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1 **Differential Roles of Hsp70 and Hsp90 in the Assembly of the Replicase Complex**
2 **of a Positive-Strand RNA Plant Virus**

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16 Running Title: Chaperone-Assisted Assembly of Viral Replicase

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19

1 **Abstract**

2 Assembly of viral replicase complexes of eukaryotic positive-strand RNA viruses is a
3 regulated process: multiple viral and host components must be assembled on
4 intracellular membranes and ordered into quaternary complexes capable of synthesizing
5 viral RNAs. However, the molecular mechanisms underlying this process are poorly
6 understood. In this study, we used a model virus, *Red clover necrotic mosaic virus*
7 (RCNMV), whose replicase complex can be detected readily as the 480-kDa functional
8 protein complex. We found that host heat shock proteins Hsp70 and Hsp90 are required
9 for RCNMV RNA replication and that they interact with p27, a viral-encoded
10 component of the 480-kDa replicase complex, on the endoplasmic reticulum membrane.
11 Using a cell-free viral translation/replication system in combination with specific
12 inhibitors of Hsp70 and Hsp90, we found that inhibition of p27-Hsp70 interaction
13 inhibits the formation of 480-kDa complex but instead induces the accumulation of
14 large complexes that is nonfunctional in viral RNA synthesis. In contrast, inhibition of
15 p27-Hsp90 interaction did not induce such large complexes, but rendered p27 incapable
16 of binding to a specific viral RNA element, which is a critical step for the assembly of
17 the 480-kDa replicase complex and viral RNA replication. Together, our results suggest
18 that Hsp70 and Hsp90 regulate different steps in the assembly of the RCNMV replicase
19 complex.

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1 INTRODUCTION

2

3 Most plant and animal viruses are positive-strand RNA viruses, which have
4 single-stranded messenger-sense genomic RNAs. These viruses often induce host
5 membrane rearrangements to form organelle-like compartments in which viral genomic
6 RNAs are replicated via negative-strand RNA intermediates by the viral replicase
7 complexes (10). Viral replicase complexes comprise multiple proteins, including viral
8 auxiliary proteins, viral RNA-dependent RNA polymerase (RdRP), and host proteins
9 (61). Viral replicase complexes have been studied extensively by characterizing their
10 RdRP activities and the functions of the viral and host components of the complexes.
11 These studies have provided important information about the mechanisms regulating
12 genome replication (15, 19, 47, 90), viral pathogenicity (68, 69), and virus–host
13 interactions (24, 25, 32, 33). However, an important question remains: how do multiple
14 viral and host components assemble properly into the replicase complex?

15 Molecular chaperones are essential for cell viability by ensuring folding of newly
16 synthesized proteins, refolding of misfolded or aggregated proteins, protein complex
17 assembly and disassembly, membrane translocation of organellar and secretory proteins,
18 protein degradation, and activities of regulatory proteins in signal transduction pathways
19 (12, 18, 51). In eukaryotic cells, the abundant and highly conserved molecular
20 chaperones heat shock proteins Hsp70 and Hsp90 play central roles in the biological
21 processes mentioned above, and the activities of Hsp70 and Hsp90 are modulated by a
22 variety of co-chaperones (37, 80). Considering their pivotal roles in cells, it is not
23 surprising that Hsp70 and Hsp90 are involved together with their co-chaperones in virus
24 infection (62). For instance, Hsp70 facilitates the assembly and disassembly of viral
25 capsids (7, 26, 46), promotes the subcellular transport of tombusvirus replicase proteins
26 and affects the activity or assembly of tombusvirus replicase complexes (71, 91),

1 controls potyvirus gene expression in cooperation with its co-chaperone CPIP (17), and
2 positively and negatively affects the genome replication of various viruses (5, 45, 87,
3 92). Hsp90 affects the early stages of *Bamboo mosaic virus* (BaMV) infection by
4 binding to the genomic RNA (20), increases the synthesis or stability of viral proteins (4,
5 8), supports the assembly and nuclear import of influenza A virus RNA polymerase
6 complex (59, 63), and tightly regulates hepatitis C virus replication in cooperation with
7 FKBP8 and hB-ind1 co-chaperones (67, 79, 89).

8 Hsp70 and Hsp90 sometimes work together in the activation or maturation of viral
9 and cellular proteins. For example, Hsp90 together with Hsp70 and a variety of
10 co-chaperones regulate the actions of steroid receptors and the responses to ligands (16).
11 It has been reported recently that Hsp70, Hsp90, and their co-chaperones facilitate the
12 incorporation of small RNAs into Argonaute proteins, which play central roles in
13 post-transcriptional gene silencing (22, 23, 31, 55). In the case of hepadnavirus reverse
14 transcriptase, Hsp70 and Hsp40 co-chaperone are essential for the specific binding of
15 the reverse transcriptase to pre-genomic RNA templates, and Hsp90 facilitates this step
16 (77, 78). However, the coordinate functions of these molecular chaperones in other
17 biological processes such as multicomponent complex assembly are poorly understood.

18 To elucidate the molecular mechanisms of the replication of positive-strand RNA
19 viruses, we used *Red clover necrotic mosaic virus* (RCNMV) as a model. RCNMV is a
20 positive-strand RNA plant virus and a member of the genus *Dianthovirus* in the family
21 *Tombusviridae*. The bipartite genomic RNAs, RNA1 and RNA2, possess neither a cap
22 structure at the 5' end (58) nor a poly(A) tail at the 3' end (48, 96). Instead, RNA1 and
23 RNA2 use distinct cap/poly(A)-independent mechanisms to produce all viral proteins
24 (30, 57, 58, 74). RNA1 encodes N-terminally overlapping replicase component proteins,
25 a 27-kDa auxiliary protein (p27), and an 88-kDa protein with an RdRP motif (p88). p88
26 is produced via programmed -1 position ribosomal frameshifting (81, 95). RNA1 also

1 encodes a coat protein (CP), which is translated from CP subgenomic RNA (76, 86). A
2 small noncoding RNA (SR1f) with a potential function in regulating RCNMV infection
3 is generated from RNA1. RNA2 encodes a movement protein that is required for viral
4 movement in plants (34, 36, 94).

5 We have developed a model to study the assembly processes of viral replicase
6 complexes by detecting the RCNMV replicase complex as a functional protein complex
7 with an apparent molecular mass of 480 kDa using blue native polyacrylamide gel
8 electrophoresis (BN-PAGE) (53). The 480-kDa replicase complexes are tightly
9 associated with the membrane of the endoplasmic reticulum (ER) and retain the ability
10 to synthesize complementary RNAs by specifically recognizing RCNMV RNAs (41,
11 53). The assembly of the 480-kDa replicase complex requires both p27–p27 and
12 p27–p88 interactions (52). In addition to p27 and p88, the 480-kDa complexes contain
13 several host proteins, whereas p27 and p88 seem to interact with both the host
14 components of the 480-kDa complex and many other host proteins including
15 chaperones, ribosomal proteins and cytoskeletal proteins, which are absent in the
16 complex (53).

17 The functions of p27 and p88 in viral RNA replication have been investigated. Both
18 p27 and p88 interact with RNA1 in a translation-coupled manner (27), and these
19 interactions are important for the *cis*-preferential replication of RNA1 (66). p27 but not
20 p88 binds specifically to the Y-shaped RNA element (YRE) of RNA2 in *trans* (27), and
21 this interaction is essential for the recruitment of RNA2 into replication (1, 21). By
22 contrast, the functions of host proteins in RCNMV RNA replication are currently
23 unknown.

24 In this study, we investigated the functions of two host molecular chaperones,
25 Hsp70 and Hsp90, in RCNMV RNA replication. Gene silencing and pharmacological
26 inhibition of Hsp70 and Hsp90 revealed that these molecular chaperones are required

1 for RCNMV RNA replication. A series of *in vivo* and *in vitro* protein interaction
2 experiments showed that both Hsp70 and Hsp90 interact with p27 via protein–protein
3 contacts on the ER membrane. Further studies using a cell-free viral
4 translation/replication system showed that when p27-Hsp70 interaction is blocked, p27
5 form large complexes that are nonfunctional in viral RNA synthesis. In contrast, in the
6 absence of p27-Hsp90 interaction, p27 was unable to bind to a viral RNA element, such
7 as YRE, which is a critical step for the assembly of the 480-kDa replicase complexes.
8 These results provide strong evidence that Hsp70 and Hsp90 have different functions in
9 regulating the assembly of the RCNMV replicase complex.

10

11 **MATERIALS AND METHODS**

12 **Molecular cloning and plasmid construction.** pUCR1 (84) and pRC2|G (97) are
13 full-length cDNA clones of RNA1 and RNA2 of RCNMV Australian strain,
14 respectively. pBI_C-CY, pBI_C-NY, pBI_N-NY and pBI_N-CY were kind gifts from
15 Dr. Takashi Araki (Kyoto University, Kyoto, Japan). Constructs described previously
16 that used in this study include pBICp35 (83), pBICp19 (83), pBICB3aGFP (35),
17 pBICRM_sG (36), pBICER:mCherry (34), pBICRC1 (84), pUBRC1 (84), pBICRC2
18 (84), pUBRC2 (84), pUBp35 (80), pUBp88 (81), pBICp27 (84), pBICp88 (84),
19 pBICp27-FLAG (53), pBICp27-HA (51), pUCp27-FLAG (52), pUCp88-T7 (52),
20 pBYL2 (52), pBINTRA6 (73), pTV00 (73), pPVX.NbHsp70c-1 (38) and
21 pBE2113-GUS (54). pET42a and pUC118 were purchased from TAKARA Bio Inc.
22 (Shiga, Japan). *Escherichia coli* DH5 α was used for the construction of all plasmids,
23 except that *E. Coli* Top10 (Invitrogen, Carlsbad, CA) was used for the construction of
24 pBYLHsp70. All plasmids constructed in this study were verified by sequencing.

25 **(i) pBYLHsp70 and pBYLHsp90.** To isolate Tobacco BY-2 *Hsp70* and *Hsp90*
26 cDNA fragments by reverse transcription-polymerase chain reaction (RT-PCR), we

1 used two sets of degenerate primers, Hsp70deg-F
2 (5'-AARAAYCARGTNGCNATGAA-3') plus Hsp70deg-R
3 (5'-CATNCGYTCDATYTCYTCYTT-3'), and Hsp90deg-F
4 (5'-AAGGCGCGCCATGGCGGABRCAGAGACGTTT-3') plus Hsp90deg-R
5 (5'-AAGGCGCGCCTTAGTCAACYTCCTCCATCTT-3'), respectively. Both 5'-rapid
6 amplification of cDNA ends (5'-RACE) and 3'-RACE techniques were carried out to
7 identify the both ends. Full-length *Hsp70* and *Hsp90* cDNAs were amplified with
8 RT-PCR using the primer pair sets, *AscI*/Hsp70-F
9 (5'-AACCGGTTGGCGCGCCATGGCAGGAAAAGGTGAAGG-3') plus
10 *AscI*/Hsp70-R (5'-AACCGGTTGGCGCGCCAACACCAACAGCTTAGTC-3'), and
11 *AscI*/Hsp90-F (5'-AAGGCGCGCCATGGCGGACACAGAGACGTTTGC-3') plus
12 *AscI*/Hsp90-R (5'-AAGGCGCGCCTTAGTCAACCTCCTCCATCTTGC3'),
13 respectively. The amplified DNAs were digested with *AscI* and inserted into pBYL2
14 that had been cut with the same restriction enzyme.

15 **(ii) pTVHsp70.** The partial fragment of *NbHsp70c-1* was amplified from
16 pPVX.NbHsp70c-1 (38) using *SmaI*/70-F
17 (5'-GGGGGGCCCCGGGTAACGAGAAGGTGCAGG-3') and *BamHI*/70-R
18 (5'-CGCGGATCCATTGGCGTCGATGTCAAAG-3'). The amplified DNA was
19 digested with *SmaI* and *BamHI*, and inserted into pTV00 that had been cut with the
20 same restriction enzymes.

21 **(iii) pBICAscII.** An *AscI* linker (5'-GATCTGGCGCGCC-3') was treated with T4
22 polynucleotide kinase (New England Biolabs, Ipswich, MA), followed by annealing.
23 The annealed linker was then inserted into pBICp35, which had been digested with
24 *BamHI*.

