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Deubiquitinases USP5 and USP13 are recruited to and regulate heat-induced stress granules by deubiquitinating activities

by

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Content

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Abstract

Stress granules are transient cytoplasmic foci induced by various stresses that contain translation-stalled mRNAs and RNA-binding proteins. They are proposed to modulate mRNA translation and stress responses. Here, I show that the deubiquitinases USP5 and USP13 are recruited to heat-induced stress granules. Heat-induced stress granules also contained K48- and K63-linked ubiquitin chains. Depletion of USP5 or USP13 resulted in elevated ubiquitin chain levels and accelerated assembly of heat-induced stress granules, suggesting that these enzymes regulate the stability of the stress granules through their ubiquitin isopeptidase activity. Moreover, disassembly of heat-induced stress granules after returning the cells to normal temperatures was markedly repressed by individual depletion of USP5 or USP13. Finally, overexpression of a ubiquitin mutant lacking the C-terminal diglycine motif caused the accumulation of unanchored ubiquitin chains and the repression of the disassembly of heat-induced stress granules. As unanchored ubiquitin chains are preferred substrates for USP5, I suggest that USP5 regulates the assembly and disassembly of heat-induced stress granules by mediating the hydrolysis of unanchored ubiquitin chains while USP13 regulates stress granules through deubiquitinating protein-conjugated ubiquitin chains.

Introduction

Stress granules (SGs) are cytoplasmic ribonucleoprotein (RNP) granules in eukaryotic cells. SGs are formed by exposure of cells to various stresses including heat stress, oxidative stress, hypoxia, starvation, and viral infection (Anderson and Kedersha, 2006; Anderson and Kedersha, 2008; Kedersha and Anderson, 2009). The function of SGs is proposed to modulate mRNA translation and stress responses, as described below. Importantly, abnormal formation of SGs is implicated in the pathogenesis of various diseases including neurodegenerative diseases (Dormann et al., 2010; Liu-Yesucevitz et al., 2010; Elden et al., 2010; Li et al., 2013; Ramaswami et al., 2013; Kim et al., 2013; Buchan et al., 2013).

1. The composition and structure of SGs

The components of SGs are eukaryotic translation initiation factors, 40S ribosome subunits, translationally silent mRNAs bound to 40S ribosomal subunits, RNA-binding proteins such as polyA-binding protein 1 (PABP1) and Ras GTPase-activating protein-binding protein (G3BP), and non-RNA-binding regulatory proteins (Nover et al., 1989; Kerdersha et al., 2002; Protter and Parker, 2016) (Fig. 1). SGs have an average diameter of 1-2 μm. The final size of SGs may be determined by the number of molecules in SGs (Tourriere et al., 2003; Gilks et al., 2004; Goehring and Hyman, 2012; Brangwynne, 2013). Stress-specific differences in the molecular composition of SGs have been reported (Kedersha et al., 1999; Aulas et al., 2017). For example, small heat shock protein HSP27 was found in SGs induced by heat stress, but not in arsenite-induced SGs (Kedersha et al., 1999).

SGs are non-uniform structures (Fig. 1). PABP1 and G3BP, two major component proteins of SGs, are non-uniform in SGs with clusters of higher concentration (Jain et al., 2016). These clusters have an average diameter of 200 nm, and are referred to as SG cores. SG cores are relatively stable and can be biochemically isolated. The surrounding areas with less concentrated of PABP1 and G3BP are referred to as the SG shell. The SG shell is formed by liquid-liquid phase separation, and the components show dynamic behavior and rapid exchange between cytosol and SGs (Souquere et al., 2009; Jain et al., 2016; Wheeler et al., 2016).

2. Functions of SGs

Forming SGs is one of mechanisms for cells to resist various environmental pressures and intracellular abnormalities. (Kedersha et al., 2013).

The sequestration of RNPs within SGs limits their interactions with polysome. Thus, the formation of SGs decreases the level of global translation (Anderson et al., 2009). Because the sequestration of RNPs also limits their interactions with degradation enzymes, SGs function as a storage of RNPs. Formation of SGs have also been suggested to promote the assembly of translation initiation complexes by increasing the local concentration of mRNAs and translation factors in SGs. Due to lack of 60S subunits, the translation initiation complexes formed in SGs are unlikely to start translation (Kedersha and Anderson, 2002). The disassembly of SGs releases the majority of mRNAs back to the translation machinery (Kedersha and Anderson, 2002). The translation initiation complexes in SGs seems to be rapidly converted into polysomes during the disassembly of SGs and translation resumes at the surface of SGs (Panas et al., 2016). Taken together, these indicates that the assembly and disassembly of SGs play an important role in the regulation of protein expression for stress responses. Interestingly, mRNAs encoding molecular chaperones and repair enzymes, which are preferentially translated during stress, are excluded from SGs by unknown mechanisms (Kedersha and Anderson, 2002).

The assembly of stress sensor/effector proteins in SGs enhances the rates of biochemical reactions in SGs that are important for the survival of cells under stress. For example, viral transfection-induced SGs recruit multiple anti-viral proteins that induce the innate immune response. The recruitment causes the activation of these proteins, thereby enhancing the viral resistance for cells (Onomoto et al., 2012; Reineke and Lloyd, 2015; Reineke et al., 2015). On the contrary, the sequestration of certain proteins within SGs limits their reactions in the other intracellular compartment. For example, sequestering some signal transducers or regulators within SGs leads to the modification of MAPK, TOR and TRAF2 pathways (Arimoto et al., 2008; Kim et al., 2005; Takahara and Maeda, 2012; Thedieck et al., 2013).

SGs may function as an intermediate compartment linking to several other organelles (Fig. 2). P-bodies are conserved cytoplasmic mRNP granules containing mRNAs associated with translational depressors and mRNA decay machinery (Sheth et al., 2003; Eystathioy et al., 2002, 2003; Ingelfinger et al., 2002; Cougot et al., 2004a, b; Yang et al., 2004; Aizer et al., 2014). Although the assembly of SGs and

P-bodies is regulated independently by different pathways, these granules can be fused under certain types of stress (Buchan et al., 2008; Kedersha et al., 2005). It indicates that some RNAs in SGs may be processed by the decay machinery in P-bodies. Lysosomes (or vacuole in yeast) are membrane-enclosed organelles in which various digestive enzymes break down all types of biological polymers. SGs can be targeted to lysosome or vacuole by autophagy system under specific conditions, where the whole structures of SGs are cleared (Buchan et al., 2013). Aggresomes are cytoplasmic aggregates of misfolded proteins. A recent report indicated that certain types of stress induces the formation of SGs containing large amounts of misfolded proteins. These SGs are then transported and assemble into aggresomes (Mateju et al., 2017).

3. Mechanisms of SG assembly and disassembly

The mechanisms of SG assembly are becoming clear (Fig. 1). Under stress, translation of most mRNAs is inhibited, polysomes are disassembled, and released mRNAs are then associated with various RNA-binding proteins. Some of the RNA-binding proteins harbor intrinsically disordered protein regions (IDPRs), such as prion-like low complexity domains (Kedersha et al., 2013, 2016). The interaction of IDPRs in different RNPs initiate the formation of RNP oligomers. They serve as nucleators of stable SG cores. Relatively weak interaction of RNAs and RNA-binding proteins surrounding SG cores induces the liquid-liquid phase separation from cytoplasm, contributing to the formation of dynamic SG shell (Han et al., 2012; Kato et al., 2012; Lin et al., 2015; Molliex et al., 2015; Panas et al., 2016; Protter and Parker, 2016; Harrison and Shorter, 2017).

SGs are not static structures in cytoplasm (Kedersha et al., 2005). Actually, SGs are attached to microtubules and the interruption of microtubule network disrupts the assembly of SGs (Ivanov et al., 2003; Chernov et al., 2009). Furthermore, microtubule motor proteins are also reported to regulate the assembly and movement of SGs (Loschi et al., 2009). These observations support the notion that SG initiation complexes travel through microtubule network while forming into typical SGs.

When stress is relieved, SGs are disassembled (Fig. 1). Of course, the decreased supply of untranslated mRNA under stress-free condition contributes to SG disassembly (Kedersha et al., 2000). However, SGs disassemble rapidly after the relief of stress, indicating that some mechanism may actively promote SG disassembly. Heat shock proteins influence the disassembly of SGs (Ganassi et al.,

2016). HSP70 localizes to SGs, and promotes the disassembly of SGs that contain large amount of protein aggregates (Gilks et al., 2004; Mateju et al., 2017). HSP70 is linked to client proteins by HSP40 co-chaperone proteins, and HSP40 also localizes to SGs. Interestingly, yeast HSP40 Ydj1 promotes SG disassembly and the recovery of translation, while the other yeast HSP40 Sis1 plays a role in the targeting of SGs to the vacuole (Walters et al., 2015). The AAA-ATPase VCP/Cdc48 is a segregase which extracts ubiquitinated proteins from protein complexes in an ATP-dependent way (Meyer et al., 2014). VCP/Cdc48 is proposed to direct SGs to autophagy by segregating some ubiquitinated proteins from SGs (Buchan et al., 2013). RNA/DNA helicases are also involved in SG disassembly; MCM and RVB helicase complexes regulate the clearance of SGs after termination of stress (Jain et al., 2016).

Mechanisms of SG assembly and disassembly may be context specific and vary among different stresses. For example, G3BP and the paralog G3BP2 are important for the formation of SGs under oxidative stress, while they are dispensable for the formation of SGs induced by osmotic-stress (Tourriere 2003; Kedersha et al., 2016).

4. Ubiquitin in SGs

It has been reported that ubiquitin is contained in SGs induced by some stressors (Kwon et al., 2007; Mateju et al., 2017). Ubiquitin is conjugated to various target proteins, which is a post-translational modification called ubiquitination. Ubiquitination is usually mediated by an enzymatic cascade that involves E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) (Fig. 3). In general, ubiquitin is conjugated through isopeptide bond between its C-terminal carboxyl group and the ε-amino group of Lys (K) residues in target proteins. Additional ubiquitin can be ligated to one of seven K residues in the ubiquitin already conjugated to target proteins, leading to the formation of ubiquitin chains. In some cases, ubiquitin can be ligated to the N-terminal methionine of another ubiquitin, forming “linear ubiquitin chains”. Recent reports also showed mixed ubiquitin chains that contain several linkage types in one chain, as well as branched chains where two ubiquitin molecules are linked to the proximal ubiquitin molecule (Komander and Rape, 2012). Based on the type of ubiquitin modification that is formed on a specific substrate, ubiquitination can be divided into monoubiquitination, multi-monoubiquitination and polyubiquitination (Fig. 3). Most of polyubiquitin chains in cells are linked through K48 or K63 of ubiquitin.

