT23* (Fig. 7). This implies that one or more other genes specifically or preferentially regulated by Mga2p play important roles in BMV RNA replication.

***DOA4*-independent depletion of free Ub inhibits Mga2p activation and BMV RNA replication.** We tested if deleting free Ub would affect Mga2p and Spt23p activation, the consequent *OLE1* expression, and BMV RNA replication as a general phenomenon not specific to deletion or inhibition of *DOA4*. Among 60 host gene deletion mutants previously found to inhibit BMV RNA replication (38), the *ufd3*Δ and *ubp6*Δ mutants have depleted free Ub levels (30, 41, 61). Ubp6p is a deubiquitinating enzyme and an accessory component of the proteasome (Fig. 1A) (5, 41). Ufd3p is involved in protein turnover via an interaction with *CDC48*, a critical component of a major pathway for delivering substrate proteins to proteasomes (30, 41, 61). Ufd3p also regulates Spt23p stability (61). Deletion of *UFD3* and *UBP6* may affect Spt23p and Mga2p activation in at least two ways. First, as in *doa4*Δ cells, decreased free Ub levels in *ufd3*Δ and *ubp6*Δ cells may inhibit ubiquitination and subsequent activation of Mga2p and Spt23p. Alternatively, or in addition, deletion of *UBP6* may affect proteasome activity and thus Mga2p and Spt23p processing (41), while deletion of *UFD3* may affect the stability of the processed, active Spt23p and Mga2p p90 forms (61).

Consistent with the results of Kushner et al. (38), BMV RNA replication was inhibited in *ufd3*Δ and *ubp6*Δ mutants to 5-fold and 10- to 20-fold, respectively (Fig. 8A). Also consistent with previous reports (30, 41, 61), free Ub levels were much reduced in both *ufd3*Δ and *ubp6*Δ mutants (Fig. 8B). While Mga2p p120 accumulated to near wt levels in *ufd3*Δ and *ubp6*Δ mutants, Mga2p p90 was much reduced (Fig. 8B), confirming that Mga2p processing was inhibited.

To test if reduced Mga2p p90 accumulation and BMV RNA replication could be restored in these mutants by supplementation with Ub, pYEP96-U was used to express Ub to wt levels in both mutants (Fig. 8B). In *ubp6*Δ cells, providing Ub increased Mga2p p90 to near wt levels. On the other hand, supplementation with Ub only slightly increased Mga2p p90 accumulation in *ufd3*Δ cells, suggesting that deletion of *UFD3* may affect both Mga2p activation and stability. Consistent with Mga2 p90 accumulation, BMV RNA replication was restored to wt levels in *ubp6*Δ cells but only increased from 20% to 35% in *ufd3*Δ cells (Fig. 8A).

DISCUSSION

The RNA replication complexes of BMV and many other positive-strand RNA viruses (17, 47, 62, 63), budding enveloped virions (10, 11, 48), and MVB intralumenal vesicles (27, 33, 53) are all topologically similar in being formed by invaginating membranes away from the cytoplasm. Just as budding of many enveloped virions depends on MVB components, prior genomewide screening showed that efficient BMV RNA replication requires MVB genes (38), and recent results show that MVB components also are transiently recruited by another positive-strand RNA virus, tomato bushy stunt virus, to peroxisome membranes, where they promote RNA replication (9).

Here we investigated how two MVB genes, the deubiquiti-

nase gene *DOA4* and the multifunctional gene *BRO1*, contribute to BMV RNA replication. The mammalian homologs of *DOA4* and *BRO1* are the genes coding for Ubpy and Alix (59, 60). Notably, Alix has emerged as a key player in the MVB-dependent budding of multiple retroviruses and some other viruses, although the precise nature of its functions in such viral budding remain under study (14, 46, 48). Nevertheless, many features of *BRO1*/ALIX and *DOA4*/Ubpy function appear conserved from yeast to mammals. Among these, in parallel to Bro1p-mediated recruitment of Doa4p for MVB cargo deubiquitination (59), recruitment of Bro1p homolog Alix to HIV-1 Gag promotes Gag deubiquitination (45).

Despite the topological similarities of membrane-bound BMV RNA replication compartments and MVB compartments, we found that the roles of *DOA4* and *BRO1* in BMV RNA replication were independent of the protein- and membrane-sorting functions of the MVB pathway. Rather, as detailed below, defects in Doa4p or Bro1p or their interactions disrupted BMV RNA replication by depleting free Ub levels and thereby inhibiting induction of *OLE1* fatty acid desaturase and other lipid synthesis genes required by BMV RNA replication (Fig. 2, 5, 6, and 7). Possible implications of these results for virus control are also considered.