25 **(iv) pBICHsp70.** The *Hsp70* sequence was amplified from pBYLHsp70 using
26 *BamHI*/Hsp70-F

1 (5'-ACGGGGATCCAAGGAGATATAACAATGGCAGGAAAAGGTGAAGGA-3')
2 and *Bam*HI-Hsp70-R (5'-ACGGGGATCCTTAGTCGACCCTCAATC-3'), digested
3 with *Bam*HI and inserted into pBICp35 that had been cut with the same restriction
4 enzyme.

5 (v) **pBICHsp90.** pBYLHsp90 was cut with *Asc*I. Then, the resulting 2.1 kilobase
6 fragment was ligated with *Asc*I-digested pBICAscII.

7 (vi) **pBICHA:cYFP and pBICmyc:nYFP.** The sequence of HA-tagged cYFP or
8 myc-tagged nYFP was amplified from pBICp27-HA:cYFP using *Stu*I-HA/cYFP-F
9 (5'-GAGAGGCCTACGGGGATCCAAGGAGATATAACAATGTACCCATACGATG
10 TTCC-3') and *Kpn*I-HA/cYFP-R, or from pBICp27-myc:nYFP using *Stu*I-myc/nYFP-F
11 (5'-GGAGAGGCCTACGGGGATCCAAGGAGATATAACAATGGAGCAGAAGCT
12 GATCAGC-3') and *Kpn*I-myc/nYFP-R, respectively. The amplified DNA fragments
13 were digested with *Stu*I and *Kpn*I, and inserted into *Stu*I/*Kpn*I-digested pBICp35 to
14 construct pBICHA:cYFP and pBICmyc:nYFP.

15 (vii) **pBICHA:cYFP-Hsp70 and pBICHA:cYFP-Hsp90.** The sequence of
16 HA-tagged cYFP was amplified from pBICHA-cYFP using *Stu*I-HA/cYFP-F and
17 *Stu*I-HA/cYFP-R (5'-GTAGGCCTCTTGTACAGCTCGTCCATGCCGAG-3'). The
18 amplified DNA was digested with *Stu*I, and cloned into *Stu*I-digested pBICHsp70 and
19 pBICHsp90 to construct pBICHA-cYFP:Hsp70 and pBIC HA-cYFP:Hsp90.

20 (viii) **pBICmyc:nYFP-Hsp70 and pBICmyc:nYFP-Hsp90.** The sequence of
21 myc-tagged nYFP was amplified from pBICmyc-nYFP using *Stu*I-myc/nYFP-F and
22 *Stu*I-myc/nYFP-R (5'-GTAGGCCTGGCCATGATATAGACGTTGTGG-3'),
23 respectively. The amplified DNA was digested with *Stu*I, and cloned into *Stu*I-digested
24 pBICHsp70 to construct pBICHA-cYFP:Hsp70 and pBICmyc-nYFP:Hsp70.

25 (ix) **pBICp27-HA:cYFP.** The sequence of C-terminal half of YFP was amplified
26 from pBI_C-CY using HA/cYFP-F

1 (5'-TACCCATACGATGTTCTTACTTGTACAGCTCGTCCATG-3') and
2 *KpnI*-HA/cYFP-R (5'-AGCGGGGTACCTTACTTGTACAGCTCGTCCATG-3'). A
3 PCR fragment was then amplified from pBICp27 using p27-22R
4 (5'-AGCAGATGGAACGTGTAG-3') and HA/p27-R
5 (5'-AGCGTAATCTGGAACATCGTATGGGTAAAAATCCTCAAGGGATTTGA-3').
6 Then, a recombinant PCR product was amplified from the mixture of these fragments
7 using p27-22R and *KpnI*-HA/cYFP-R, digested with *EcoRI* and *KpnI*, and inserted into
8 the corresponding region of pBICp27.

9 **(x) pBICp27-myc:nYFP.** The sequence of N-terminal half of YFP was amplified
10 from pBI_C-NY using myc/nYFP-F
11 (5'-GAGCAGAAGCTGATCAGCGAGGAGGACCTGGCCGGTGGTGGAGGAGCC
12 GGC-3') and *KpnI*-myc/nYFP-R
13 (5'-AGCGGGGTACCTTAGGCCATGATATAGACGTTG-3'). A PCR fragment was
14 then amplified from pBICp27 using p27-22R and myc/p27-R
15 (5'-CAGGTCCTCCTCGCTGATCAGCTTCTGCTCAAATCCTCAAGGGATTTGA
16 -3'). Then, a recombinant PCR product was amplified from the mixture of these
17 fragments using p27-22R and *KpnI*-myc/nYFP-R, digested with *EcoRI* and *KpnI*, and
18 inserted into the corresponding region of pBICp27.

19 **(xi) pBICp88-HA:cYFP.** The sequence of C-terminal half of YFP was amplified
20 from pBI_C-CY using HA/cYFP-F and *KpnI*/HA/cYFP-R, and a PCR fragment was
21 amplified from pBICp88 using p88-167R (5'-AGTGCGAGCTTCGTTGG-3') and
22 HA/p88-R
23 (5'-AGCGTAATCTGGAACATCGTATGGGTATCGGGCTTTGATTAGATCTTTG-3
24 '). Then, a recombinant PCR product was amplified from the mixture of these fragments
25 using p88-167R and *KpnI*-HA/cYFP-R, digested with *XhoI* and *KpnI*, and inserted into
26 the corresponding region of pBICp88.

1 **(xii) pBICp88-myc:nYFP.** The sequence of N-terminal half of YFP was amplified
2 from pBI_C-NY using myc:nYFP-F and *KpnI*-myc/nYFP-R, and a PCR fragment was
3 amplified from pBICp88 using p88-167R and myc/p88-R
4 (5'-CAGGTCCTCCTCGCTGATCAGCTTCTGCTCTCGGGCTTTGATTAGATCTTT
5 G-3'). Then, a recombinant PCR product was amplified from the mixture of these
6 fragments using p88-167R and *KpnI*-myc/nYFP-R, digested with *XhoI* and *KpnI*, and
7 inserted into the corresponding region of pBICp88.

8 **(xiii) pBICsGFP-Hsp70 and pBICsGFP-Hsp90.** The GFP sequence was
9 amplified from pBICRM_sG using *StuI*-sGFP-F
10 (5'-GGAGAGGCCTACGGGGATCCAAGGAGATATAACAATGGTGAGCAAGGG
11 CGAGGAGCTG-3') and *StuI*-sGFP-R
12 (5'-CCGTAGGCCTCTTGTACAGCTCGTCCATG-3'), digested with *StuI* and inserted
13 into *StuI*-digested pBICHsp70 and pBICHsp90 to construct pBICsGFP-Hsp70 and
14 pBICsGFP-Hsp90, respectively.

15 **(xiv) pBICDRm-p27.** A PCR fragment from pDsRed-monomer-Actin was
16 amplified using Bam/DRm-F
17 (5'-GGGGATCCGGATGGACAACACCGAGGACGTCATC-3') and p27/DRm-R
18 (5'-ATTTATAAAACCCATGCCCCCTGGGAGCCGGAGTGGCGG-3'), and a PCR
19 fragment from pUCR1 was amplified using DRm/p27-F
20 (5'-CACTCCGGCTCCCAGGGGGGCATGGGTTTTATAAATCTTT-3') and
21 *KpnI*/p27-R (5'-GGGGTACCCTAAAAATCCTCAAGGGATTT-3'). Then, a
22 recombinant PCR product was amplified from the mixture of these two fragments by
23 the use of Bam/DRm-F and *KpnI*/p27-R, digested with *Bam*HI and *KpnI*, and inserted
24 into the corresponding region of pBICp35.

25 **(xv) pBICp27-DRm.** A PCR fragment from pBICp27 was amplified using
26 p27-47R (5'-AGATGACATGGGAAAGG-3') and DRm-p27-R

1 (5'-CCATGCCCCCAAATCCTCAAGGGATTTGA-3'), and a PCR fragment from
2 pBICER-DRm (35) was amplified using p27-DRm-F (5'-
3 TTTTGGGGGCATGGACAACACCGAGGACGT-3') and *KpnI*-DRm-R
4 (5'-CGGGGTACCCTACTGGGAGCCGGAGTGGCGGG-3'). Then, a recombinant
5 PCR product was amplified from the mixture of these two fragments by the use of
6 p27-47R and *KpnI*-DRm-R, digested with *EcoRI* and *KpnI*, and inserted into the
7 corresponding region of pBICp27.

8 **(xvi) pUEGFP2.** pBE2113-GUS (54) was cut with *SacI*, treated with T4 DNA
9 polymerase, cut with *SmaI*, and self-ligated to eliminate GUS gene. The small
10 *HindIII/EcoRI* fragment of the resultant plasmid was inserted into the *HindIII/EcoRI*
11 site of pUC118 (Takara Bio), creating pUC2113. A GFP gene was PCR-amplified from
12 pBICB3aGFP (35) using the *XbaI*/5LGFP5' primer
13 (5'-GGTGGCTCTAGAAAGGAGATATAACAATGAGTAAAGGAGAAGAAGT-3')
14 and the *Bam*/GFP3' primer (5'-GGGGGGATCCTTATTTGTATAGTTCATCC-3').
15 The amplified fragment was cut with *XbaI* and *Bam*HI and inserted into the
16 *XbaI/Bam*HI site of pUC2113, creating pUEGFP2.

17 **(xvii) pUBp27-HA.** A PCR fragment was amplified from pBICp27-HA using
18 *Bam*HI-p27-F
19 (5'-GGAGAGGCCTACTCTAGAGGATCCGGATGGGTTTTATAAATCTT-3') and
20 *KpnI*-p27-HA-R
21 (5'-TTCAGCGGGGTACCCTAAGCGTAATCTGGAACATCGTATGGGTA-3'),
22 digested with *Bam*HI and *KpnI*, and inserted into corresponding region of pUBP35.

23 **(xviii) pUBp88-HA.** The sequence of p88 was amplified from pUBp88 using
24 p88-*Xho*I-F (5'-CCTGTCGATGTACTCGAGAAGGTGGCGTTT-3') and p88-HA-R
25 (5'-TTAAGCGTAATCTGGAACATCGTATGGGTATCGGGCTTTGATTA-3'). And
26 a PCR fragment from pUBp88 was amplified using p88-HA-F

1 (5'-TACCCATACGATGTTCCAGATTACGCTTAAGGTACCCCGCTGAA -3') and
2 M13-rev (5'-CAGGAAACAGCTATGACCATG-3'). Then, a recombinant PCR product
3 was amplified from the mixture of these fragments using p88-*XhoI*-F and M13-rev,
4 digested with *XhoI* and *KpnI*, and inserted into the corresponding region of pUBp88.

5 ***In vitro* transcription.** The plasmids with the prefixes 'pUC' or 'pRC' were
6 digested with *SmaI*. The linearized plasmids were used as templates for *in vitro*
7 transcription by T7 RNA polymerase as described previously (58). If required, capped
8 transcripts were prepared using the ScriptCap m⁷G capping system according to the
9 manufacturer's instruction (Epicentre Biotechnologies, Madison, WI).

10 **Inhibitor treatments.** 2-phenylethynylsulfonamide (PES), Methanesulfonamide
11 (MS), geldanamycin (GDA) and MG132 were purchased from EMD Biochemicals, Inc.
12 (Gibbstown, NJ), Wako (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and Merck
13 (Darmstadt, Germany), respectively. The inhibitors were diluted from stock solutions in
14 dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For the control experiments, an
15 equivalent concentration of DMSO was applied.

16 **Protoplast experiments.** Protoplasts were prepared from tobacco BY-2 suspension
17 cultured cell as described previously (84). BY-2 protoplasts ($\sim 3 \times 10^5$) were inoculated
18 with *in vitro*-transcribed RNA1 and RNA2 (1 μ g each), or with RCNMV virion (2 μ g),
19 or with pUBRC1 and pUBRC2 (5 μ g each), or with pUBp27-HA, pUBp88-HA, and *in*
20 *vitro*-transcribed RNA2 (10 μ g, 5 μ g, and 500 ng, respectively) as described previously
21 (21). The inoculated protoplasts were incubated at 17 °C for 16 h in the presence of PES,
22 MS, or GDA. Viral RNAs were detected by northern blotting.