K48-linked chains induce the proteasomal degradation of target proteins, and K63-linked chains induce their endocytosis, lysosomal degradation and formation of protein complexes for signal transduction and DNA repair (Kulathu and Komander, 2012). However, target proteins, linkage types, and roles of ubiquitin chains in SGs were unknown.

5. Deubiquitinating enzymes in SGs

Deubiquitination is the reverse process of ubiquitination, which is accomplished by deubiquitinating enzymes (DUBs) (Nijman et al., 2005). Based on which part of the ubiquitin chain is cleaved, DUB activities can be divided into three: exo-activity (hydrolyzing ubiquitin chains from the end), endo-activity (cleaving ubiquitin chains in the middle) or DUB activity at the base of a chain (*i.e.,* removal of monoubiquitination) (Heideker and Wertz, 2015) (Fig. 3). There are ~100 DUBs encoded by the human genome, which can be divided into five families: ubiquitin C-terminal hydrolases (UCH), ubiquitin specific proteases (USP), ovarian tumor proteases (OTU), Machado-Joseph disease proteases (MJD) and JAB1/MPN/Mov34 metalloenzymes (JAMM) (Heideker and Werts, 2015). DUBs vary in the sequence of catalytic domains even within the same family, and often harbor multiple domains other than catalytic domains. These cause the difference in their protein-protein interactions, target recognitions and ubiquitin chain linkage preferences. The catalytic activity of a DUB and its substrate recognition is regulated by various ways, such as its transcription and translation, post-translational modification, proteolytic cleavage, localization, and association with proteins including E2s, E3s and co-factors. DUBs have numerous physiological functions including regulation of protein stability, ubiquitin recycling, negative regulation of ubiquitin signals and so on (Komander et al., 2009).

Previous reports demonstrated that ubiquitin-specific protease 10 (USP10) is recruited to SGs upon various stresses (Ohn et al., 2008; Wang et al., 2012; Takahashi et al., 2013; Kedersha et al., 2016; Nostramo et al., 2015). USP10 is one of the USP family proteins, and essential for SG assembly. However, this function of USP10 is independent of its DUB activity, and dependent of its interaction with G3BP (Takahashi et al., 2013; Kedersha et al., 2016).

In this study, I found that two deubiquitinating enzymes, USP5 and USP13, localize to SGs under some stress conditions. USP5, also known as isopeptidase T, is

a DUB in the USP family. USP5 harbors the catalytic core composed of the Cys- and His-boxes, as well as 2 tandem UBA domains between the Cys- and His-boxes in the primary sequence (Buchberger, 2002), and ZnF-UBP domain in the N-terminal region (Bonnet et al., 2008). In general, UBA domain and ZnF-UBP domain are ubiquitin-binding domains. In USP family, UBA domains often facilitate substrate binding onto the enzymes, while ZnF-UBP domains activate the catalytic reaction or regulate other biological functions (Dikic et al., 2009; Bonnet et al., 2008). In USP5, the ZnF-UBP domain specifically recognizes the free C-terminal Gly-Gly sequence of ubiquitin. Thus, USP5 exhibits isopeptidase specificity toward ubiquitin chains that are not attached to any substrate (that is, unanchored ubiquitin chains). In cells, USP5 regulates the cellular level of unanchored ubiquitin chains (Wilkinson et al., 1995; Amerik et al., 1997; Reyes-Turcu et al., 2006; Dayal et al., 2009). The function of USP5 in SGs was unclear.

USP13 shows about 80% sequence similarity with USP5, and has the same domain architecture as USP5 (Timms et al., 1998). However, the ZnF-UBP domain of USP13 lacks binding ability to the free C-terminus of ubiquitin (Zhang et al., 2011). Therefore, USP13 exhibits isopeptidase activity toward ubiquitin chains conjugated to target proteins. USP13 deubiquitinates and stabilizes STAT1, by which USP13 regulates interferon (IFN) signaling (Yeh et al., 2013). Interestingly, STAT1 is reported to be recruited to SGs (Jain et al., 2016). USP13 also deubiquitinates and stabilizes Beclin1 (Liu et al., 2011). Beclin1 can regulates autophagy as a component of Vps34 complexes, and autophagy is involved in the clearance of SGs (Buchan et al., 2013). However, target proteins and functions of USP13 in SGs were unknown.

6. The aim and outline of this study

As described above, ubiquitin is present in SGs induced by some stressors. However, regulatory mechanisms of ubiquitin chain levels in SGs and roles of ubiquitin chains in SGs remained unclear. The aim of this study is to elucidate them.

In this study, I found that USP5 and USP13 are recruited to heat-induced SGs. I examined the stress-type specificity of the recruitment of USP5, USP13, and ubiquitin chains. I also investigated the molecular mechanism underlying their recruitment. Next, I examined the roles of USP5 and USP13 in the regulation of ubiquitin chain levels in heat-induced SGs, and in the SG assembly and disassembly. I provided

evidence suggesting that the stability of SGs is regulated by ubiquitin chains and their hydrolysis by these deubiquitinases.

Materials and Methods

1. Plasmid preparation

The cDNAs for human USP5 and USP13 were purchased from GE Healthcare (Chicago, IL) and Kazusa DNA Research Institute (Kisarazu, Japan), respectively, and inserted into the N-terminally Flag epitope-tagged mammalian expression vector pME-Flag (Kato et al., 2000). The expression plasmid for Flag-tagged ubiquitin was provided by Dr. Toshiaki Suzuki (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Mutations were introduced into these cDNAs using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). siRNA-resistant cDNAs for USP5 and USP13 were constructed by the introduction of silent mutations into the siRNA target sequences.

2. Cell culture and SG induction

HeLa cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37°C. To induce SGs, cells were incubated: (1) at 44°C for 45–60 min, (2) with 0.5 mM sodium arsenite (Wako Pure Chemical Industries, Osaka, Japan) for 45 min, (3) with 0.2 mM MG132 (Sigma-Aldrich, St Louis, MO) for 3 h, (4) with 20 μM CCCP (Nacalai Tesque, Kyoto, Japan) for 1.5 h, (5) with 5 μg/ml puromycin (Wako) and 100 μM VER-155008 (Sigma-Aldrich) for 3 h or (6) with 400 ng/ml poly(I:C) mixed with a transfection reagent polyethylenimine (cat. no. #24765, batch #644175, Polysciences, Warrington, PA) for 7 h. To examine whether the nuclear export of USP5 and USP13 occurs during heat stress, 50 nM leptomycin B (LC Laboratories, Woburn, MA) was added into the culture medium 30 min before the incubating temperature was raised to 44°C.

3. DNA and siRNA transfection

Plasmid DNA transfection was performed using the FuGene6 transfection reagent (Promega, Madison, WI) or polyethylenimine following the manufacturer's instructions. For siRNA transfection, synthetic duplex siRNAs were purchased from GE Healthcare [siUSP5-1 and siUSP5-2, single siRNAs targeting a specific sequence of human USP5 (D-006095-02 and J-006095-10); siUSP13-1, a mixture of four

siRNAs targeting different sequences of human USP13 (M-006064-00); siUSP13-2 and siUSP13-3, single siRNAs targeting human USP13 (D-006064-01 and D-006064-02); and siCont, a negative control siRNA (D-001210-02-05)]. siRNA transfection was performed using DharmaFECT 1 transfection reagent (GE Healthcare) following the manufacturer's instructions.

4. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100 in PBS, and blocked with 5% fetal bovine serum in PBS. Cells were then incubated with rabbit anti-USP5 (3 μg/ml; catalog no. #10473-1-AP, batch #00000702, Proteintech, Chicago, IL), rabbit anti-USP13 (8 μg/ml; catalog. no. #HPA004827, batch #A37295, Sigma-Aldrich), mouse anti-PABP1 (10 μg/ml; clone 10E10, catalog no. #ab6125, batch #GR114685-30, Abcam, Cambridge, UK), mouse anti-G3BP (0.5 μg/ml; clone 23/G3BP, catalog no. #611126, batch #6302844, BD Transduction Laboratories, San Jose, CA), mouse anti-ubiquitin (1 μg/ml; clone FK2, catalog no. #D058-3, batch #043, MBL, Nagoya, Japan), rabbit anti-K48-linked ubiquitin chain (1:100; clone Apu2, catalog no. #05-1307, batch #2145090, EMD Millipore, Billerica, MA), rabbit anti-K63-linked ubiquitin chain (1:100; clone Apu3, catalog no. #05-1308, batch #2063204, EMD Millipore), rabbit anti-Flag (0.8 μg/ml; catalog no. #F7425, batch #093M4798, Sigma-Aldrich), rabbit anti-Beclin (1:100; catalog no. #PD017, MBL), and rabbit anti-STAT1 (1:100; catalog no. #9172, Cell Signaling Technology) antibodies. Secondary antibodies were Alexa Fluor 488- and 594-conjugated antimouse IgG and anti-rabbit IgG antibodies (1:1000; Thermo Fisher Scientific, Waltham, MA). Nuclei were stained with DAPI (0.1 mg/ml, Nacalai Tesque) during incubation with secondary antibodies. Fluorescence images were captured using a laser-scanning confocal microscope LSM780 with the imaging software ZEN 2012 and a Plan-Apochromat 40×/1.4 NA oil DIC M27 lens (Carl Zeiss, Oberkochen, Germany). For super-resolution microscopy, the structured illumination microscopic system (ELYRA S.1, Carl Zeiss) was used.

5. Quantification

The quantification of SG size, number and ratio of cells positive for SG was performed using wide-field views of microscopy using 5×5 tile scanning. The number

of G3BP-positive foci and the size of each focus was analyzed by ImageJ (NIH, Bethesda, MA). SG-bearing cells (with more than three G3BP-positive foci with a diameter of >0.1 μm) in each image were counted, and the proportion of the total cell number was calculated. Numbers of SGs per cell were calculated by dividing numbers of G3BP-positive foci (with 0.01∼3 μm² in size) in each image by the total cell number. Areas of all G3BP-positive foci (with 0.01–3 μm² in size) in each image were measured to determine the SG size. FK2-positive SGs were analyzed with the ImageJ line profile tool, and the maximum values of each SGs were measured as FK2 staining intensity. Thirty FK2-positive SGs were analyzed in each image.