***DOA4*-, *BRO1*-, and Ub-dependent activation of lipid synthesis genes.** Multiple results showed that the BMV RNA replication defects in *doa4*Δ and *bro1*Δ cells were linked to free Ub levels and the activation of lipid synthesis/modification genes, but not to the membrane-shaping functions of the MVB pathway. BMV RNA replication required Doa4p with a catalytically active deubiquitinase (Fig. 1B), a function that is dispensable for invaginating intralumenal vesicles to form MVBs (59) but essential to maintain normal free Ub levels (69). In keeping with this, across varied circumstances in *doa4*Δ cells there was a tight correlation between BMV RNA replication and the levels of free Ub (Fig. 2), the Ub-dependent activation of lipid synthesis regulator Mga2p (Fig. 5B), and the accumulation of *OLE1* fatty acid desaturase mRNA (Fig. 6A). The mechanistic linkage between these observations was confirmed by showing that the BMV RNA replication defect in *doa4*Δ cells was complemented by supplementation of free Ub levels (Fig. 2), which restored activation of Mga2p (Fig. 5B), or by feeding the UFA products of Ole1p fatty acid desaturase (Fig. 6B), thus bypassing Ub levels, Mga2p activation, and *OLE1* expression.

BRO1, which was also required for BMV RNA replication, provides multiple functions to the MVB pathway. For example, unlike *DOA4*, deletion of *BRO1* blocks the formation of normal MVB membrane compartments, yielding instead aberrant "class E" endosomes that are flattened cisternae without intralumenal vesicles (27, 33, 53). However, using mutations that selectively ablate specific functions, we found that *BRO1* contributes to BMV RNA replication indirectly by interacting with Doa4p to stimulate its deubiquitinase activity and the accumulation of free Ub (Fig. 3B). Thus, in *bro1*Δ cells, BMV RNA replication was restored by expressing a Bro1p C-terminal fragment, *bro1-C*, that neither supports formation of functional MVB membrane compartments nor facilitates Doa4p recruitment to MVB sites, but interacts with and activates the Doa4p catalytic domain (59) and stimulates free Ub levels (Fig. 3B). Conversely, BMV RNA replication in *bro1*Δ cells was not restored by expressing the *bro1-2* C-terminal-truncation mutant (Fig. 3B), which supports intralumenal vesicle and MVB compartment formation but does not stimulate Doa4p's deubiquitinase activity (59). In keeping with this, the *doa4*^{AAFA} mutant, which disrupts the Doa4p side of the activating interaction with wt Bro1p, similarly blocked BMV RNA replication in *doa4*Δ cells (Fig. 3C). Thus, Doa4p's deubiquitinating activity, not Bro1p's possible role in inducing membrane curvature, is required for supporting BMV RNA replication.

Thus, all of the above results indicated that *DOA4* and *BRO1* primarily affected BMV RNA replication through their effects on free Ub levels and the Ub-dependent activation of lipid synthesis regulators Mga2p and Spt23p. While this underlying linkage between Ub levels, lipid synthesis, and BMV RNA synthesis was clear, some variations were noted between the levels of free Ub and associated effects on BMV RNA replication in the *doa4*Δ and *bro1*Δ mutants (Fig. 2A and 3B). One likely explanation for some variations in both Ub levels and inhibition of BMV RNA replication would be variations in culture growth conditions, which alter free Ub levels in the *doa4*Δ mutant. Specifically, Ub levels in the *doa4*Δ strain reach ~1/3 of those in wt cells during log-phase growth, but fall to only ~1/10 of those in wt cells in the stationary phase (69).

We further tested and validated the linkage of free Ub levels, lipid synthesis activation, and BMV RNA replication by using mutations in other host genes that modulate free Ub levels. As predicted, overexpressing *RFU1*, which inhibits Doa4p's enzymatic activity and depletes free Ub (34), markedly reduced *OLE1* transcript levels (results not shown) and BMV RNA replication in wt cells (Fig. 1C). Moreover, *DOA4*-independent inhibition of BMV RNA replication in *ubp6*Δ and *ufd3*Δ cells also strongly correlated with decreased free Ub levels and deficient Mga2p activation (Fig. 8).