23 ***Agrobacterium* infiltration.** The plasmids containing the prefixes 'pBIC' and
24 'pTV' were introduced by electroporation into *Agrobacterium tumefaciens* GV3101
25 (pMP90) and *A. tumefaciens* GV3101 (pSoup), respectively. *Agrobacterium*
26 suspensions were mixed at a final OD₆₀₀ of 0.2 each for Bimolecular fluorescence

1 complementation (BiFC) experiments, OD₆₀₀ of 0.4 each for subcellular localization
2 assays, and OD₆₀₀ of 0.5 for gene silencing. *Agrobacterium* suspensions harboring an
3 empty vector, pBICp35, were used as the filler. The mixtures were infiltrated into *N.*
4 *benthamiana* leaves essentially as described by Takeda *et al.* (84).

5 **Silencing of *Hsp70* and *Hsp90* in *N. benthamiana* plants.** Appropriate
6 combinations of silencing vectors were expressed by *Agrobacterium* infiltration in 3- to
7 4-weeks-old *N. benthamiana* plants as described previously (73). At 10 days post
8 agroinfiltration (dai), the leaves above the agroinfiltrated leaves were inoculated with *in*
9 *vitro* transcribed RNA1 and RNA2. The inoculated plants were incubated at 22 °C for 2
10 days. Total RNAs were extracted using TRIzol reagent (Invitrogen), treated with RQ1
11 RNase-free DNase (Promega, Madison, WI), purified by phenol/chloroform and
12 chloroform extractions and precipitated with ethanol. Viral RNAs were detected by
13 northern blotting. The mRNA levels of *NbHsp70c-1* (6, 38) were examined by
14 semiquantitative RT-PCR using Nb70c-F
15 (5'-CTAGAATCCCAAAGGTGCAACAGC-3') and Nb70c-R
16 (5'-CTTCTCATCTTTCACAGTGTTCTC-3'). The mRNA levels of *NbHsp90* were
17 examined as described previously (85). As a control to show the equal amounts of
18 complementary DNA templates in each reaction, ribulose 1,5-biphosphate carboxylase
19 small subunit gene (*RbcS*), a constitutively expressed gene, was amplified by RT-PCR
20 using NbRbcS-F (5'-CCTCTGCAGTTGCCACC-3') and NbRbcS-R
21 (5'-CCTGTGGGTATGCCTTCTTC-3').

22 **BiFC and subcellular localization assays.** Appropriate combinations of Yellow
23 fluorescent protein (YFP) fragments-fused proteins or fluorescent protein-fused proteins
24 were expressed in *N. benthamiana* leaves by *Agrobacterium* infiltration. Fluorescence
25 of, YFP, green fluorescent protein (GFP), DsRed monomer (DRm), and mCherry were
26 visualized with confocal microscopy at 3 days post infiltration (dpi) for subcellular

1 localization assay and at 4 dpi for BiFC. Protein expression was confirmed by western
2 blotting. Accumulations of viral RNAs were analyzed by northern blotting.

3 **Confocal microscopy.** The fluorescence signals of GFP, YFP, DRm, and mCherry
4 were observed using an Olympus FluoView FV500 confocal microscope (Olympus
5 Optical Co., Tokyo, Japan) equipped with an argon laser, a He:Ne laser, and a 40× Plan
6 Apo oil immersion objective lens. The samples were excited with the argon laser for
7 GFP/YFP, and with the He:Ne laser for DsRed-monomer/mCherry. We used a dichroic
8 mirror, DM488/543, a beam splitter, SDM560, and two emission filters, BA505-525 for
9 GFP/YFP, and BA560IF for DsRed-monomer/mCherry. Scanning was performed in
10 sequential mode to minimize signal bleed-through. Images were processed using Adobe
11 Photoshop CS3 software.

12 **Glutathione S-transferase (GST) pull-down assays.** *E. coli* Rossetta 2 (DE3)
13 (Invitrogen) transformed by pET42a, pET42a-Hsp70 or pET42a-Hsp90 was grown
14 overnight at 37 °C in LB medium containing kanamycin (100 µg/ml). The overnight
15 cultures of the transformed *E. coli* were diluted to 1:50 in LB medium containing
16 kanamycin (100 µg/ml). After incubation for 2h at 37 °C, protein expression was
17 induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside. The cells
18 expressing GST, GST-Hsp70 or GST-Hsp90 were cultured at 28 °C for 1 h, 4h or 4h,
19 respectively. The induced cells were harvested by centrifugation at 5,000×g for 5 min.

20 Cells collected from 5 ml (pET-42a) and 10 ml (pET42a-Hsp70 and pET42a-Hsp90)
21 of medium were resuspended in 500 µl of phosphate buffer saline (140 mM NaCl, 2.7
22 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄), and sonicated on ice to disrupt the
23 cells. After sonication, Triton-X 100 was added at the final concentration of 0.5% and
24 centrifuged at 15,000×g for 10 min at 4°C. The supernatant was added to 12.5 µl bed
25 volume of equilibrated Glutathione Sepharose 4B (GE Healthcare, Little Chalfont,
26 Buckinghamshire, UK), and incubated at 4°C for 1 h with gentle rotation. The resin was

1 washed three times with 1 ml of binding buffer (50 mM Tris-HCl pH 7.4, 150 mM
2 NaCl, 10 mM 2-mercaptoethanol, and 0.5% Triton X-100). After washing, the resin was
3 incubated for 2 h at 4°C in 200 µl of binding buffer containing 500 ng of the purified
4 His-p27-FLAG (52). After incubation, the resin was washed three times with 500 µl of
5 binding buffer. The bound proteins were eluted by addition of Laemmli sample buffer
6 (42), followed by incubation for 3 min at 95°C. Protein samples were subjected to
7 SDS-PAGE and then blotted onto a PVDF membrane. The separated proteins were
8 analyzed by western blotting, and stained with Ponceau S.

9 **Cell-free *in vitro* translation and replication experiments.** The cell extracts of
10 evacuated BY-2 protoplasts (BYL) were prepared and the *in vitro*
11 translation/replication reactions were performed essentially as described previously (28,
12 40, 52, 53). BN-PAGE analysis was performed as described previously (53), except
13 that the total protein samples solubilized with 0.5% Triton X-100 were subjected to
14 BN-PAGE. StreptoTag affinity purification was performed essentially as described
15 previously (27). Immunopurification of p27-FLAG is described below.

16 **Immunopurification of p27-FLAG.** p27-FLAG was expressed in BYL by adding
17 an *in vitro* transcript to a concentration of 20 nM. After 2 h of incubation, BYL
18 expressing p27-FLAG was incubated on ice for 30 min with 500 µM of PES, MS or
19 GDA. At this time, ADPβS or ATPγS, which stabilizes the binding of Hsp70 or Hsp90
20 to substrate proteins, respectively, was also added to concentrations of 2 mM. Then, 10
21 µl bed volume of ANTI-FLAG M2-Agarose Affinity Gel (Sigma-Aldrich) was added to
22 the BYL and further incubated for 90 min with occasional mixing. The resin was
23 washed three times with 200 µl of TR buffer (40) supplemented with 500 mM NaCl and
24 0.1% Triton-X-100 for the analysis of p27-Hsp70 interaction, or with TR buffer
25 supplemented with 0.1% Triton X-100 for the analysis of p27-Hsp90 interaction. The
26 bound proteins were eluted by addition of Laemmli sample buffer, followed by

1 incubation for 3 min at 95°C. Protein samples were subjected to SDS-PAGE, followed
2 by western blotting with appropriate antibodies.

3 **Northern blot analysis.** Northern blot analysis was performed essentially as
4 previously described (28). The digoxigenin-labelled RNA probes specific for the 3'
5 untranslated regions (UTRs) of RCNMV RNA1 and RNA2, and the full-length
6 negative-strand RNA1 and RNA2 were described previously (56, 57). The RNA signals
7 were detected with a luminescent-image analyzer (LAS-1000 plus; Fuji Photo Film,
8 Japan), and the signal intensities were quantified using the NIH Image program.

9 **Western blot analysis.** Western blot analysis was performed essentially as
10 described previously (53). Protein samples were subjected to SDS-PAGE or BN-PAGE
11 and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P,
12 Millipore, Bedford, MA). Anti-p27 rabbit polyclonal antibody (84), anti-NtHsp90 rabbit
13 polyclonal antibody (82), anti-Hsp70/Hsc70 mouse monoclonal antibody (Stressgen,
14 Victoria, BC, Canada), anti-c-myc mouse polyclonal antibody (Santa Cruz
15 Biotechnology Inc., Santa Cruz, CA), anti-HA rat monoclonal antibody (Roche
16 Diagnostics, Penzberg, Germany), and anti-FLAG M2 mouse monoclonal antibody
17 (Sigma-Aldrich) were used as the primary antibodies. Alkaline phosphatase
18 (AP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Beverley, MA),
19 AP-conjugated anti-mouse IgG antibody (KPL, Gaithersburg, MD), AP-conjugated
20 anti-rat IgG antibody (Santa Cruz Biotechnology Inc.), horseradish peroxidase
21 (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology), and
22 HRP-conjugated anti-mouse IgG antibody (KPL) were used as the secondary antibodies.
23 To detect p88-T7, AP-conjugated anti-T7 monoclonal antibody (Merck) was used.
24 Signals were detected using a luminescent-image analyzer (LAS-1000 plus), and the
25 signal intensities were quantified using the NIH Image program.

26 **Accession numbers.** The GenBank accession numbers for the *Nicotiana tabacum*

1 Hsp70 and Hsp90 cDNA sequences reported in this paper are AB689673 and
2 AB689674, respectively.

3

4

1 RESULTS

2 **Hsp70 and Hsp90 are host factors required for RCNMV RNA replication.** In a
3 previous study, we identified many host proteins that were co-purified with RCNMV
4 replicase complexes from virus-infected *Nicotiana benthamiana* tissues. These included
5 Hsp70- and Hsp90-related proteins (53). This led us to investigate whether Hsp70 and
6 Hsp90 are involved in RCNMV infection. We applied *Tobacco rattle virus*
7 (TRV)-based gene silencing to downregulate cytosolic *Hsp70* and *Hsp90* in *N.*
8 *benthamiana* plants. The TRV vectors harboring the partial fragment of *Hsp70*
9 (TRV:Hsp70) (38) or *Hsp90* (TRV:Hsp90) (85) were expressed by
10 *Agrobacterium*-mediated expression. As a control, the empty TRV vector (TRV:00)
11 (73) was expressed. The newly developed leaves were inoculated with *in vitro*
12 transcribed RCNMV RNA1 and RNA2 at 10dai. Note that any morphological defects
13 such as chlorotic and stunted phenotypes were not observed at this stage (Fig.1A),
14 although such phenotypes became visible after 16 dpi (data not shown). Total RNA was
15 extracted from the inoculated leaves two days after inoculation (at 12 dpi).
16 Semiquantitative RT-PCR analysis confirmed the specific reduction of *Hsp70* and
17 *Hsp90* mRNAs in plants infiltrated with TRV:Hsp70 and TRV:Hsp90, respectively (Fig.
18 1A and B, lower panels). Importantly, the accumulation of RCNMV RNAs was
19 impaired in *Hsp70*- and *Hsp90*-silenced plants as assessed by northern blotting (Fig. 1A
20 and B, upper panels). These results suggest that Hsp70 and Hsp90 play positive roles
21 during RCNMV infection.

22 To test whether Hsp70 and Hsp90 function in RCNMV RNA replication in a single
23 cell, we treated tobacco BY-2 protoplasts with specific inhibitors of Hsp70 or Hsp90,
24 PES or GDA, respectively. These inhibitors have been used successfully to analyze the
25 functions of Hsp70 and Hsp90 in plant and animal systems (22, 23, 31, 43, 44, 82). MS,
26 an analogue of PES, was used as a negative control for PES. Protoplasts inoculated with

1 *in vitro* transcribed RNA1 and RNA2 were incubated in the presence of various
2 amounts of the inhibitors. Northern blot analysis showed that PES had an inhibitory
3 effect on the accumulation of RCNMV RNAs; 40 μ M of PES inhibited viral RNA
4 accumulation by about 95%, but MS had no effect (Fig. 2A and B). GDA had little
5 effect on the accumulation of RCNMV RNAs (data not shown). However, GDA
6 inhibited viral RNA accumulation when protoplasts were inoculated with the plasmids
7 that transcribe RCNMV RNAs under the control of *Cauliflower mosaic virus* 35S
8 promoter: 50 μ M of GDA inhibited the accumulation of RCNMV RNAs by about 70%
9 (Fig. 2C and D). Importantly, GDA did not reduce the accumulation of GFP mRNAs
10 (Fig. 2C), indicating that the reduction of viral RNA accumulations was not caused by
11 the inhibitory effect of GDA on CaMV 35S promoter-driven transcription under the
12 assay condition. The inhibitory effect of GDA on viral RNA accumulations was also
13 observed in protoplasts inoculated with RCNMV virionS (Fig. 2E and F). Overall, these
14 data suggest that both Hsp70 and Hsp90 are required for RCNMV RNA replication in a
15 single cell.