6. Immunoblotting

Cells were lysed in 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, and the supernatants were collected after centrifugation. Primary antibodies for immunoblotting were: anti-USP5 (0.3 μg/ml; catalog no. #10473-1-AP, batch #00000702, Proteintech), anti-USP13 (0.4 μg/ml; catalog no. #HPA004827, batch #A37295, Sigma-Aldrich), anti-ubiquitin (1:1000; clone P4G7, catalog no. #MMS-258R, Covance, Princeton, NJ), anti-Flag (2–5 μg/ml; clone M2, catalog no. #F3165, batch #SLBN2445V, Sigma-Aldrich), anti-α-tubulin (1:2500; catalog no. #ab15246, batch #GR121903-2, Abcam), anti-G3BP (1:500; clone 23/G3BP, catalog no. #611126, batch #6302844, BD Transduction Laboratories, San Jose, CA), and anti-PABP1 (1:200; clone 10E10, catalog no. #ab6125, batch #GR114685-30, Abcam, Cambridge, UK) antibodies. Secondary antibodies were peroxidase-conjugated anti-mouse-IgG and anti-rabbit-IgG antibodies (GE Healthcare). Blots were detected by using ECL Western Blotting Detection Reagents (GE Healthcare) and ImageQuant LAS 4000mini chemiluminescence detection system (GE Healthcare).

7. Immunoprecipitation

Cell lysates were incubated with primary antibodies (anti-USP5 antibody, 0.3 μg; anti-USP13 antibody, 0.4 μg) and 20 μl Protein A Sepharose 4 Fast Flow resin (GE Healthcare) at 4℃ with rotation for 3 h. Beads were washed 4 times with lysis buffer

and subsequently resuspended and boiled in 40 μl 1×SDS-PAGE sample buffer for immunoblotting.

Results

1. USP5 and USP13 are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008 and SGs induced by polyinosinic-polycytidylic acid

In the process of studying the function of USP5 and USP13, two structural paralogues of DUBs, I found that they exhibit mostly nuclear localization in human cervical cancer HeLa cells under normal conditions (Fig. 4A), and also found that they can be observed in cytoplasmic punctate structures upon raising the incubating temperature. When cells were incubated at 44°C for 1 h and stained for endogenous USP5 and USP13 together with major SG component, PABP1 or G3BP, a fraction of USP5 as well as USP13 co-localized with PABP1 and G3BP on the puncta (Fig. 4B). It indicates that USP5 and USP13 are recruited to SGs induced by heat stress. For an unknown reason, USP13 was more clearly detected in the SGs than USP5.

As SGs are formed by a variety of cellular stresses, I examined whether these DUBs are also recruited to SGs upon other stress. Treatment of cells with an oxidative agent arsenite, a proteasome inhibitor MG132, or a mitochondrial membrane-depolarizing agent carbonyl cyanide m-chlorophenyl hydrazine (CCCP), also induced PABP1-positive SGs (Fig. 4C, D, E). However, co-staining of the cells with anti-USP5 or anti-USP13 antibody revealed that both DUBs co-localize with PABP1 on SGs in these cells at much lower levels, if at all, than in heat-stressed cells (Fig. 4C, D, E).

Puromycin, which dissociates polysomes and generates polysome-free mRNA, can efficiently induce SGs in the presence of HSP70 inhibitor VER-155008 that increases misfolded proteins (Bounedjah et al., 2014; Fig. 4F). A fraction of USP5 and USP13 were localized to these SGs (Fig. 4F). These results suggested that USP5 and USP13 are preferentially recruited to heat-induced SGs as well as SGs induced by puromycin and VER-155008.

I also examined viral infection-induced SGs using Polyinosinic-polycytidylic acid [poly(I:C)], a virus-mimetic synthetic dsRNA (Onamoto et al., 2012). As shown in Fig. 4G, poly(I:C) treatment induced G3BP-positive SGs, and a fraction of USP5 and USP13 were localized to the SGs (Fig. 4G).

2. Ubiquitin chains are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008 and SGs induced by poly(I:C)

Recruitment of USP5 and USP13 to heat-induced SGs suggested the presence of their substrates (*i.e.*, unanchored ubiquitin chains for USP5 and protein-conjugated ubiquitin chains for USP13) in the SGs. I therefore double-stained heat-stressed cells with the FK2 anti-ubiquitin antibody together with anti-USP5 or anti-USP13 antibody. FK2 recognizes conjugated ubiquitin (including ubiquitin chains) but not free monoubiquitin (Fujimuro and Yokosawa, 2005). As shown in Fig. 5A, FK2 stained USP5- and USP13-positive SGs, indicating that conjugated ubiquitin is recruited to heat-induced SGs.

To elucidate the linkage type of ubiquitin chains in the SGs, I next stained heat-stressed cells with linkage-specific anti-ubiquitin chain antibodies. Although antibodies against K48- and K63-linked ubiquitin chains exhibited clearly different staining patterns in cells under normal conditions (Fig. 5B), both antibodies stained SGs in heat-stressed cells, showing the presence of K48- and K63-linked ubiquitin chains in the SGs (Fig. 5C). I were not able to determine whether the ubiquitin chains are unanchored or conjugated to target proteins because anti-ubiquitin antibodies that can distinguish the difference were unavailable.

The linkage-specific anti-ubiquitin chain antibodies did not stain SGs induced by arsenite, MG132 or CCCP (Fig. 5D, E, F), but stained SGs induced by puromycin and VER-155008 (Fig. 5G). In cells treated with poly(I:C), the linkage-specific anti-ubiquitin chain antibodies also showed punctate pattern in the cytoplasm, and their size was much smaller than that of G3BP-positive SGs (Fig. 5H). A fraction of these puncta was merged with G3BP-positive SGs. These results indicated that poly(I:C)-induced SGs contain small amounts of ubiquitin chains.

3. USP5 and USP13 are adjacent to PABP1-, G3BP-, or ubiquitin-positive regions within heat-induced SGs

In confocal microscopy analyses, USP5 and USP13 did not appear to co-localize completely with PABP1, G3BP or ubiquitinated proteins in heat-induced SGs (Fig. 4B and Fig. 5A). To determine the more precise localization pattern of these DUBs in the SGs, I performed super-resolution microscopy analysis using structured illumination microscopy. I stained heat-stressed cells with anti-USP5 or anti-USP13 antibody together with anti-PABP1, anti-G3BP, or FK2 anti-ubiquitin antibody. In Fig. 6, each panel shows a single SG, clearly indicating that PABP1 and G3BP are non-uniform, confirmed the core-shell structure of heat-induced SGs. Ubiquitin is also

non-uniform in SGs, and most of USP5 (Fig. 6A) and USP13 (Fig. 6B) within SGs were detected at regions with less concentration of PABP1, G3BP and ubiquitin. These results demonstrated that USP5 and USP13 are localized to the SG shell and small portion of USP5 and USP13 within SGs are co-localized with ubiquitin.

In order to confirm that USP5 and USP13 are localized to SG shell, I performed co-immunoprecipitation analysis of them with SG core proteins, G3BP and PABP1. The result showed that neither PABP1 nor G3BP is co-immunoprecipitated with USP5 or USP13 (Fig. 7). It suggested that USP5 and USP13 are not components of SG cores, which is consistent with my super-resolution microscopy analysis.

4. A fraction of USP5, USP13 and ubiquitin chains are translocated from the nucleus to SGs in response to heat stress

I examined whether USP5 and USP13 are translocated from the nucleus in response to heat stress. Treatment of cells with leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export, reduced the recruitment of USP5 and USP13 to SGs (Fig. 4B and Fig. 8), indicating that a fraction of USP5 and USP13 in the SGs are translocated from the nucleus. A similar translocation of a nuclear protein to SGs under stress conditions was reported (Gao et al., 2015). Most of K48-linked ubiquitin chains were also translocated from the nucleus in response to heat-stress (Fig. 5B, C). However, LMB treatment had little effect on heat-induced translocation of ubiquitin chains (Fig. 8), indicating that ubiquitin chains are translocated in CRM1-independent manner.

5. The USP domain has the ability to recruit USP13 to heat-induced SGs

I tried to determine the domain or motif in these USP5 and USP13 responsible for their recruitment to heat-induced SGs (Fig. 9). When cells expressing Flag-tagged USP5 or USP13 were heat-stressed, I confirmed that both Flag-USP5 and Flag-USP13 were localized to G3BP-positive SGs as assessed by staining with anti-Flag antibody (Fig. 9A). As observed for endogenous USP5 and USP13 (Fig. 4B), the SG localization was more clearly observed for Flag-USP13 than for Flag-USP5. I therefore chose USP13 and constructed its mutants lacking ZnF-UBP domain and/or the tandem UBA domains (ΔZnF, ΔUBA and ΔZnFΔUBA; Fig. 10B). When these mutants were expressed in cells, all of them were detected in heat-induced,

G3BP-positive SGs (Fig. 9C), suggesting that these domains are not necessary for the recruitment of USP13. I also constructed other USP13 mutants, which lack USP domain or consist only of USP domain (ΔUSP and USP; Fig. 9B). ΔUSP was not successfully expressed, probably because USP domain is important for the stability of USP13. The mutant consisting only of USP domain was detected in heat-induced SGs (Fig. 9C), suggesting that USP domain itself has the ability to recruit USP13 to heat-induced SGs.

6. Depletion of USP5 or USP13 elevates the level of conjugated ubiquitin in heat-induced SGs

I examined whether USP5 and USP13 hydrolyze ubiquitin chains in heat-induced SGs. I carried out knockdown experiments by transfection of small interfering RNAs (siRNAs; siUSP5 and siUSP13 for USP5 and USP13, respectively). Immunoblotting analyses confirmed efficient knockdown of these DUBs (Fig. 10A). To evaluate the effect on the level of conjugated ubiquitin, regardless of whether they are unanchored chains or conjugated to target proteins, I stained cells with FK2 anti-ubiquitin antibody. Compared with the level of conjugated ubiquitin in heat-induced SGs in control cells, those in cells transfected with siUSP5, siUSP13, or both were elevated (Fig. 10B, C). In siUSP5-transfected cells, the FK2 staining level was also significantly elevated in the cytoplasm, implying the presence of USP5 substrates (*i.e.*, unanchored ubiquitin chains) in the cytoplasm (Fig. 10B). These results suggested that USP5 and USP13 exhibit DUB activity and regulate the ubiquitin chain levels in heat-induced SGs.

7. Depletion of USP5 or USP13 accelerates assembly of heat-induced SGs

I examined the roles of these DUBs in the assembly of heat-induced SGs. To evaluate the effects of their knockdown on SG formation rate, I stained cells after 45 min of incubation at 44°C. At this time point, G3BP-positive SGs were observed in cells transfected with siUSP5, siUSP13, or both, suggesting that USP5 and USP13 are dispensable for the formation of heat-induced SGs (Fig. 11A). Importantly, quantitative image analyses revealed that percentage of cells with SGs, number of SGs per cell, and SG size were increased in cells transfected with siUSP5, siUSP13, or both (Fig. 11B, C, D), showing that depletion of USP5 or USP13 accelerates

assembly of heat-induced SGs. Depletion of USP5 or USP13 by alternative siRNAs also accelerated assembly of heat-induced SGs (Fig. 12), excluding the possibility of siRNA off-target effects.