In both plant cells and yeast, subgenomic RNA4 accumulation is much more sensitive than genomic RNA3 accumulation to slight variations in inoculation, growth conditions, host genotype, etc. In keeping with this, *DOA4* deletion inhibited RNA4 accumulation more significantly than RNA3 accumulation, as shown in Fig. 1B and elsewhere. Thus, while both RNA3 synthesis and RNA4 synthesis are inhibited by *DOA4* deletion and restored by supplying Ub or unsaturated fatty acids, they do have different sensitivities to these and other effects.

Mga2p-regulated genes beyond *OLE1* contribute to BMV RNA replication. *OLE1* mutations inhibit BMV RNA replication ≥20-fold, but are completely complemented by supplying the UFA products of *OLE1* in the medium (40). In contrast, the BMV RNA replication defect in *doa4*Δ cells was only partially complemented by supplementing cells with UFA (Fig. 6B), implying that *OLE1* was not the only BMV-required gene affected. Consistent with this, Mga2p and Spt23p control many genes involved in fatty acid, sphingolipid, and sterol metabolism (7), and BMV RNA replication depends on many lipid synthesis modification and transport genes beyond *OLE1* (38). Furthermore, although both accumulated wt levels of *OLE1* mRNA, BMV RNA replication was inhibited 5-fold in *mga2*Δ cells but by only ~25% in *spt23*Δ cells.

Accordingly, genes other than *OLE1* that are preferentially controlled by Mga2p might account for the residual inhibition of BMV RNA replication that is not complemented by UFA

feeding (Fig. 7). Mga2p was predominantly expressed over Spt23p in the BY4743 strain used here (Fig. 5B), which may partly account for the greater defect in *mga2*Δ cells. Additionally, the promoters of multiple lipid metabolism genes are more strongly bound by Mga2p than Spt23p, including genes involved in synthesizing sterols important for the replication of other positive-strand RNA viruses, including hepatitis C virus (HCV) (73) and tomato bushy stunt virus (64).

Relevance to bromovirus replication in plants. The results presented here obtained with yeast reveal new aspects of host function required for BMV RNA replication and interconnections between key pathways required by BMV, including unsaturated fatty acid synthesis and Ub-dependent proteasomal processing. As noted above, these pathways show broad conservation across eukaryotes, and other recent results from our laboratory show that BMV RNA replication in plants also depends critically on both of these pathways. We find, e.g., that inhibiting of proteasome function suppresses BMV RNA replication in barley protoplasts (B. Gancarz and P. Ahlquist, unpublished data), confirming that proteasome-dependent pathways are essential for BMV replication in cells of a natural plant host. Moreover, in a particularly direct connection to the present results, we find that knocking down fatty acid desaturase activity dramatically inhibits BMV RNA replication in *N. benthamiana* (X. Wang, A. Diaz, and P. Ahlquist, unpublished data). Thus, as was originally found (39, 40) and further extended here using yeast, membrane lipid composition and unsaturated fatty acids in particular are also crucial for BMV RNA replication in a plant host. Together, these results and recent studies of the involvement of sterol synthesis in tombusvirus replication (64) show that multiple membrane-associated pathways linked to virus replication in yeast are also essential in natural hosts.

Relationship to other positive-strand RNA viruses. Membrane lipid synthesis and composition are critical for RNA replication by many if not all positive-strand RNA viruses (13, 32, 38, 64, 66, 72–74). Inhibition of one or more aspects of fatty acid synthesis, e.g., inhibits replication of many viruses, including poliovirus (23), Semliki Forest virus (52), cowpea mosaic virus (12), BMV (39, 40), HCV (32), and *Drosophila* C virus (16). Similarly, cholesterol metabolic pathways are required by many viruses, such as HCV, for entry, RNA replication, and egress (73). Accordingly, the results presented here with a variety of host genes highlight broader potentials to control viral RNA replication and other aspects of infection by manipulating lipid synthesis and its regulation in novel ways. Advancement of understanding of the lipid dependencies of specific virus replication steps and the cellular regulatory circuits that control different aspects of lipid metabolism and their interaction with other pathways should enhance the potential for virus control while minimizing host toxicity. The wide importance of these pathways suggests the possibility of approaches effective against multiple viruses, and the broad conservation of many features of lipid regulation from yeast to humans (1, 20, 56) should facilitate generalizing such strategies across a range of practically important hosts.

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