16 **Hsp70 and Hsp90 interact with p27 within the virus-induced aggregated**
17 **structures of the ER in the context of viral RNA replication.** Because Hsp70 and
18 Hsp90 are well-known protein chaperones, these chaperones might bind directly or
19 indirectly to p27 or p88 or both. To characterize these interactions in living cells, we
20 used a BiFC assay . We isolated cDNAs encoding Hsp70 and Hsp90 were isolated from
21 tobacco BY-2 cultured cells as described in Materials and Methods. We first tested
22 whether p27 interacts with Hsp70 and Hsp90. p27 fused to the N- or C-terminal half of
23 YFP at the C-terminus was expressed together with Hsp70 or Hsp90 fused to the other
24 halves of YFP at the N-terminus (e.g., p27-nYFP plus cYFP-Hsp70 or cYFP-Hsp90,
25 p27-cYFP plus nYFP-Hsp70 or nYFP-Hsp90) in the presence of p88, RNA2, mCherry
26 containing an ER targeting signal (ER-mCherry) (34), and the silencing suppressor p19

1 of *Tomato bushy stunt virus* (TBSV) in *N. benthamiana* by *Agrobacterium* infiltration.
2 At 4dai, large aggregated fluorescent structures of YFP were detected (Fig. 3A and B,
3 left panels). This YFP fluorescence was merged with the large aggregated fluorescent
4 structures of ER-mCherry (Fig. 3A and B, middle and right panels), a characteristic
5 feature of morphological changes of ER induced by RCNMV infection (88)
6 (Kusumawanegara *et al.*, in press). Little or no YFP fluorescence was detected in
7 control experiments, in which YFP fragments, p27, Hsp70, or Hsp90 were expressed
8 separately instead of their fusion protein counterparts (Fig. 3C and D). Western blot
9 analysis confirmed the accumulation of YFP fragment-fused proteins in
10 *Agrobacterium*-infiltrated leaves (Fig. 3E and F). Northern blot analysis showed that
11 p27-cYFP but not p27-nYFP supported the accumulation of positive- and
12 negative-strand RNA2 (Fig. 3E and F), indicating that p27-cYFP participated in the
13 replication of RNA2 under the assay conditions. Together, these results suggest that p27
14 interacts with Hsp70 and Hsp90 *in planta* in the absence and presence of viral RNA
15 replication.

16 We also used BiFC to test whether p88 interacts with Hsp70 and Hsp90, but we
17 failed to observe these interactions. Appropriate expression of C-terminally YFP
18 fragment-fused p88 in combination with the other YFP fragment-fused Hsp70 or Hsp90
19 showed no or little YFP fluorescence (data not shown). We note that both p88
20 derivatives were functional in supporting the replication of RNA2, although
21 C-terminally cYFP-fused p88 did not accumulate to detectable levels (data not shown).
22 Thus, it appears that the interactions of p88 with Hsp70 and Hsp90 do not occur, or, if
23 they occur, the interactions are too weak to be detected by BiFC.

24 Because the reconstitution of YFP is irreversible (49), the above BiFC experiments
25 did not rule out the possibility that the reconstitution of YFP occurred only in the
26 cytoplasm, and that the YFP signals detected in the aggregated ER structures reflected

1 artificial tethering of Hsp70 and Hsp90 to p27 localized to the ER. To check whether
2 Hsp70 and Hsp90 co-localize with p27 without the artificial tethering, we first
3 examined the subcellular localization of Hsp70 and Hsp90 in the absence of RCNMV
4 infection by expressing GFP-fused Hsp70 (GFP-Hsp70) or Hsp90 (GFP-Hsp90)
5 together with ER-mCherry in *N. benthamiana*. At 3dai, fluorescence of GFP-Hsp70 and
6 GFP-Hsp90 was observed in the nucleus and cytoplasm (Fig. 4A and C, left panels).
7 Fluorescence of ER-mCherry showed an ER distribution that included the nuclear
8 envelope (Fig. 4A and C, middle panels). The fluorescence of GFP-Hsp70 and
9 GFP-Hsp90 was merged only partially with the fluorescence of ER-mCherry (Fig. 4A
10 and C, right panels), indicating that Hsp70 and Hsp90 hardly localized to the ER in the
11 absence of RCNMV infection. However, when co-expressed with C-terminally
12 DRm-fused p27 (p27-DRm), p88, and RNA2, the large aggregated fluorescence of
13 GFP-Hsp70 and GFP-Hsp90 was detected in addition to the cytoplasmic and nuclear
14 fluorescence (Fig. 4B and D, left panels), and it merged with the fluorescent aggregates
15 of p27-DRm (Fig. 4B and D, middle and right panels). These results show that
16 GFP-Hsp70 and GFP-Hsp90 co-localized with p27-DRm in the large aggregated
17 structures of the ER. We note that the p27-DRm was functional in supporting the
18 replication of RNA2 (data not shown). Together, these data suggest that Hsp70 and
19 Hsp90 are recruited by p27 to the large aggregate structures of the ER in the context of
20 viral RNA replication.

21 We used an *in vitro* GST pull-down assay to characterize further the interactions of
22 p27 with Hsp70 and Hsp90. We purified p27 with an N-terminal His-tag and a
23 C-terminal FLAG-tag (His-p27-FLAG), as described previously (52). N-terminally
24 GST-fused Hsp70 (GST-Hsp70) or Hsp90 (GST-Hsp90) captured on glutathione-bound
25 beads was incubated with purified His-p27-FLAG. After extensive washing, the bound
26 proteins were analyzed by western blotting with an anti-FLAG antibody.

1 His-p27-FLAG was pulled down by both GST-Hsp70 and GST-Hsp90, but not by GST
2 alone, which was used as a negative control (Fig. 5A). Thus, like other substrate
3 proteins, Hsp70 and Hsp90 bind to p27 via protein–protein contacts.

4 **Hsp70 and Hsp90 promote the assembly of the 480-kDa replicase complex of**
5 **RCNMV by binding to p27.** Because PES and GDA block interactions of Hsp70 and
6 Hsp90 with their client proteins, respectively (23, 31, 43, 44), we hypothesized that
7 these chemicals inhibit RCNMV RNA replication by blocking the interactions of Hsp70
8 and Hsp90 with p27. To test this, we used BYL, an *in vitro* translation/replication
9 system (40). BYL has been used successfully to recapitulate the RNA replication
10 processes of RCNMV (1, 21, 27-29, 52, 53, 57, 74, 81). We first tested the effects of
11 PES and GDA on the interactions of p27 with Hsp70 and Hsp90, respectively. BYL
12 expressing C-terminally FLAG-tagged p27 (p27-FLAG), which is functional in
13 RCNMV RNA replication (53), was subjected to immunopurification with anti-FLAG
14 antibodies in the presence of inhibitors. Western blot analysis showed that PES and
15 GDA blocked the co-purification of endogenous Hsp70 and Hsp90 with p27-FLAG,
16 respectively (Fig. 5B and C). These results confirmed the inhibitory effects of PES and
17 GDA on the interactions of p27 with Hsp70 and Hsp90, respectively.

18 Next, we tested the effects of PES and GDA on the negative-strand RNA synthesis
19 of RCNMV RNAs. Both chemicals inhibited the accumulation of p27 and
20 negative-strand RNAs in BYL (Fig. 6), suggesting that Hsp70 and Hsp90 are required
21 for the translation/accumulation of p27 protein as well as the negative-strand RNA
22 synthesis. We then designed an assay using BYL to investigate the roles of Hsp70 and
23 Hsp90 in RCNMV RNA replication other than in protein translation and folding (Fig.
24 7A). This assay is based on the replication mechanism of RNA2 that exploits p27 and
25 p88 supplied *in trans* in contrast to the translation-coupled replication of RNA1 (27, 66).
26 This assay enabled us to monitor the assembly of the 480-kDa replicase complex and

1 the subsequent negative-strand synthesis of RNA2 after the completion of translation
2 and folding of p27 and p88 proteins. In this assay, p27-FLAG and C-terminally
3 T7-tagged p88 (p88-T7) were translated individually in BYL. These BYL were mixed
4 together following the addition of the protein synthesis inhibitor cycloheximide. RNA2
5 was then added with PES or GDA to the mixed BYL, followed by further incubation.
6 Northern blot analysis showed dose-dependent inhibition of the negative-strand RNA2
7 synthesis by PES and GDA (Fig. 7B, E and F). Western blot analysis on the protein
8 complexes separated by BN-PAGE revealed that PES and GDA inhibited the formation
9 of the 480-kDa replicase complex in a dose-dependent manner (Fig. 7B-D).
10 Interestingly, PES but not GDA increased the accumulation of large complexes (~1024
11 kDa) (Fig. 7B-D). MS had no effects on the accumulation of the 480-kDa complexes
12 and negative-strand RNA2 (Fig. 7B). PES affected the stability of p88-T7 (Fig. 7B-C).
13 However, this reduction in p88-T7 was not the major reason for the inhibitory effect of
14 PES on the negative-strand synthesis of RNA2 because more negative-strand RNA2
15 accumulated in the presence of MS than in the presence of PES even when the amount
16 of p88-T7 in MS-treated BYL was adjusted to be similar to or even less than that of
17 PES-treated BYL (Fig. 7G). Taken together, these results suggest that Hsp70 and Hsp90
18 facilitate the assembly of RCNMV replicase complex likely through interactions with
19 p27 and thereby promote subsequent negative-strand RNA synthesis.

20 **Hsp90 regulates the assembly of the 480-kDa replicase complex by promoting**
21 **the specific binding of p27 to YRE.** We have shown previously that the addition of
22 RNA2 increases the accumulation of the 480-kDa complexes in BYL (53) and that
23 mutations in p27 that compromise the binding activity of p27 to YRE of RNA2 affect
24 the assembly of the 480-kDa complexes in BYL (21). An aptamer-based pull-down
25 assay using p27 produced in BYL showed the interaction between p27 and YRE,
26 whereas no such interaction was observed using purified recombinant p27 produced in

1 *Escherichia coli* in an electrophoretic mobility shift assay (27), suggesting that an
2 unknown plant factor(s) is required for the binding of p27 to YRE. These findings led
3 us to hypothesize that the interaction between p27 and YRE is a key step in the
4 assembly of the 480-kDa replicase complex on RNA2 and that this step is regulated by
5 Hsp70 and/or Hsp90.

6 To test this hypothesis, we first used an RNA2 mutant (RNA2 LM8) that is unable
7 to interact with p27 (27) to evaluate its ability to support the assembly of the 480-kDa
8 complex. After addition of cycloheximide, BYL expressing p27-FLAG was mixed with
9 BYL expressing p88-T7, as illustrated in Fig. 7A. Wild-type RNA2 or RNA2 LM8 was
10 then added to the mixed BYL, followed by further incubation. Northern blot analysis
11 confirmed the requirement of p27-YRE interaction for the negative-strand synthesis of
12 RNA2 (Fig. 8A) (1, 21, 27). Western blot analysis in combination with BN-PAGE
13 showed that the accumulation of the 480-kDa complex was increased by the addition of
14 wild-type RNA2, whereas little increase in the accumulation of the 480-kDa complex
15 was observed when RNA2 LM8 was added (Fig. 8A). These results indicate that the
16 binding of p27 to YRE is one of the key steps for the assembly of the 480-kDa replicase
17 complex.