8. Depletion of USP5 or USP13 represses disassembly of heat-induced SGs

I found that when cells were incubated at 44°C for 1 h, the percentage of cells with SGs were elevated to 90% or more in control cells, similar levels to those in cells transfected with siUSP5, siUSP13, or both (Fig. 13A, B). At this time point, the numbers of SGs per cell were also similar between control cells and cells transfected with siUSP5, siUSP13, or both (Fig. 13C), while the SG sizes were still increased in cells transfected with siUSP5, siUSP13, or both (Fig. 13D). I examined the roles of these DUBs in the disassembly of heat-induced SGs. After cells were incubated at 44°C for 1 h, cells were returned to the normal temperature (37°C) for 1 h. The percentage of cells with SGs was reduced to 14% in control cells, while it was still 60% or more in cells transfected with siUSP5, siUSP13, or both (Fig. 14), suggesting that depletion of USP5 or USP13 represses disassembly of heat-induced SGs. Depletion of USP5 or USP13 by alternative siRNAs also repressed disassembly of heat-induced SGs (Fig. 15), excluding the possibility of siRNA off-target effects. In addition, I performed rescue experiments, and found that re-expression of these DUBs in the knockdown cells partially restored the SG disassembly (Fig. 16). The restoration was partial presumably because, in cells re-expressing USP5 or USP13, it took a longer time for SGs to be disassembled due to increased cellular stress by plasmid transfection and overexpression of these DUBs. Re-expression of catalytically inactive mutants of USP5 and USP13 failed to restore the SG disassembly (Fig. 16), showing the importance of their deubiquitinating activities in the regulation of SG disassembly. I also examined the effects of depletion of USP5 or USP13 on the assembly and disassembly of arsenite-induced SGs, and no effects were observed (Fig. 17).

9. Accumulation of unanchored ubiquitin chains represses disassembly of heat-induced SGs

As USP5, but not USP13, selectively hydrolyzes unanchored ubiquitin chains (Wilkinson et al., 1995; Amerik et al., 1997; Zhang et al., 2011), I examined whether

the accumulation of unanchored ubiquitin chains represses the disassembly of heat-induced SGs. Overexpression of ubiquitin with a mutation in the C-terminal diglycine motif (e.g., ubiquitin$^{G75A/G76A}$) results in the formation of unanchored ubiquitin chains in cells, because such a mutant can serve as the proximal ubiquitin of ubiquitin chains formed with endogenous ubiquitin molecules but these ubiquitin chains cannot be conjugated to target proteins (Hodgins et al., 1992; Amerik et al., 1997; Dayal et al., 2009). Moreover, these unanchored ubiquitin chains accumulate, because they are resistant to the hydrolysis by USP5 due to their lack of C-terminal diglycine motif in the proximal ubiquitin which is required for the substrate recognition by USP5 (Wilkinson et al., 1995). Actually, in cells overexpressing N-terminally Flag-tagged ubiquitin$^{G75A/G76A}$, immunoblotting with anti-ubiquitin antibody (clone P4G7) or anti-Flag antibody detected 3 bands (Fig. 18A, arrowheads). These are most likely unanchored ubiquitin monomers, dimers, and trimers, based on their migration rates compared with those of purified K48- and K63-linked ubiquitin chains. In contrast, in cells overexpressing Flag-tagged WT ubiquitin, I observed anti-Flag antibody-positive signals at high-molecular-weight region (Fig. 18A, middle panel, asterisk). These are most likely polyubiquitin-protein conjugates, based on their migration rates compared with endogenous polyubiquitin-protein conjugates detected by anti-ubiquitin antibody (Fig. 18A, top panel, asterisk).

I performed confocal microscopy analysis of cells expressing Flag-tagged WT ubiquitin or ubiquitin$^{G75A/G76A}$. When cells were continuously cultured at 37°C, WT ubiquitin as well as ubiquitin$^{G75A/G76A}$ was detected in the cytoplasm and nucleus. When cells were incubated at 44°C for 55 min, nuclear signals of WT ubiquitin were reduced but those of ubiquitin$^{G75A/G76A}$ were not obviously affected. The reason for this difference was unclear. G3BP-positive SGs were observed at similar levels in both cells, and a part of WT ubiquitin as well as ubiquitin$^{G75A/G76A}$ was detected within the SGs (Fig. 18B). After the recovery incubation at 37°C, the percentage of cells with SGs was 35% in cells expressing WT ubiquitin, while it was 76% in cells expressing ubiquitin$^{G75A/G76A}$ (Fig. 18B, C), indicating that accumulation of unanchored ubiquitin chains represses disassembly of heat-induced SGs.

10. The substrate of USP13 in SGs needs further investigation

To identify the substrate of USP13 in SGs, I focused on known USP13 substrates, Beclin1 and STAT1. Beclin1 can be deubiquitinated and stabilized by USP13 (Liu et al., 2011). Beclin1 can regulates autophagy as a component of Vps34 complexes, and autophagy is involved in the clearance of SGs (Buchan et al., 2013). Thus, I suspected that USP13 may regulate the stability of SGs through Beclin1. By immunofluorescence, I found that a fraction of Beclin1 was recruited to G3BP-positive SGs induced by heat stress. USP13 knockdown did not obviously reduce Beclin1 levels in the SGs (Fig. 19A), suggesting that Beclin1 in the SGs is not a substrate of USP13. STAT1 can be deubiquitinated and stabilized by USP13 (Yeh et al., 2013), and is identified as one of SG core proteins (Jain et al., 2016). I found that a fraction of STAT1 was recruited to heat-induced SGs, but USP13 knockdown did not reduce STAT1 levels in the SGs (Fig. 19B). It suggested that STAT1 in the SGs is not a substrate of USP13. Thus, further screening is needed to identify USP13 substrates involved in the SG regulation.

Discussion

Ubiquitination and deubiquitination are implicated in a broad range of cellular processes as posttranslational modifications. Ubiquitin is contained in SGs (Kwon et al., 2007), but its specific role was unknown. Here I provide evidences indicating that structural but not functional paralogues of DUBs, USP5 and USP13, are re-localized to SGs upon heat stress and play roles in the destabilization of SGs most likely through hydrolyzing ubiquitin chains and protein-ubiquitin conjugates, respectively (Fig. 20).

1. Presence of USP5, USP13, and ubiquitin chains in SGs under specific stress conditions

Stress-specific differences in the molecular composition and assembly of SGs have been reported (Kedersha et al., 1999; Aulas et al., 2017). While a recent proteome analysis in human osteosarcoma U2-OS cells identified USP5 in arsenite-induced SGs (Jain et al., 2016), I found that USP5 and USP13 are recruited preferentially to heat-induced SGs as well as SGs induced by puromycin and VER-155008 and SGs induced by poly(I:C), rather than other stresses (Fig. 4). In addition, K48- and K63-linked ubiquitin chains, which may be unanchored or conjugated to some proteins, were also preferentially recruited to heat-induced SGs as well as SGs induced by puromycin and VER-155008 and SGs induced by poly(I:C) (Fig. 5), suggesting a specific role for ubiquitin chains and their hydrolysis in the regulation of SGs induced by these stresses.

My super-resolution microscopy analysis and immunoprecipitation experiment demonstrated that USP5 and USP13 are localized to the SG shell (Fig. 6 and Fig. 7). It is proposed that components in the SG shell are highly mobile, while those in the cores are less dynamic (Jain et al., 2016). Thus, USP5 and USP13 in the SGs may be mobile and transiently associate with other SG components. My analysis also revealed that small portion of USP5 and USP13 within the SGs are co-localized with ubiquitin (Fig. 6), suggesting that these DUBs transiently associate with and hydrolyze ubiquitin chains in SGs.

2. Mechanisms underlying the accumulation of ubiquitin chains in SGs

Mechanisms underlying the accumulation of ubiquitin chains in SGs under certain stress conditions are obscure, but I propose possible models in Fig. 20A. Heat-induced translocation of K48-linked ubiquitin chains from the nucleus must contribute to their accumulation in the SGs, while the transport mechanism remains unclear. Because ubiquitin chains by themselves autonomously assemble under high temperature conditions (Morimoto et al., 2015), heat stress can promote the assembly of pre-existing ubiquitin chains, which may also contribute to the intense accumulation of ubiquitin in heat stress-induced SGs.

In addition, it is reported that, in cells expressing misfolding-prone model proteins, heat stress induces ubiquitin-positive SGs whereas arsenate or proteasome inhibition induces SGs devoid of ubiquitin (Mateju et al., 2017), which is consistent with my observation. Interestingly, this report also showed that concomitant treatment with HSP70 inhibitor triggers the accumulation of ubiquitin in the SGs induced by arsenate or proteasome inhibitor (Mateju et al., 2017). Thus, I speculate that stressors such as arsenate and proteasome inhibitor may induce the recruitment of ubiquitinated proteins to the SGs to a lesser extent than heat stress, but their accumulation in SGs is prevented by the action of HSP70.

A cytoplasmic viral RNA sensor protein RIG-I, which associates with ubiquitin chains in response to viral infection, is recruited to SGs induced by viral infection (Onamoto et al., 2012). It is consistent with my observation that poly(I:C)-induced SGs contain ubiquitin chains. The amounts of ubiquitin chains in the SGs were less than those in SGs induced by heat stress or by puromycin and VER-155008 (Fig. 5). The reason may be that viral infection induces the ubiquitination of specific proteins including RIG-I in a controlled manner, whereas stressors such as heat stress cause misfolding and ubiquitination of large amounts of cellular proteins, nonspecifically. The makeup of viral infection-induced SGs seems to differ fundamentally from SGs induced by heat stress and other stresses.

3. Mechanisms underlying the recruitment of USP5 and USP13 to SGs

What is the mechanism of recruitment of USP5 and USP13 to heat-induced SGs? I propose a possible model in Fig. 20B. USP5 harbors four ubiquitin binding domains: USP domain which contains the enzyme active site, ZnF-UBP domain, and two UBA domains (Reyes-Turcu et al., 2008). USP13 also has these domains while the ZnF-UBP domain lacks the ubiquitin-binding ability (Zhang et al., 2011). My

experiments using USP13 mutants demonstrated that neither the ZnF-UBP domain nor the tandem UBA domains are necessary for the recruitment of USP13 to heat-induced SGs, and that the USP domain itself has the ability to recruit USP13 to the SGs (Fig. 10). Therefore, it is possible that the USP domain recruits these DUBs to the SGs through the interaction with ubiquitin chains in the SGs. This notion is supported by the observation that USP5 and USP13 were also recruited to ubiquitin-positive SGs induced by puromycin and VER-155008, as well as by poly(I:C).