18 We next used a pull-down assay based on the high affinity of an RNA aptamer
19 termed ‘STagT’ with streptomycin to test the effects of PES and GDA on the interaction
20 between p27 and YRE (2, 9). This RNA-based pull-down assay in combination with the
21 membrane-depleted BYL (BYLS20), which can support negative-strand synthesis of
22 RCNMV RNAs, was used to analyze the interaction between p27 and YRE (21, 27).
23 STagT-fused YRE was incubated with BYLS20 expressing p27-FLAG in the presence
24 of the inhibitors. The p27-FLAG proteins interacting with STagT-fused YRE were
25 pulled down by streptomycin-conjugated beads. Similar amounts of bait RNAs were
26 purified and indicated by ethidium bromide staining. Interestingly, western blotting with

1 anti-FLAG antibodies showed that GDA but not PES inhibited the co-purification of
2 p27-FLAG with STagT-fused YRE (Fig. 8B). These results suggest that the interaction
3 of p27 with Hsp90, but not with Hsp70, is critical for the interaction between p27 and
4 YRE of RNA2, which then promotes the formation of the 480-kDa replicase complex.
5 These results also illustrate the differential contributions of Hsp70 and Hsp90 in the
6 assembly of the 480-kDa replicase complex.

7

8 **DISCUSSION**

9 In this study, we showed that Hsp70 and Hsp90 play critical roles in different steps
10 of the assembly of the RCNMV replicase complex likely through interactions with p27.
11 Inhibition of the p27–Hsp70 interaction resulted in the assembly of p27 into large
12 complexes that were nonfunctional in viral RNA synthesis. By contrast, inhibition of
13 p27–Hsp90 interaction lost the ability of p27 to bind to viral genomic RNA2, which is
14 critical for the assembly of the 480-kDa replicase complex. These findings reveal the
15 regulatory mechanism for the assembly of viral replicase complexes and suggest the
16 potential roles of Hsp70 and Hsp90 in controlling the assembly of ribonucleoprotein
17 complexes.

18 **Hsp70 regulates the correct assembly of the RCNMV replicase complex.** Hsp70
19 interacts with viral replicase proteins of various eukaryotic positive-strand RNA viruses
20 (5, 11, 64, 91). For example, Pogany *et al.* (71) successfully reconstituted the RdRP
21 activities of TBSV replicase complexes using viral replicase proteins, p33 and p92,
22 produced in *E. coli* with the membrane fractions of yeast extracts and purified yeast
23 Hsp70. They suggested that Hsp70 plays an integral role in the early replication process
24 of TBSV, including the assembly of TBSV replicase complexes.

25 In the present study, we analyzed the assembled RCNMV replicase complex itself
26 and found that inhibition of p27–Hsp70 interaction by PES causes the formation of

1 large aggregates containing p27 and reduces accumulation of the 480-kDa replicase
2 complex, which in turn impairs viral RNA synthesis (Fig. 7). These data lead us to
3 propose that one of the major roles of Hsp70 in viral RNA replication is to control the
4 proper assembly of viral replicase complexes.

5 How does Hsp70 regulate the assembly of the RCNMV replicase complex? Based
6 on the accumulation of nonfunctional large complexes upon the inhibition of
7 p27–Hsp70 interaction, it is possible that the Hsp70 prevents the aggregation of p27
8 protein and thereby facilitate the assembly of the RCNMV replicase complex. In fact, it
9 has been reported that Hsp70 family proteins promote the assembly of cellular
10 multiprotein complexes such as immunoglobulin G antibody and synaptic SNARE
11 complex by preventing aggregations of their component proteins (13, 75). Alternatively,
12 the large complexes could be intermediates for the assembly of the RCNMV replicase
13 complex and Hsp70 might assist the assembly process from these complexes to
14 functional replicase complexes. Such functions are reported for the chaperones
15 dedicated to the assembly of proteasome (60). Further studies are needed to gain
16 mechanistic insights into how Hsp70 regulates the assembly of RCNMV replicase
17 complex.

18 **Hsp90 regulates the key interaction between p27 and YRE, which is required**
19 **for the assembly of the RCNMV replicase complex.** Gene silencing in plants and
20 pharmacological inhibition in protoplasts showed that Hsp70 and Hsp90 are host factors
21 required for RCNMV replication (Figs. 1 and 2). Interestingly, however, an inhibitory
22 effect of GDA in protoplast experiments was observed when RCNMV RNAs were
23 supplied from plasmids or virions but not when *in vitro* synthesized RCNMV RNAs
24 were directly introduced (Fig. 2C-F and data not shown). These results suggest that
25 Hsp90 is required for plasmid or virion-dependent processes such as efficient
26 transcription and virion uncoating. An alternative, but not mutually exclusive

1 interpretation is that Hsp90 plays a role in a very early step in the RCNMV replication
2 process because time required for transcription from plasmids or virion uncoating must
3 delay the initiation of viral RNA replication. The latter interpretation was supported by
4 the finding that GDA inhibits two distinct steps in the early replication process of
5 RCNMV *in vitro* (Figs. 6-8). The first step is the accumulation of p27 protein (Fig. 5).
6 Inhibitory effect of GDA on p27 accumulation as well as viral RNA accumulation was
7 also observed when BY-2 protoplasts were inoculated with plasmids that express
8 hemagglutinin (HA)-tagged p27 and p88 together with RNA2 (data not shown). The
9 roles of Hsp90 in the accumulation of RCNMV replicase proteins are discussed later.
10 The second step is the binding of p27 to YRE of RNA2, which is a critical step in the
11 assembly of the 480-kDa replicase complex (Fig. 8). A similar mode of assembly was
12 proposed for TBSV replicase complexes: p33 auxiliary protein interacts with the
13 internal replication element (IRE) located in the coding region of p92 RdRP (72), and
14 this interaction is required for the RdRP activities of the TBSV replicase complexes in
15 yeast and its cell-free extracts (70-72). However, unlike p27, p33 can bind to the IRE
16 without other viral and host proteins (72). Thus, it appears that these two related viruses
17 have evolved different strategies for the interactions of viral replicase proteins with
18 replication templates and subsequent assembly of the viral replicase complexes.

19 Recently, Huang *et al.*, (20) showed that Hsp90 of *N. benthamiana* binds to the
20 3'UTR of BaMV genomic RNA and is required for the efficient accumulation of the
21 genomic RNA during the early stages of infection. This finding suggests a potential
22 function of Hsp90 in regulating viral RNA replication through interaction with viral
23 RNAs. However, it is unlikely that Hsp90 promotes the assembly of 480-kDa replicase
24 complex by binding to RCNMV RNA2, because we failed to detect any interactions
25 between Hsp90 and RNA2 in BYL based on StagT-aptamer pull-down assays (data not
26 shown). It is still possible that Hsp90 binds to RNA1 as well as p27 and/or p88, which

1 may facilitate translation and subsequent assembly of the replicase complex.
2 Interestingly, Lsm1-7 complex, which is involved in mRNA degradation in Processing
3 Bodies, is required for translation and replication of *Brome mosaic virus* (BMV)
4 genomic RNAs in yeast (3, 50, 65). Lsm1-7 complex binds directly to tRNA-like
5 structures and the intergenic region of BMV RNA3 (14).

6 **Roles of Hsp70 and Hsp90 in the translation/stability of RCNMV replicase**
7 **proteins.** PES and GDA decreased the accumulation of p27 in BYL when the inhibitors
8 were present throughout the experiments (Fig. 6). However, the deleterious effects were
9 not observed when these inhibitors were added after the addition of cycloheximide (Fig.
10 7B to D), suggesting that Hsp70 and Hsp90 are dispensable for the stability of p27 after
11 translation. It is likely that Hsp70 and Hsp90 promote the synthesis of p27 as seen for
12 the synthesis of flock house virus protein A (4, 92). Alternatively, it is possible that the
13 translating p27 is protected from degradation by a ribosome-associated chaperone
14 system, in which Hsp70 is thought to interact directly with nascent polypeptides
15 emerging from ribosomes (37, 93). Co-translational binding of Hsp70 and/or Hsp90
16 might facilitate the subsequent function of these chaperones during the assembly of the
17 RCNMV replicase complex.

18 By contrast, PES decreased the stability of p88 in BYL (Fig. 7B and D). The
19 proteasome inhibitor MG132 did not restore the stability of p88 (data not shown),
20 suggesting that Hsp70 might protect p88 from proteasome-independent degradation.
21 However, it is also possible that the effect of PES on the stability of p88 is an artifact of
22 the *in vitro* condition, in which p88 tends to form aggregates because of its
23 overexpression (53). This situation never occurs during the actual infection process of
24 RCNMV because p88 is translated by a programmed -1 ribosomal frameshifting at a
25 quite low frequency (39, 81) and because the translation and accumulation of p88 are
26 tightly coupled to viral RNA replication (27, 53, 66).

1 In conclusion, this study has demonstrated the multiple roles of Hsp70 and Hsp90 in
2 the early replication process of RCNMV. These chaperones promote the translation of
3 p27 and interact directly with p27. These interactions lead to the recruitment of Hsp70,
4 Hsp90, and RNA2 to the ER membrane (Figs. 3, 4 and 8) (21, 27), where Hsp70 and
5 Hsp90 regulate the assembly of the 480-kDa replicase complex (Figs. 7 and 8). Finally,
6 the properly assembled 480-kDa replicase complex initiates viral RNA replication via
7 complementary RNA synthesis (53).

8

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1 **FIGURE LEGENDS**

2 **Fig. 1.** Knock-down of Hsp70 or Hsp90 mRNA levels via gene silencing inhibits
3 accumulation of RCNMV RNAs in *N. benthamiana* plants. (A) The *Tobacco rattle*
4 *virus* (TRV) vector harboring the partial fragment of *N. benthamiana Hsp70c-1*
5 (TRV:Hsp70) or *Hsp90* (TRV:Hsp90) was expressed in *N. benthamiana* by
6 *Agrobacterium* infiltration. The empty TRV vector (TRV:00) was used as a control.
7 Pictures were taken at 10 days after agroinfiltration (dai). Note that the infiltrated plants
8 show no morphological defects at this stage. (B and C) The newly developed leaves
9 were inoculated with RCNMV RNA1 and RNA2 10 days after agroinfiltration. Total
10 RNAs were extracted from the inoculated leaves 2 days after inoculation. Accumulation
11 of RCNMV RNAs was analyzed by northern blotting. Ethidium bromide (EtBr)-stained
12 ribosomal RNAs (rRNA) are shown below the northern blots as loading controls. Hsp70
13 and Hsp90 mRNA levels were assessed by RT-PCR with primers that allow the
14 amplification of the regions of Hsp70 and Hsp90 not present in the TRV:Hsp70 and
15 TRV:Hsp90, respectively. RT-PCR results of the ribulose 1,5-biphosphate carboxylase
16 small subunit gene (*RbcS*) gene demonstrate equal amounts of total RNAs used for the
17 RT and the equal efficiency of RT-reaction in the samples.

18

19 **Fig. 2.** Inhibitors of Hsp70 or Hsp90 impair RCNMV RNA replication in a single cell.
20 (A and B) Tobacco BY-2 protoplasts were inoculated with *in vitro*-transcribed RNA1
21 and RNA2. The inoculated protoplasts were incubated for 16 h at 17 °C in the presence
22 of various concentrations of 2-phenylethynesulfonamide (PES), a specific inhibitor of
23 Hsp70. Methanesulfonamide (MS) is a non-functional analogue of PES. Total RNAs
24 were analyzed by northern blotting. EtBr-stained rRNAs as loading controls are shown
25 below the northern blots (A). The accumulation levels of RNA1 and RNA2 from three
26 separate experiments using PES were quantified using NIH Image and are plotted in the

1 graphs (B). (C and D) BY-2 protoplasts were inoculated with pUBRC1 and pUBRC2,
2 which transcribe RNA1 and RNA2, respectively, under the control of the *cauliflower*
3 *mosaic virus* (CaMV) 35S promoter. pUEGFP, which expresses GFP mRNA driven by
4 the CaMV 35S promoter, was also inoculated as an internal control. The inoculated
5 protoplasts were incubated for 16 h at 17 °C in the presence of various concentrations of
6 geldanamycin (GDA), a specific inhibitor of Hsp90. Total RNAs were analyzed by
7 northern blotting. EtBr-stained rRNAs are shown as loading controls (C). The
8 accumulation levels of RNA1 and RNA2 from three separate experiments were
9 quantified using NIH Image and are plotted in the graphs (D). (E and F) BY-2
10 protoplasts were inoculated with RCNMV virions, and incubated with GDA as
11 described above. Total RNAs were analyzed by northern blotting. EtBr-stained rRNAs
12 are shown as loading controls (E). The accumulation levels of RNA1 and RNA2 from
13 three separate experiments were quantified using NIH Image and are plotted in the
14 graphs (F).