4. Roles of USP5, USP13, and ubiquitin chains in the regulation of SG stability

Depletion of USP5 or USP13 elevates ubiquitinated protein levels in heat-induced SGs (Fig. 10), accelerates the SG assembly (Fig. 11 and Fig. 12), and represses the SG disassembly (Fig. 14 and Fig. 15). Moreover, re-expression of catalytically inactive mutants of USP5 and USP13 failed to restore SG disassembly (Fig. 16). Taken together, I believe that during heat stress, the supply of ubiquitinated proteins to SGs may exceed the deubiquitinating capacity of these USP5 and USP13, causing the accumulation of ubiquitin chains in SGs. When cells are returned to the normal temperature, the supply of ubiquitinated proteins diminishes and ubiquitinated protein levels in SGs are reduced by these DUBs, contributing to the SG disassembly.

Although both USP5 and USP13 are recruited to heat-induced SGs, my results indicate that these DUBs have non-redundant roles in the SGs because depletion of USP5 or USP13 individually exhibited significant effects on the assembly and disassembly of heat-induced SGs. USP5 has substrate specificity toward unanchored ubiquitin chains (Wilkinson et al., 1995; Amerik et al., 1997). In contrast, USP13 has a structurally different ZnF-UBP domain lacking affinity for unanchored ubiquitin chains, and selectively hydrolyzes ubiquitin chains attached to its target proteins (Zhang et al., 2011). Thus, I propose that USP5 and USP13 act on different steps in the process of SG assembly/disassembly, and hydrolysis of unanchored ubiquitin chains by USP5 and that of protein-conjugated ubiquitin chains by USP13 are both required for the regulation of SG stability. Due to the lack of anti-ubiquitin antibodies that specifically stain unanchored chains in immunofluorescence, I was not able to examine whether unanchored ubiquitin chains are indeed present in heat-induced SGs and if their level is elevated upon USP5 depletion. I therefore took another approach using a C-terminal mutant of ubiquitin, and provided the evidence that elevating the

unanchored ubiquitin chain levels in heat-induced SGs represses their disassembly (Fig. 18), supporting my proposal that hydrolysis of unanchored ubiquitin chains is required for the SG disassembly. On the other hand, my result indicated that Beclin1 and STAT1 are not the substrates of USP13 in SGs (Fig. 19). Thus, it is important to determine what kinds of proteins are deubiquitinated by USP13 in the SGs.

USP10, known as Ubp3 in yeast, is another DUB recruited to SGs upon various stress including heat (Ohn et al., 2008; Wang et al., 2012; Takahashi et al., 2013; Kedersha et al., 2016; Nostramo et al., 2015). However, USP10/Ubp3 is required for the assembly, but not disassembly, of SGs through its catalytic activity in the budding yeast (Nostramo et al., 2015), while this DUB is dispensable for SG assembly/disassembly in the fission yeast (Wang et al., 2012). Mammalian USP10 is essential for SG assembly, independently of its DUB activity and dependently of its interaction with G3BP (Takahashi et al., 2013; Kedersha et al., 2016). Therefore, the roles for USP10/Ubp3 in SGs are clearly different from those for USP5 and USP13.

5. Mechanisms by which the hydrolysis of ubiquitin chains by USP5 and USP13 can promote the SG disassembly

I propose possible mechanisms by which the hydrolysis of ubiquitin chains by USP5 and USP13 can promote the SG disassembly (Fig. 20C). One possible mechanism is that USP5-induced hydrolysis of unanchored ubiquitin chains may inhibit the transport of unfolded proteins into SGs. In cells, most of unfolded proteins are ubiquitinated, and the ubiquitin chains are often released from unfolded proteins by DUBs, ataxin-3 and Poh1, resulting in the formation of the complexes of unanchored ubiquitin chains and unfolded proteins (Ouyang et al., 2012; Hao et al., 2013). These complexes are known to be transported by HDAC6-dynein motor complex, through the recognition of the unanchored ubiquitin chains by the ZnF-UBP domain of HDAC6 (Ouyang et al., 2012; Hao et al., 2013). Interestingly, HDAC6 is required for the formation of SGs (Kwon et al., 2007), and SGs seem to contain significant levels of unfolded proteins (Mateju et al., 2017). These suggest that the complexes of unanchored ubiquitin chains and unfolded proteins may accumulate at SGs by the action of HDAC6-dynein motor complex, and USP5-induced hydrolysis of the unanchored ubiquitin chains may inhibit the accumulation.

Another possibility is that USP5 and USP13 hydrolyze ubiquitin chains in SGs and prevent the formation of ubiquitin chain aggregates in SGs, which promote the

SG destabilization. A recent report demonstrates that ubiquitin chains have a tendency to form insoluble fibrils at high temperatures *in vitro* and in cells, and that this property of ubiquitin chains depends on the chain length, but not on their linkage types or whether they are conjugated to target proteins (Morimoto et al., 2015). Thus, heat-induced aggregation of ubiquitin chains may be involved in the stabilization of SGs, and the hydrolysis of ubiquitin chains in SGs by USP5 and USP13 may decrease the ubiquitin chain aggregates, resulting in the destabilization of SGs.

6. Conclusions and perspectives

Taking all data together, I concluded that;

- Large amounts of ubiquitin chains are contained in SGs induced by specific stress conditions, such as heat stress.
- USP5 and USP13 are preferentially recruited to heat-induced SGs, possibly through the interaction between their USP domain and ubiquitin chains in the SGs.
- In SGs, USP5 hydrolyzes unanchored ubiquitin chains and USP13 hydrolyzes ubiquitin chains conjugated to uncharacterized proteins. Both reactions are required for the efficient destabilization of SGs.

To my knowledge, this study is the first report demonstrating that ubiquitin chains in SGs play an important role in the stabilization of SGs. Mutations in proteins associated with neurodegenerative diseases have been implicated in abnormal assembly/disassembly of SGs (Dormann et al., 2010; Elden et al., 2010; Liu-Yesucevitz et al., 2010; Buchan et al., 2013; Kim et al., 2013). Thus, it is of not only cell biological but also pathophysiological and medical importance to elucidate the more precise molecular mechanisms by which USP5 and USP13 facilitate SG destabilization. I anticipate that my future work will propose USP5 and USP13 as a novel therapeutic target in diseases caused by aberrant SG formation.

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Fig. 1

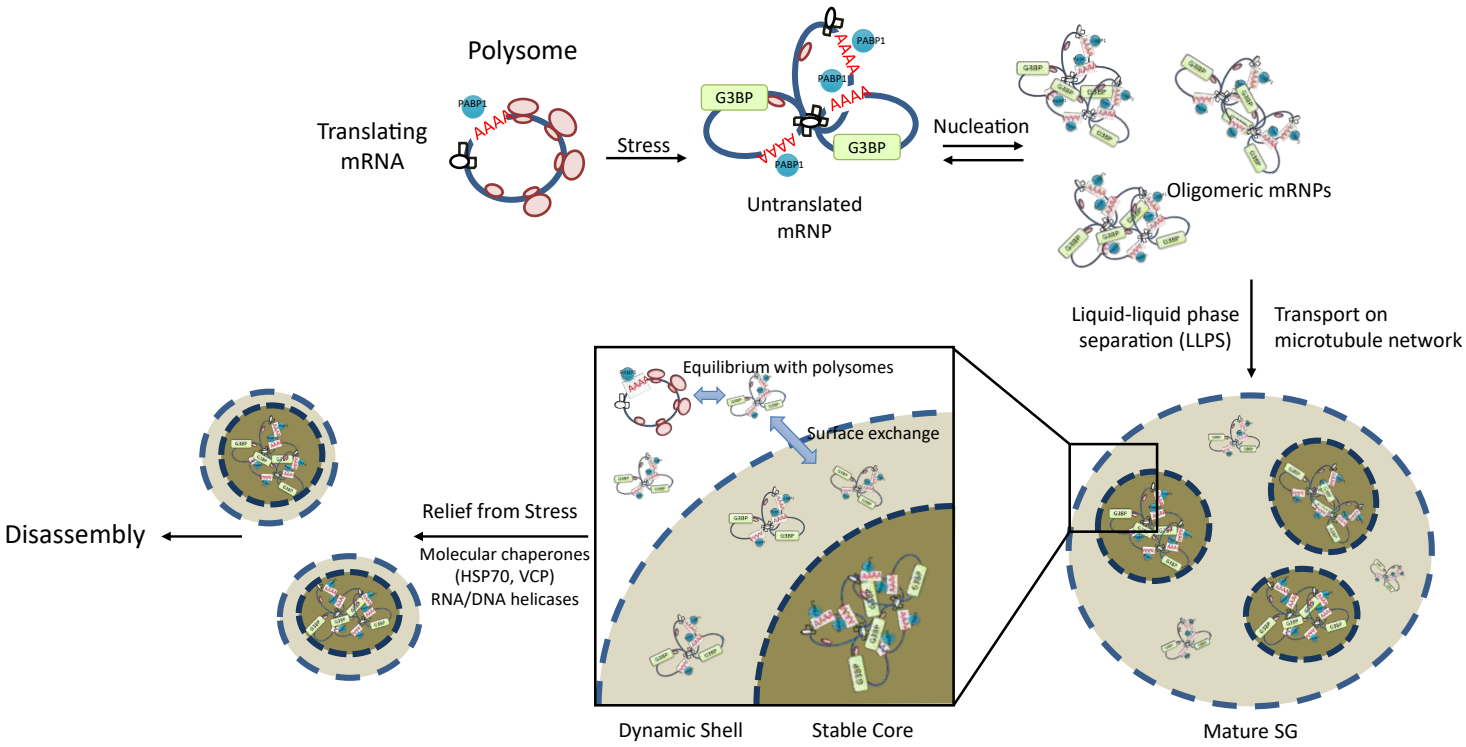

Fig. 1. The assembly and disassembly of SG
Cellular stresses cause the dissociation of polysomes, resulting in the generation of excess untranslated mRNPs. High local concentration of mRNP nucleates oligomeric mRNPs. The interaction of RNAs and RNA-binding proteins surrounding the oligomers causes liquid-liquid phase separation (LLSP). These oligomers and the compartment made by LLSP are thought to be SG cores and SG shell, respectively. SG cores are relatively stable, while SG shell has more frequent material exchange with cytoplasm. When cells are relieved form stress, SGs are disassembled. This process may be regulated by molecular chaperons and RNA/DNA helicases.

Fig. 2

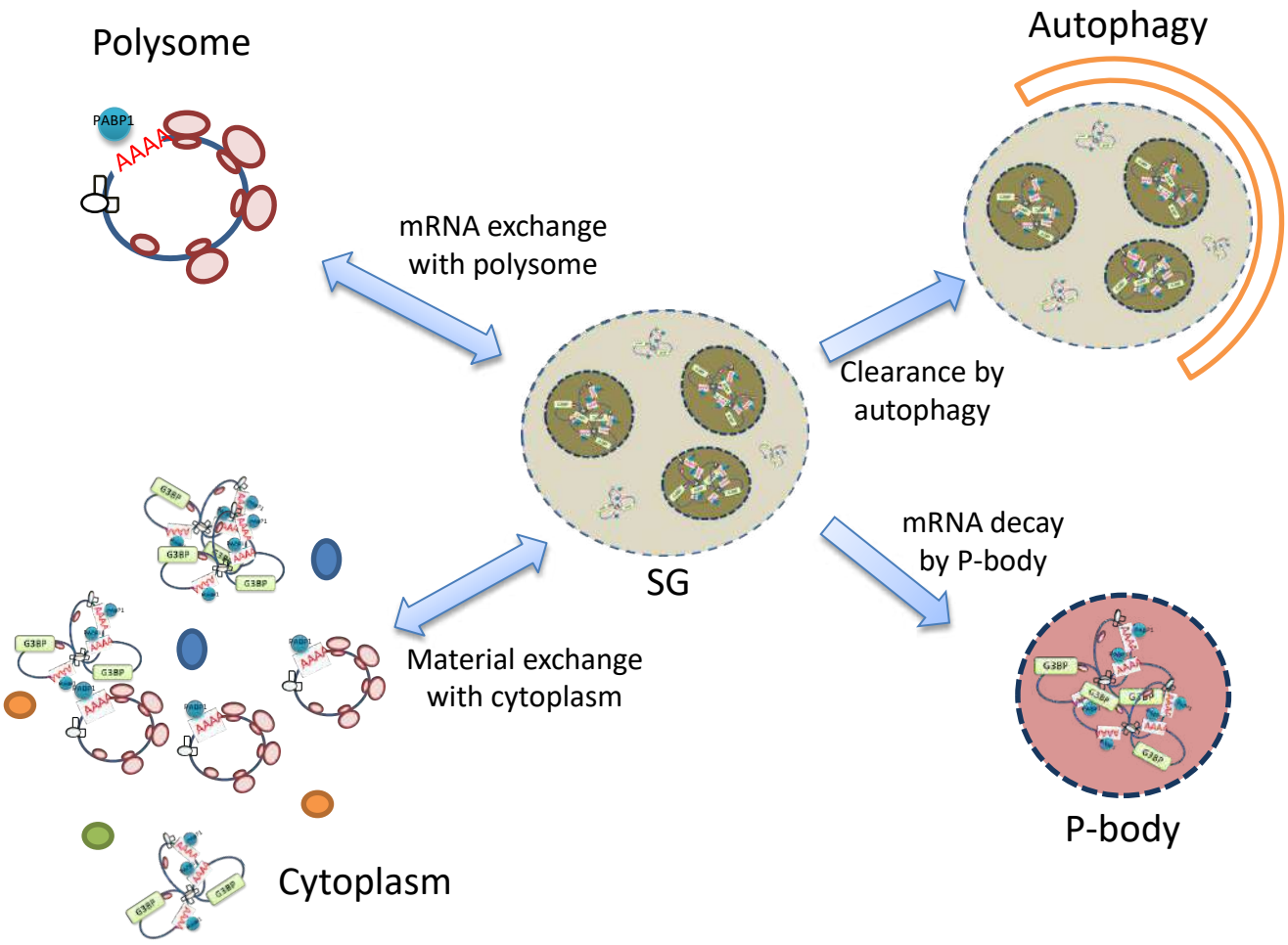

Fig. 2. The interaction of SGs with cytoplasm and other organelles
SGs are dynamic sites of mRNP remodeling. SGs frequently exchange components with cytoplasm, which helps to modulate mRNA translation and some reactions in cytoplasm. Upon the formation of SGs, mRNAs are assembled from polysomes into SGs. During the disassembly of SGs, part of mRNAs can be returned back to polysomes and start translating again. SGs can also physically interact with P-bodies, which are mRNA decay machinery. The interaction may allow the degradation of some mRNAs in SGs. In addition, SGs can be cleared by autophagy system.

Fig. 3

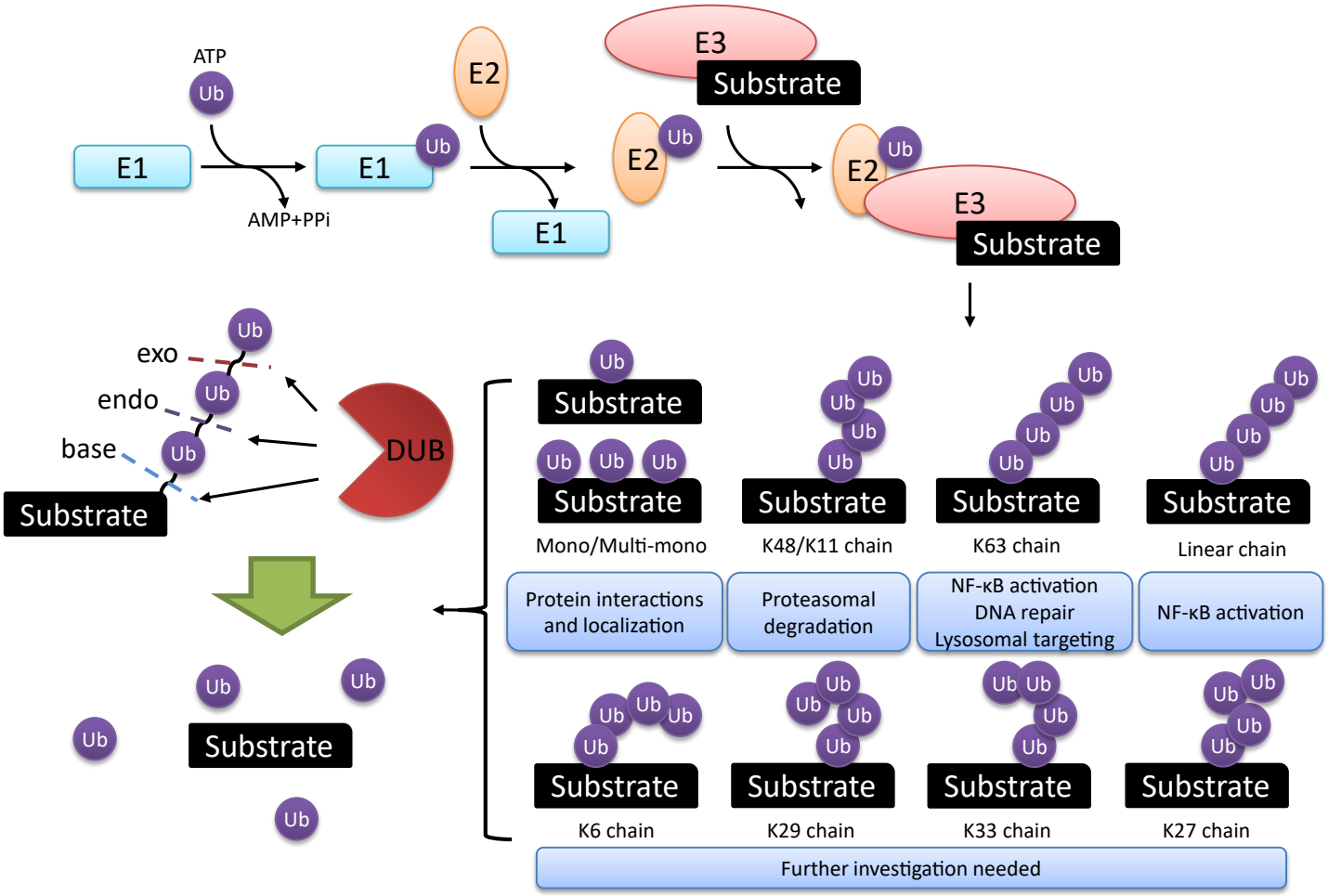

Fig. 3. Schematic diagram of ubiquitination and deubiquitination
The sequential activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) mediates the formation of isopeptide bond between the carboxyl group of Gly76 residue of ubiquitin and ε-amino group of Lys residue in substrate or in another ubiquitin molecule. Substrates can be monoubiquitinated, multi-monoubiquitinated and polyubiquitinated. Polyubiquitin chains can be classified by ubiquitin linkage types. Different types of ubiquitin chains are involved in different cellular processes. Ubiquitin chains are cleaved by deubiquitinating enzymes (DUB). DUBs show exo-activity, endo-activity or cleavage activity at the base of a chain. DUBs have numerous physiological functions including regulation of protein stability and ubiquitin recycling.