15

16 **Fig. 3.** p27 interacts with Hsp70 and Hsp90 *in planta*. (A and B) Bimolecular
17 fluorescence complementation (BiFC) analysis of the interactions of p27 with Hsp70
18 and Hsp90. p27 fused to the N- or C-terminal half of YFP at the C-terminus (p27-nYFP
19 and p27-cYFP) was expressed together with Hsp70 or Hsp90 fused to the other half of
20 YFP at the N-terminus (cYFP-Hsp70, nYFP-Hsp70, cYFP-Hsp90, and nYFP-Hsp90) in
21 the presence of p88, RNA2, ER-mCherry, and the suppressor p19 of *Tomato bushy*
22 *stunt virus*, in *N. benthamiana* leaves by *Agrobacterium* infiltration. Right panels show
23 the merging of mCherry and YFP as yellow color. White arrowheads indicate the large
24 fluorescent aggregates. Bars, 20 μm. (C and D) Control experiments for the BiFC
25 analysis. YFP fragments and p27, Hsp70 or Hsp90 were separately expressed instead of
26 their fusion protein counterparts in the presence of p88, RNA2, ER-mCherry and p19 in

1 *N. benthamiana* leaves by *Agrobacterium* infiltration. Merged images of the YFP and
2 ER-mCherry visualized by confocal microscopy at 4 days post agroinfiltration are
3 shown. White arrowheads indicate the large fluorescent aggregates. Bars, 20µm. (E and
4 F) Functional analysis of YFP fragment-fused p27 in viral RNA replication. Total
5 RNAs and proteins extracted from agroinfiltrated leaves were analyzed by northern and
6 western blotting, respectively. EtBr-stained rRNAs as loading controls were shown
7 below northern blots.

8

9 **Fig. 4.** RCNMV RNA replication affects the subcellular localization of Hsp70 and
10 Hsp90. Green fluorescent protein (GFP)-fused Hsp70 (GFP-Hsp70) plus ER-mCherry
11 (A), GFP-Hsp70 plus C-terminally DsRed monomer-fused p27 (p27-DRm) plus p88
12 plus RNA2 (B), GFP-fused-Hsp90 (GFP-Hsp90) plus ER-mCherry (C), and
13 GFP-Hsp90 plus p27-DRm plus p88 plus RNA2 (D) were expressed in *N. benthamiana*
14 leaves by *Agrobacterium* infiltration. Fluorescence was visualized by confocal
15 microscopy. The merging of the green and red fluorescence is shown as yellow color.
16 Bars, 20 µm.

17

18 **Fig. 5.** p27 interacts with Hsp70 and Hsp90 *in vitro*. (A) Glutathione resin-bound
19 GST-fused Hsp70 (GST-Hsp70) or GST-Hsp90 was incubated with the purified
20 recombinant N-terminally His and C-terminally FLAG-tagged p27 (His-p27-FLAG).
21 After washing, pulled-down complexes were subjected to SDS-PAGE and blotted onto
22 a membrane. Pulled down His-p27-FLAG was detected by western blotting with
23 anti-FLAG antibody. After detection, the separated proteins on the membrane were
24 visualized with Ponceau S staining (Ponceau S). (B and C) A capped transcript that
25 expresses p27-FLAG (20 nM) was added to BYL. After 2 h of incubation at 17 °C,
26 BYL expressing p27-FLAG was incubated for 30 min at 17 °C with DMSO, PES (500

1 μM), or MS (500 μM) in the presence of 2 mM of a non-hydrolysable analogue of ADP
2 (ADP βS) for the analysis of p27–Hsp70 interaction (B). Alternatively, BYL expressing
3 p27-FLAG was incubated for 30 min at 17 °C with DMSO or GDA (500 μM) in the
4 presence of 2 mM of a non-hydrolysable analogue of ATP (ATP γS) and 3 mM of
5 MgCl_2 for the analysis of p27–Hsp90 interaction (C). The BYL was then mixed with a
6 10 μl bed volume of anti-FLAG M2 antibody agarose, and incubated further on ice for
7 90 min. After washing, Hsp70 and Hsp90 co-purified with p27-FLAG were analyzed by
8 western blotting with appropriate antibodies.

9

10 **Fig. 6.** PES and GDA inhibit accumulations of p27 and negative-strand RNAs in BYL.
11 BYL was incubated for 4 h at 17 °C with *in vitro* synthesized RNA1 (7.5 nM) and
12 RNA2 (20 nM) in the presence of various concentrations of inhibitors. Accumulations
13 of negative-strand RNA1 and RNA2 were detected by northern blotting. Accumulations
14 of p27 were analyzed by western blotting with anti-p27 antisera. EtBr-stained rRNAs
15 and CBB-stained cellular proteins are shown as loading controls.

16

17 **Fig. 7.** Hsp70 and Hsp90 promote the assembly of the RCNMV replicase complex. (A)
18 Depiction of the assay system using BYL to monitor the assembly of the 480-kDa
19 replicase complex and the synthesis of negative-strand RNA2 independently of the
20 translation and folding of p27 and p88 replicase proteins. [1] Capped transcripts
21 expressing p27-FLAG (20 nM) or p88-T7 (20 nM) were incubated separately in BYL
22 for 2 h at 17 °C. [2] After the addition of cycloheximide at a concentration of 100 $\mu\text{g}/\text{ml}$,
23 BYL expressing p27-FLAG and BYL expressing p88-T7 were mixed at 2:1 ratio. [3]
24 RNA2 (20 nM) was added together with DMSO, PES, MS, or GDA to the mixed BYL,
25 and incubated for an additional 2 h at 17 °C. (B) Effects of PES (500 μM) and GDA
26 (100 μM) on the accumulation of 480-kDa complexes and the negative-strand synthesis

1 of RNA2. Accumulation of negative-strand RNA2 was analyzed by northern blotting.
2 EtBr-stained rRNAs are shown below the northern blots as loading controls.
3 Accumulation of the 480-kDa replicase complexes were analyzed by western blotting
4 with anti-p27 antisera after the separation of protein complexes by blue native
5 polyacrylamide gel electrophoresis (BN-PAGE). Accumulation of p27-FLAG and
6 p88-T7 was analyzed by western blotting with anti-FLAG and anti-T7 antibodies,
7 respectively, after separation of the denatured proteins by SDS-PAGE. CBB-stained
8 cellular proteins are shown below the western blots as loading controls. (C-F)
9 Dose-dependent effects of PES and GDA on the accumulation of the 480-kDa replicase
10 complex and negative-strand RNA synthesis. BYL expressing p27-FLAG and p88-T7
11 were prepared and mixed as described in (A). Then, the mixed BYL was incubated with
12 RNA2 (20 nM) for 2 h at 17 °C in the presence of increasing amounts of PES (C and D)
13 or GDA (D and E). Western blots with anti-FLAG antibodies in combination with
14 BN-PAGE detected the 480-kDa replicase complexes and nonfunctional large
15 complexes. Western blots in combination with SDS-PAGE detected p27-FLAG and
16 p88-T7 components. CBB-stained cellular proteins are shown as loading controls.
17 Northern blots showed accumulations of negative-strand RNA2. EtBr-stained rRNAs
18 are shown below the northern blots as loading controls. (G) Effects of the reduced
19 accumulation of p88 on the negative-strand RNA2 synthesis. BYL expressing
20 p27-FLAG, BYL expressing p88-T7, and mock-treated BYL were mixed at 2:1:0,
21 2:0.67:0.33, 2:0.5:0.5, and 2:0.33:0.67 in the presence of MS, and BYL expressing
22 p27-FLAG and BYL expressing p88-T7 were mixed at 2:1 in the presence of PES (from
23 left to right). Accumulation of viral RNAs and proteins were analyzed by northern and
24 western blotting, respectively. EtBr-stained rRNAs and CBB-stained cellular proteins
25 are shown below the northern and western blots as loading controls, respectively.

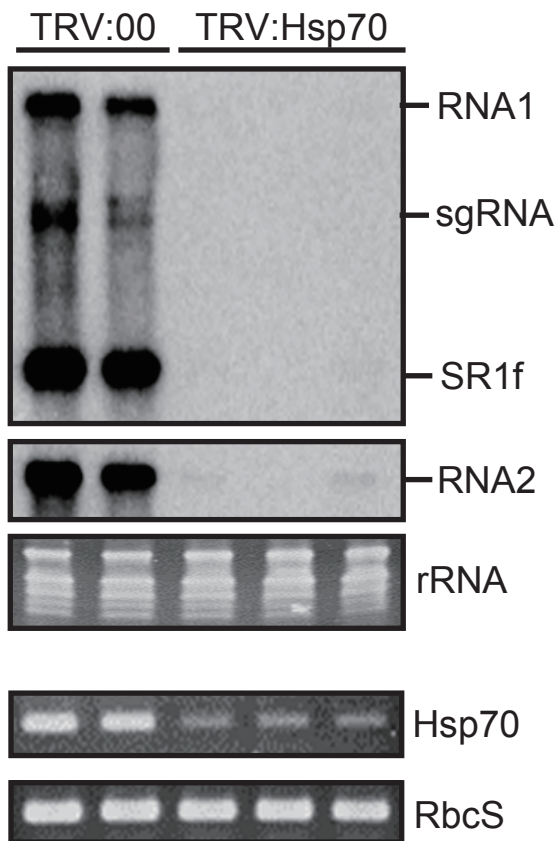
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1 **Fig. 8.** Hsp90 regulates the specific binding of p27 to the Y-shaped RNA element
2 (YRE) of RNA2, which is critical for the assembly of the RCNMV replicase complex.
3 (A) Effects of a mutation in YRE that inhibits the interaction between p27 and YRE on
4 the assembly of the 480-kDa complex. BYL expressing p27-FLAG and BYL expressing
5 p88-T7 were mixed as shown in Fig. 7A. RNA2 or its mutant, LM8, which cannot
6 interact with p27 (27), was added to the mixed BYL and the mixture was incubated for
7 2 h. Accumulation of viral RNAs was analyzed by northern blotting. Accumulation of
8 the 480-kDa complexes and its viral components was analyzed by western blotting in
9 combination with BN-PAGE and SDS-PAGE, respectively. (B) Effects of inhibitors on
10 the binding of p27 with YRE. Membrane-depleted BYL (BYLS20) expressing
11 p27-FLAG was incubated for 20 min on ice with StagT-fused YRE in the presence of
12 PES, MS or GDA (500 μ M each). Heparin was added to the BYLS20 at a final
13 concentration of 20 μ g/ml and the mixture was incubated for 40 min. The BYLS20 was
14 subjected to Strepto-tag affinity purification, followed by western blotting with
15 anti-FLAG antibody. The affinity-purified YRE was detected with EtBr staining.