Fig. 4

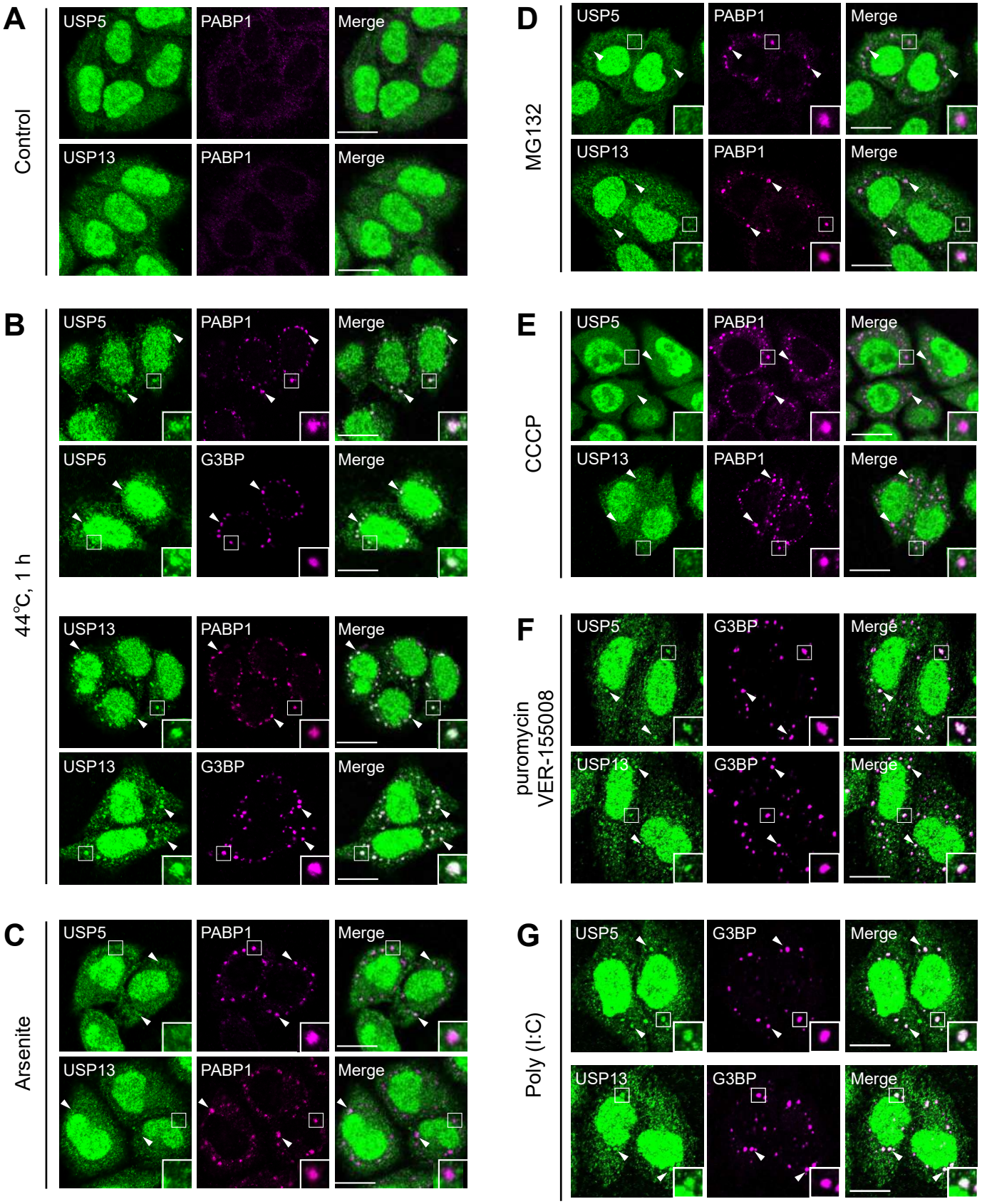

Fig. 4. USP5 and USP13 are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008 and SGs induced by poly(I:C)

HeLa cells were cultured under normal conditions (A), subjected to heat stress (44°C for 1 h) (B), or treated with arsenite (0.5 mM for 45 min) (C), MG132 (0.2 mM for 3 h) (D), CCCP (20 μM for 1.5 h) (E), or puromycin and VER-155008 (5 μg/ml and 100 μM, respectively, for 3 h) (F), or transfected with poly(I:C) (400 ng/ml) using a transfection reagent polyethylenimine (G). Cells were then stained with anti-USP5 or anti-USP13 antibody together with anti-PABP1 or anti-G3BP antibody. Arrowheads indicate typical SGs. Insets show higher magnification images of regions indicated by squares. Bars, 20 μm. The experiments were repeated more than 3 times with similar results.

Fig. 5

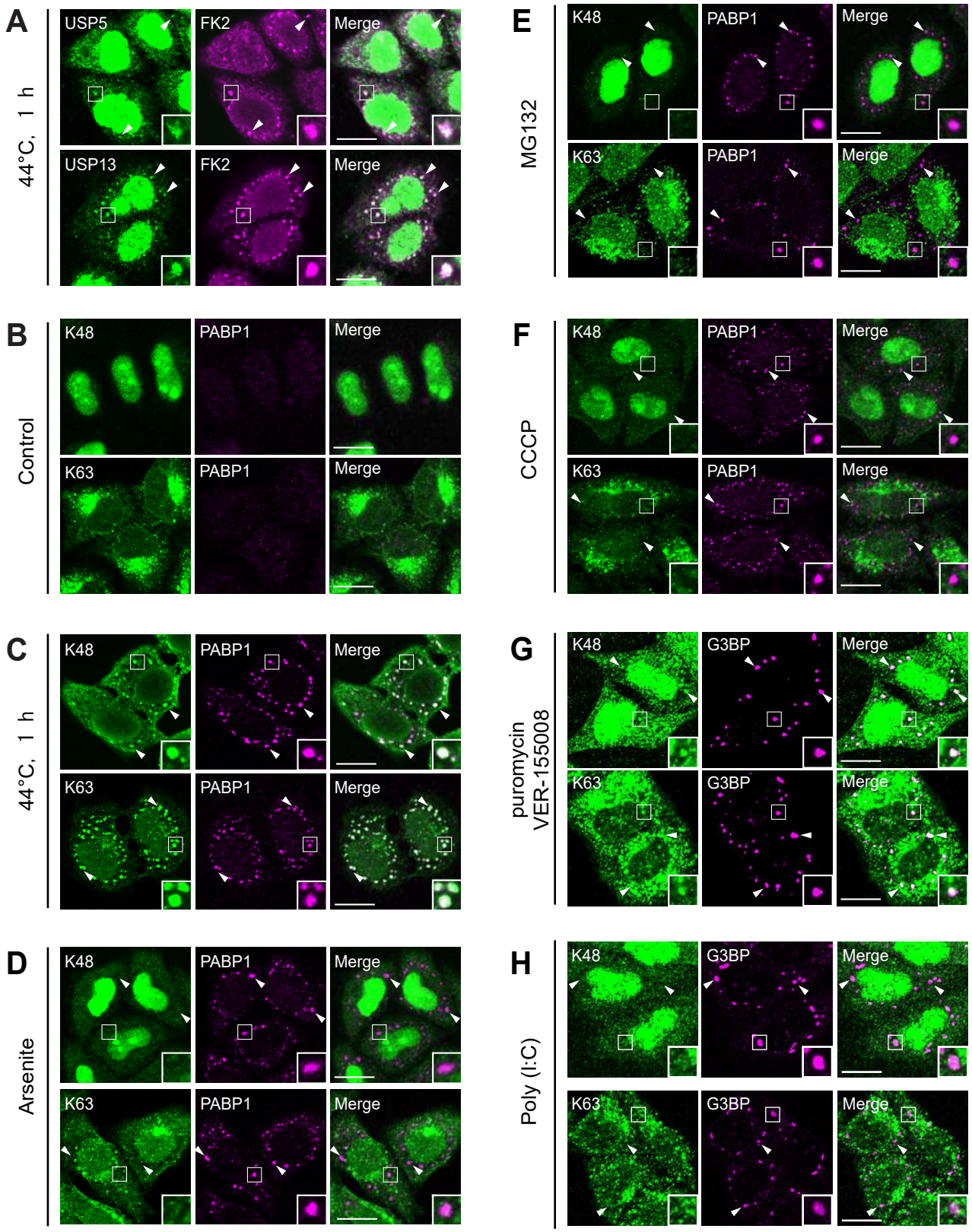

Fig. 5. Ubiquitin chains are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008 and SGs induced by poly(I:C)

(A) Cells were subjected to heat stress (44°C for 1 h), and stained with anti-USP5 or anti-USP13 antibody together with the FK2 anti-ubiquitin antibody. (B-G) Cells were cultured under normal conditions (B), subjected to heat stress (44°C for 1 h) (C), or treatment with arsenite (0.5 mM for 45 min) (D), MG132 (0.2 mM for 3 h) (E), CCCP (20 μM for 1.5 h) (F), or puromycin and VER-155008 (5 μg/ml and 100 μM, respectively, for 3 h) (G), or transfected with poly(I:C) (400 ng/ml) (H). Cells were then stained with antibody against K48- or K63-linked ubiquitin chains together with anti-PABP1 or anti-G3BP antibody. Arrowheads indicate typical SGs. Insets show higher magnification images of regions indicated by squares. Bars, 20 μm. The experiments were repeated more than 3 times with similar results.

Fig. 6

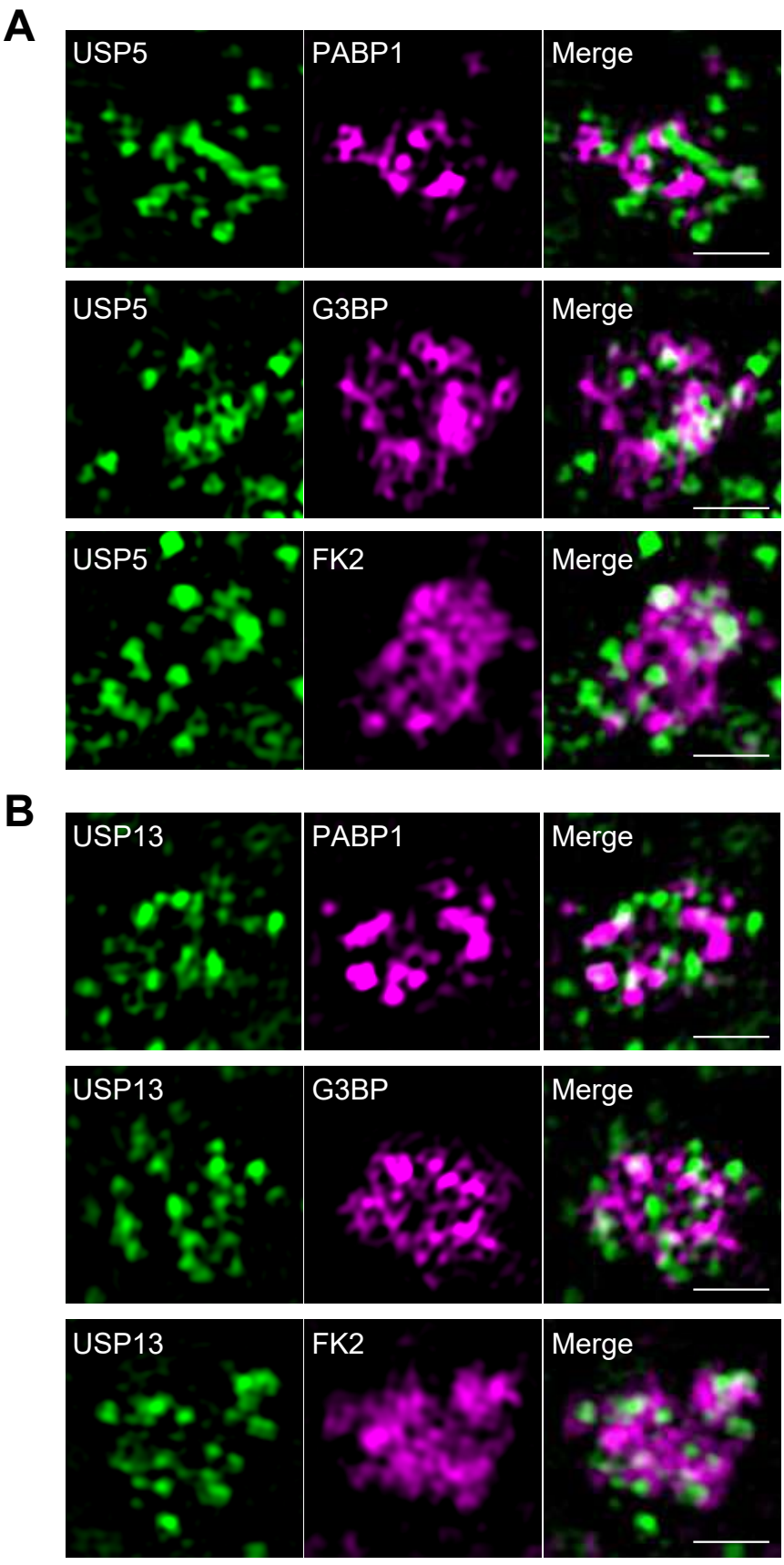

Fig. 6. USP5 and USP13 are adjacent to PABP1-, G3BP-, or ubiquitin-positive regions within heat-induced SGs

Cells were subjected to heat stress (44°C for 1 h), stained with anti-USP5 antibody (A) or anti-USP13 antibody (B) together with anti-PABP1, anti-G3BP, or FK2 anti-ubiquitin antibody, and observed using structured illumination microscopy. Each panel shows a single SG. Bars, 1 μm. The experiments were repeated 3 times with similar results.