A



B



C

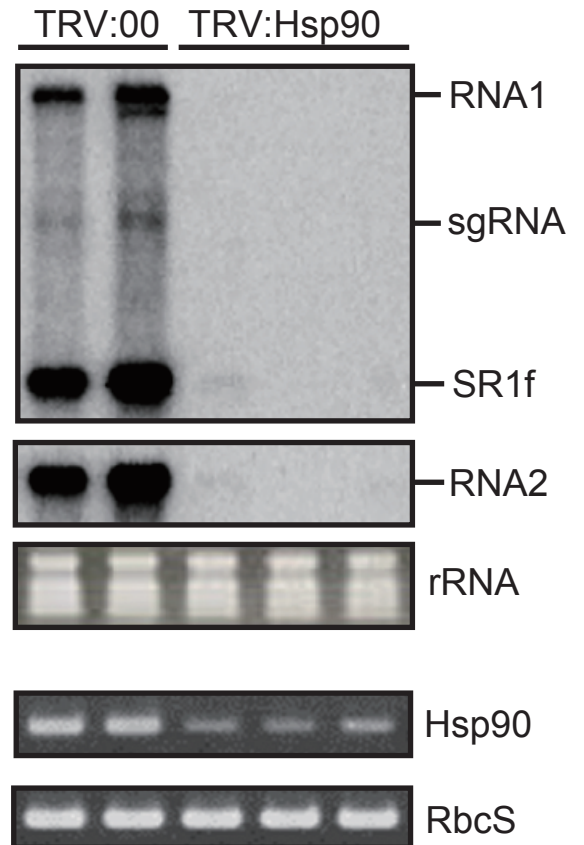


Figure 1

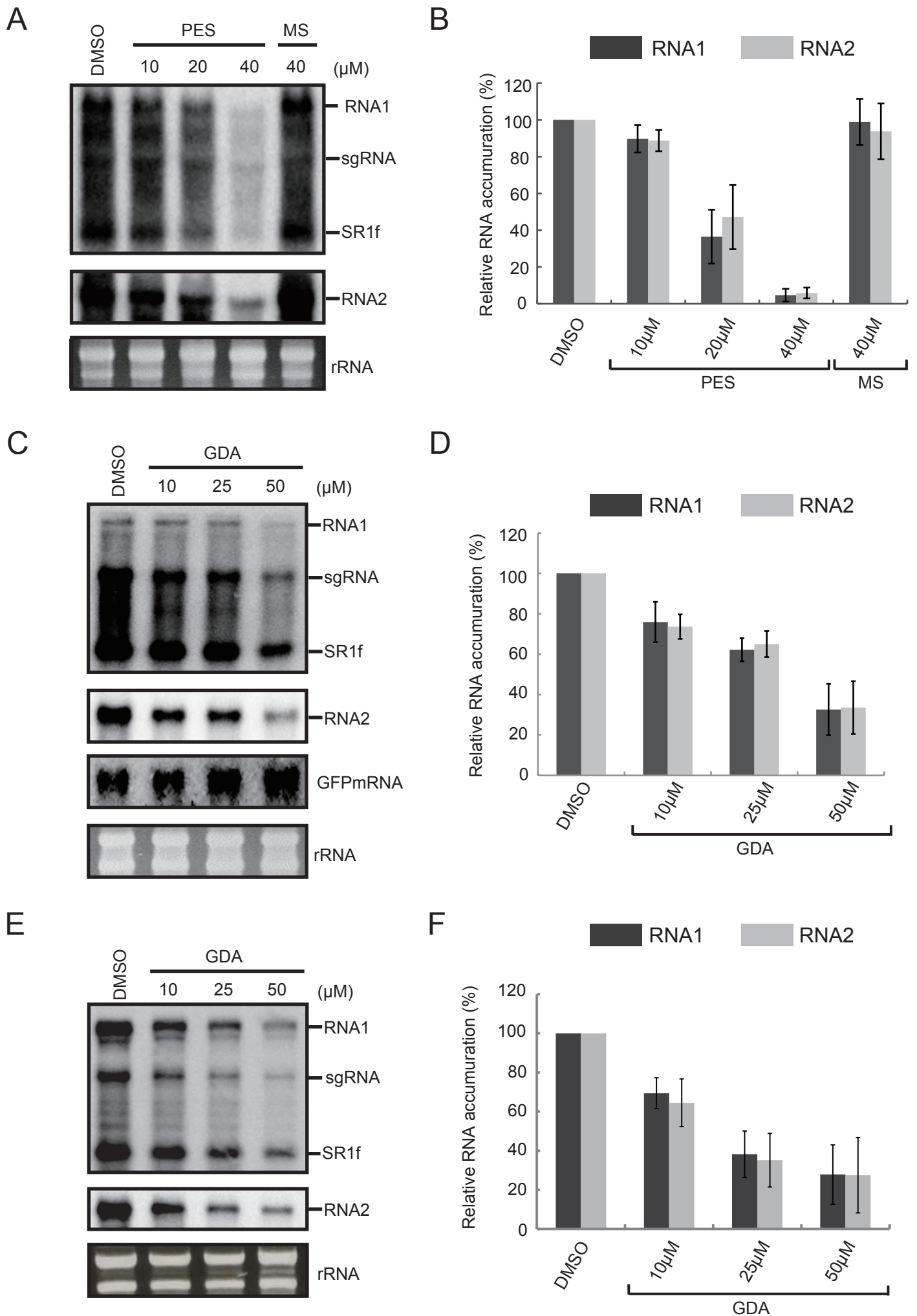


Figure 2

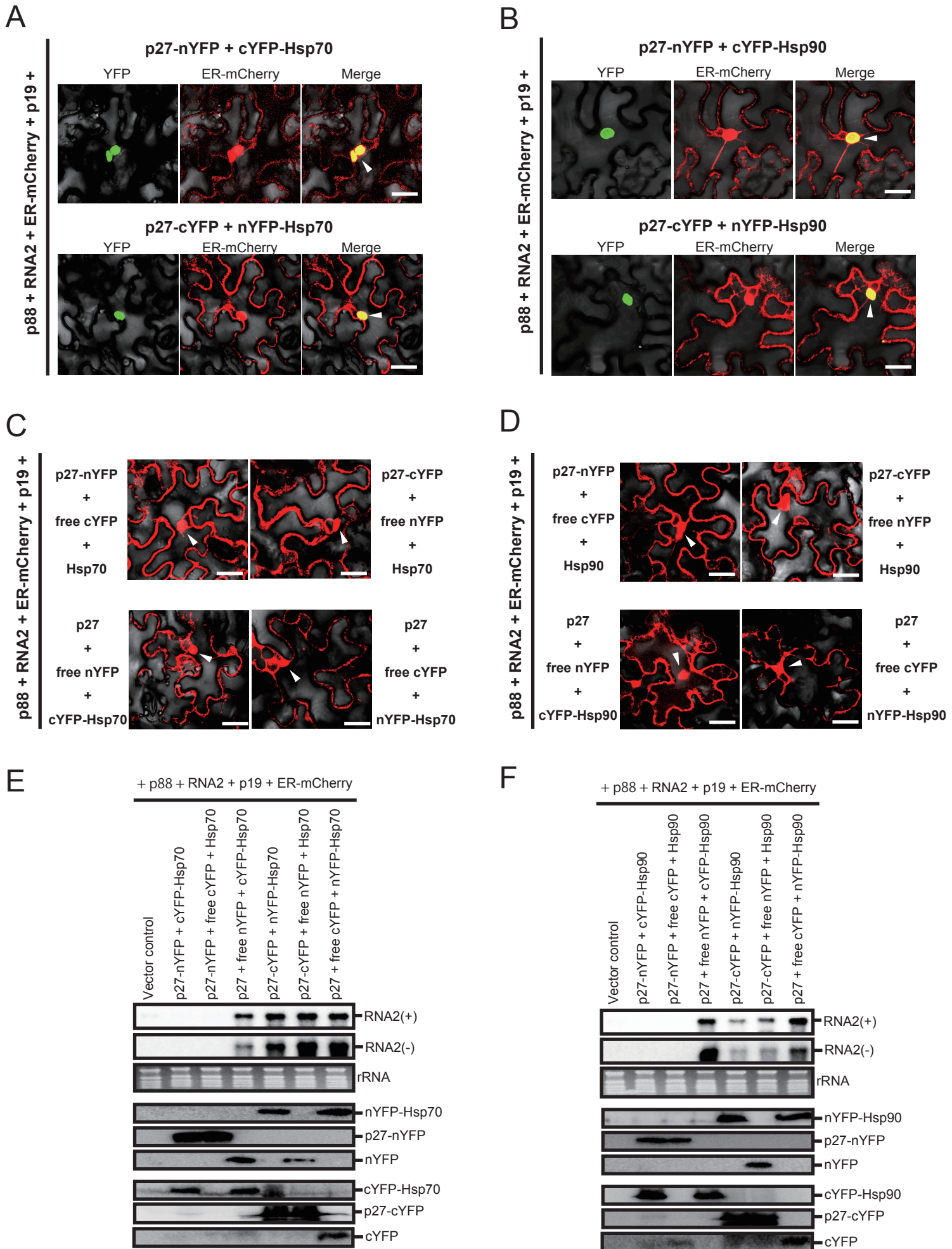
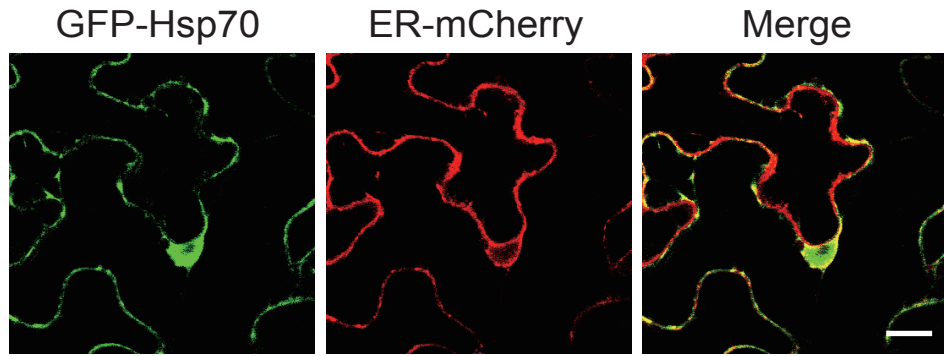
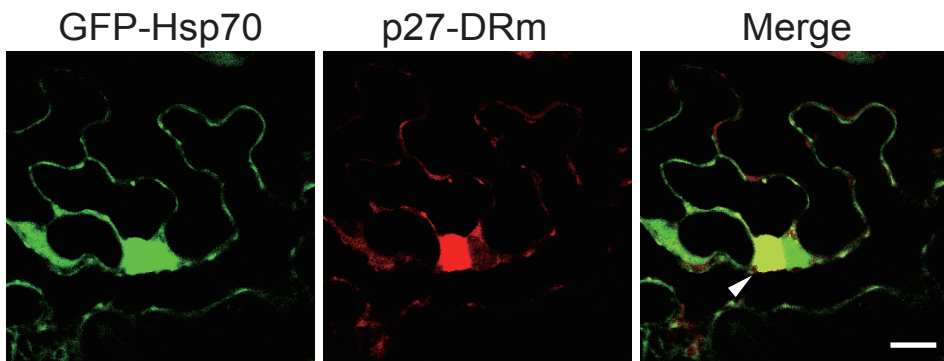


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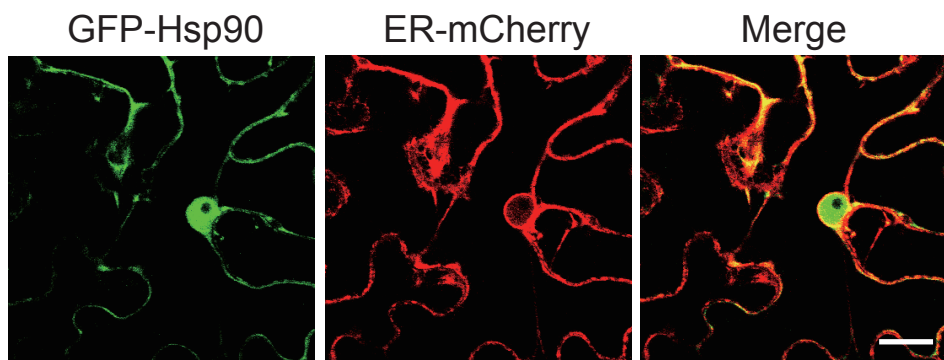
A. GFP-Hsp70 + ER-mCherry



B. GFP-Hsp70 + p27-DRm + p88 + RNA2



C. GFP-Hsp90 + ER-mCherry



D. GFP-Hsp90 + p27-DRm + p88 + RNA2

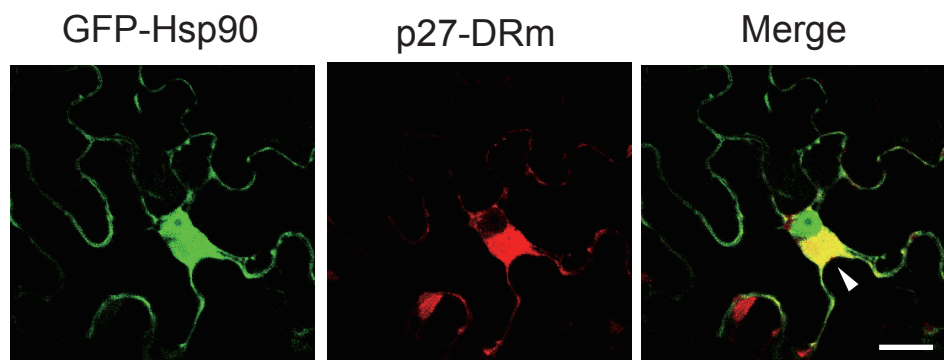
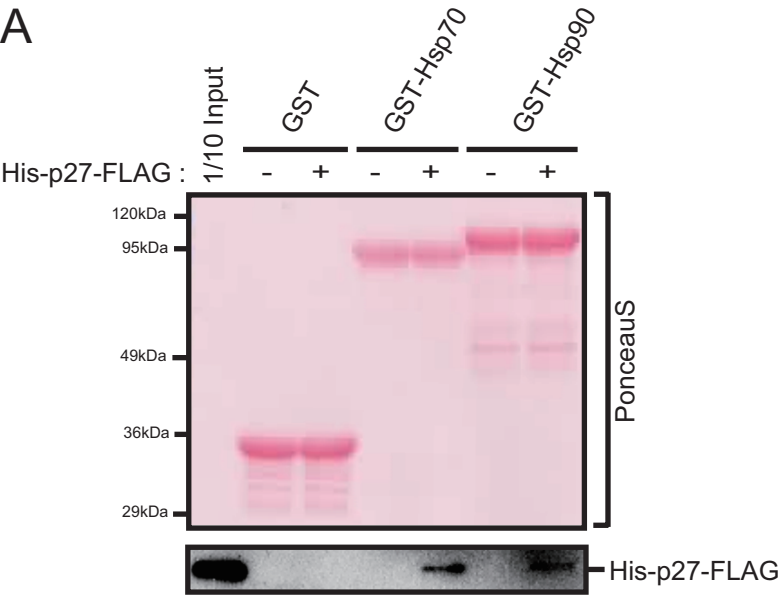
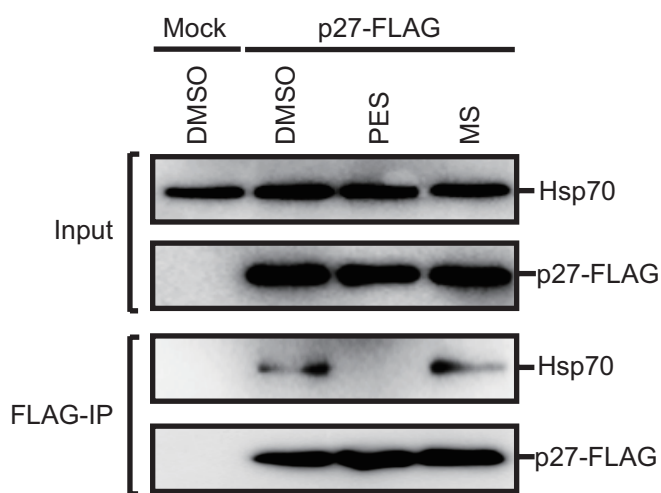
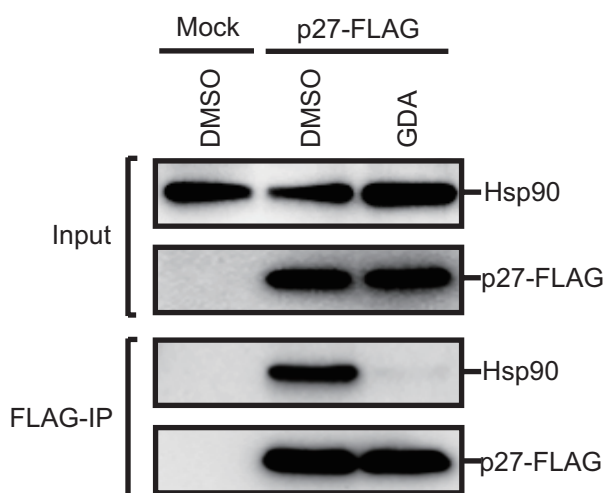


Figure 4

A**B****C****Figure 5**

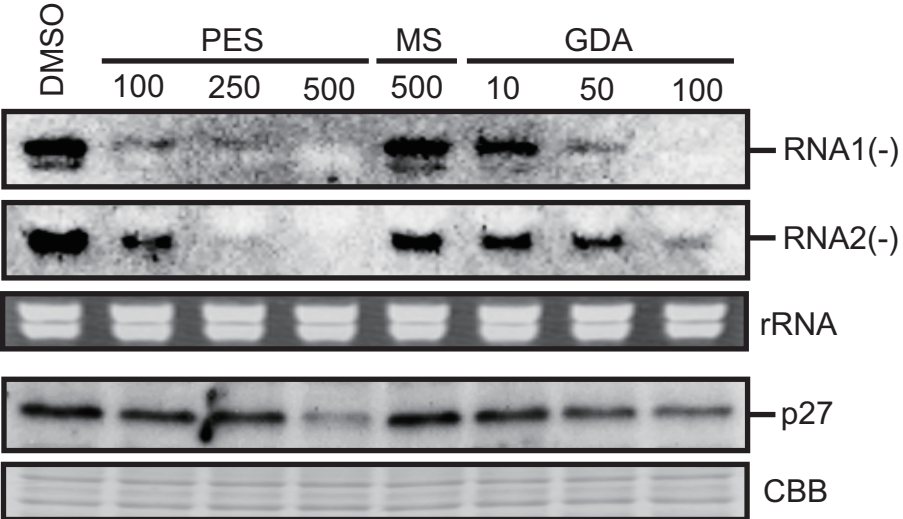


Figure 6

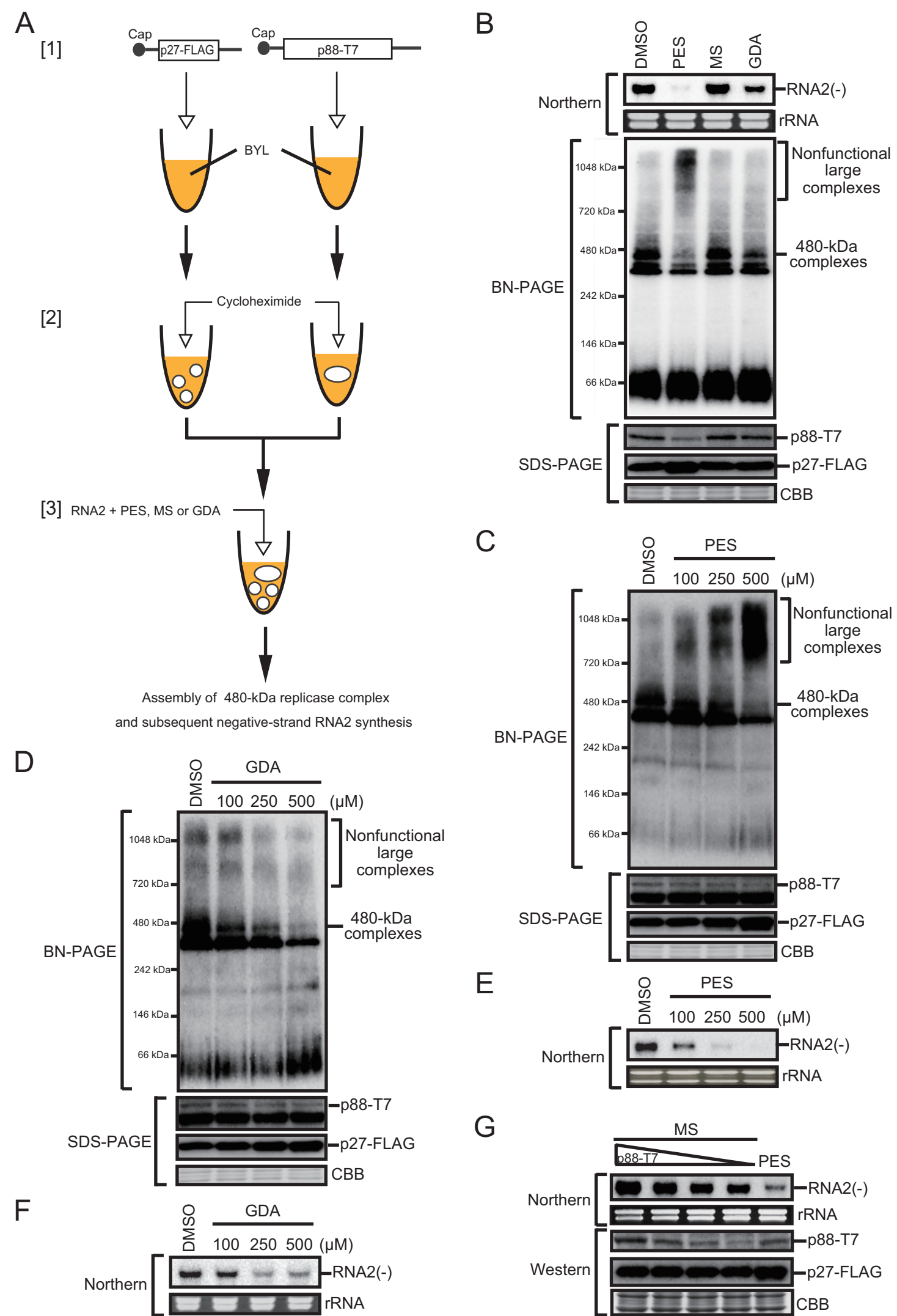
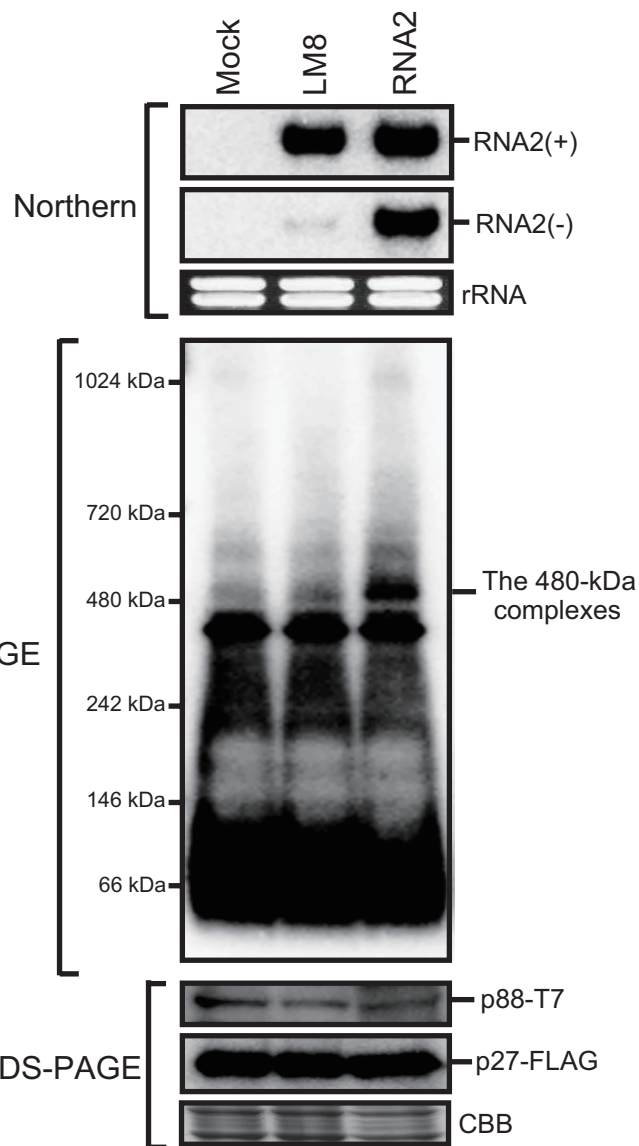
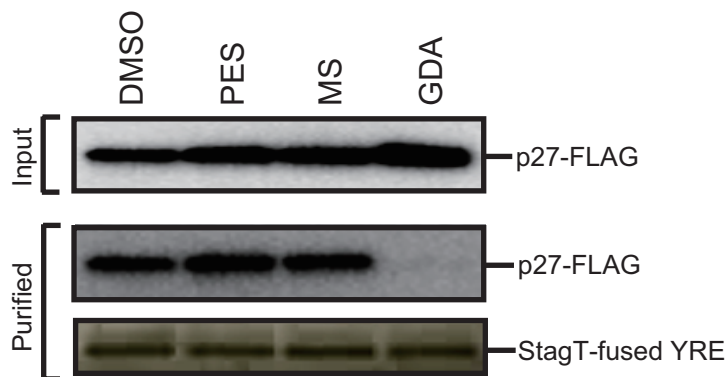


Figure 7

A**B****Figure 8**