Fig. 7

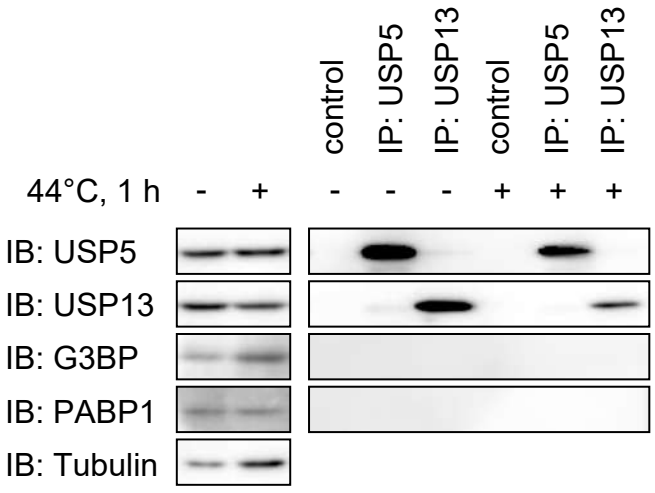

Fig. 7. Neither USP5 nor USP13 is stably interacted with PABP1 and G3BP
HeLa cells were cultured at 37°C or subjected to heat stress (44°C for 1 h). The cell lysates were subjected to the immunoprecipitation with anti-USP5 antibody or anti-USP13 antibody. The lysates and immunoprecipitates were subjected to immunoblotting with indicated antibodies.

Fig. 8

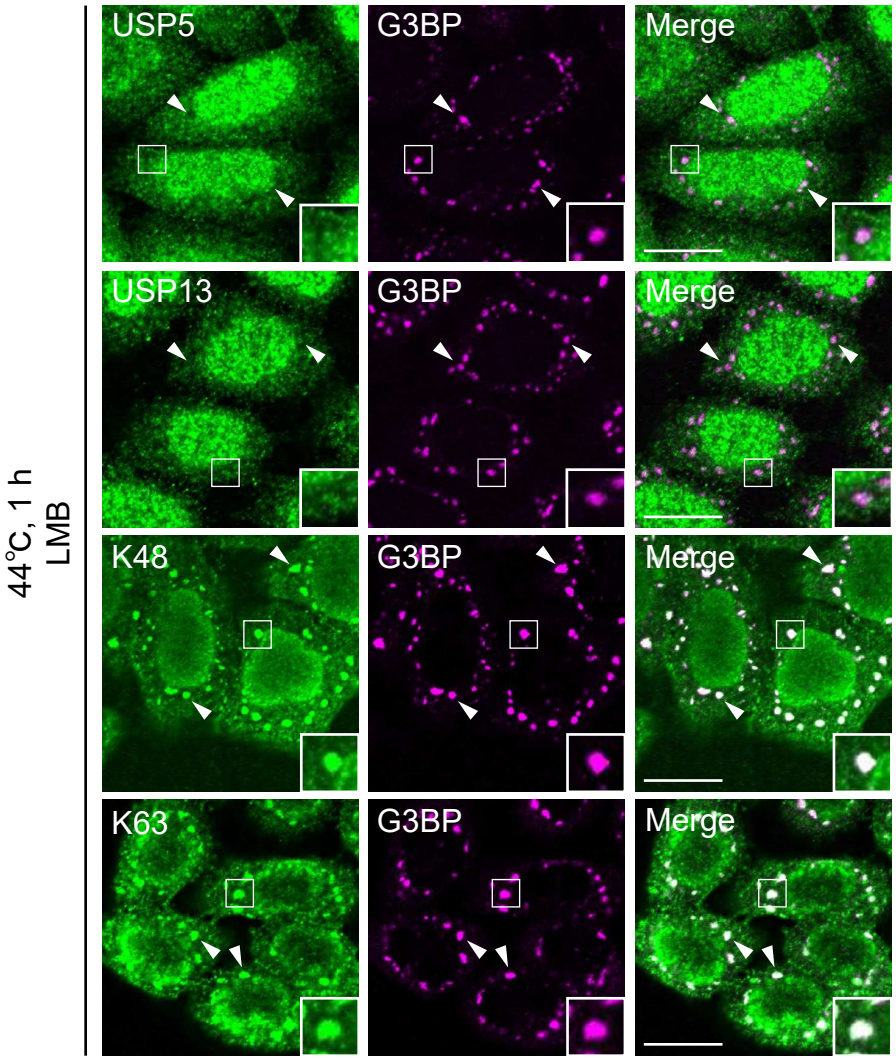

Fig. 8. A fraction of USP5 and USP13 in heat-induced SGs are translocated from the nucleus
Cells were cultured at 44°C for 1 h in the presence of 50 nM LMB, and stained with anti-USP5, anti-USP13, anti-K48-linked ubiquitin chain, or K63-linked ubiquitin chain antibody together with anti-G3BP antibody. Arrowheads indicate typical SGs. Insets show higher magnification images of regions indicated by squares. Bars, 20 μm. The experiments were repeated more than 3 times with similar results.

Fig. 9

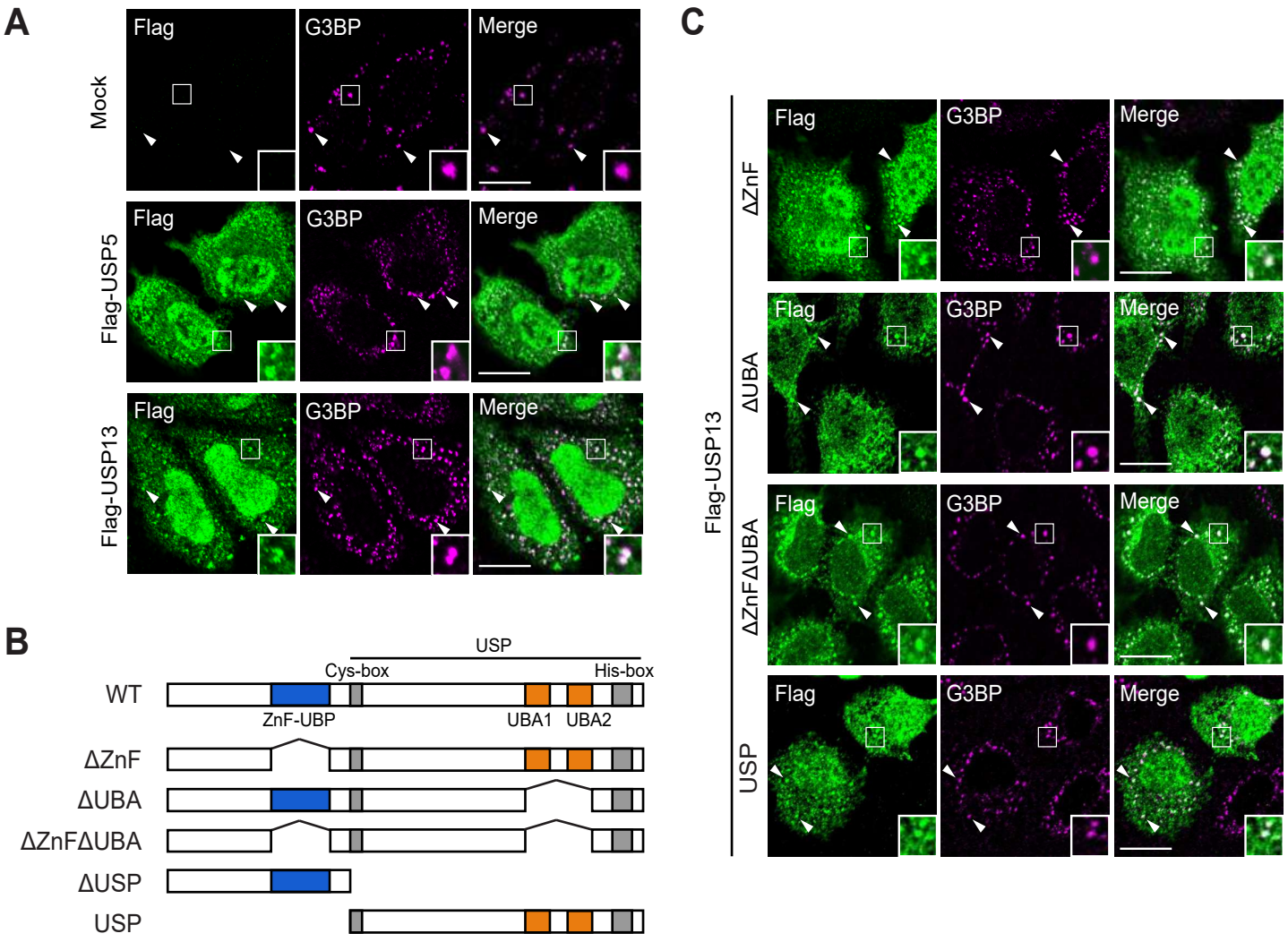

Fig. 9. The USP domain of USP13 has the ability to recruit USP13 to heat-induced SGs
(A) Cells were transfected with Flag-tagged USP5 or USP13, subjected to heat stress (44°C for 1 h), and then double-stained with anti-Flag and anti-G3BP antibodies. (B) The conserved domain structure of USP5 and USP13, and USP13 mutants used in C. ΔZnF, a deletion of amino acids 210 to 265 (aa210-265); Δ UBA, a deletion of aa654-766;ΔUSP, aa1-336; USP, aa337-863. (C) Cells were transfected with Flag-tagged USP13 mutants, subjected to heat stress (44°C for 1 h), and then double-stained with anti-Flag and anti-G3BP antibodies. In A and C, arrowheads indicate typical SGs, and insets show higher magnifition images of regions indicated by squares. Bars, 20 μm. The experiments were repeated more than 3 times with similar results.

Fig. 10

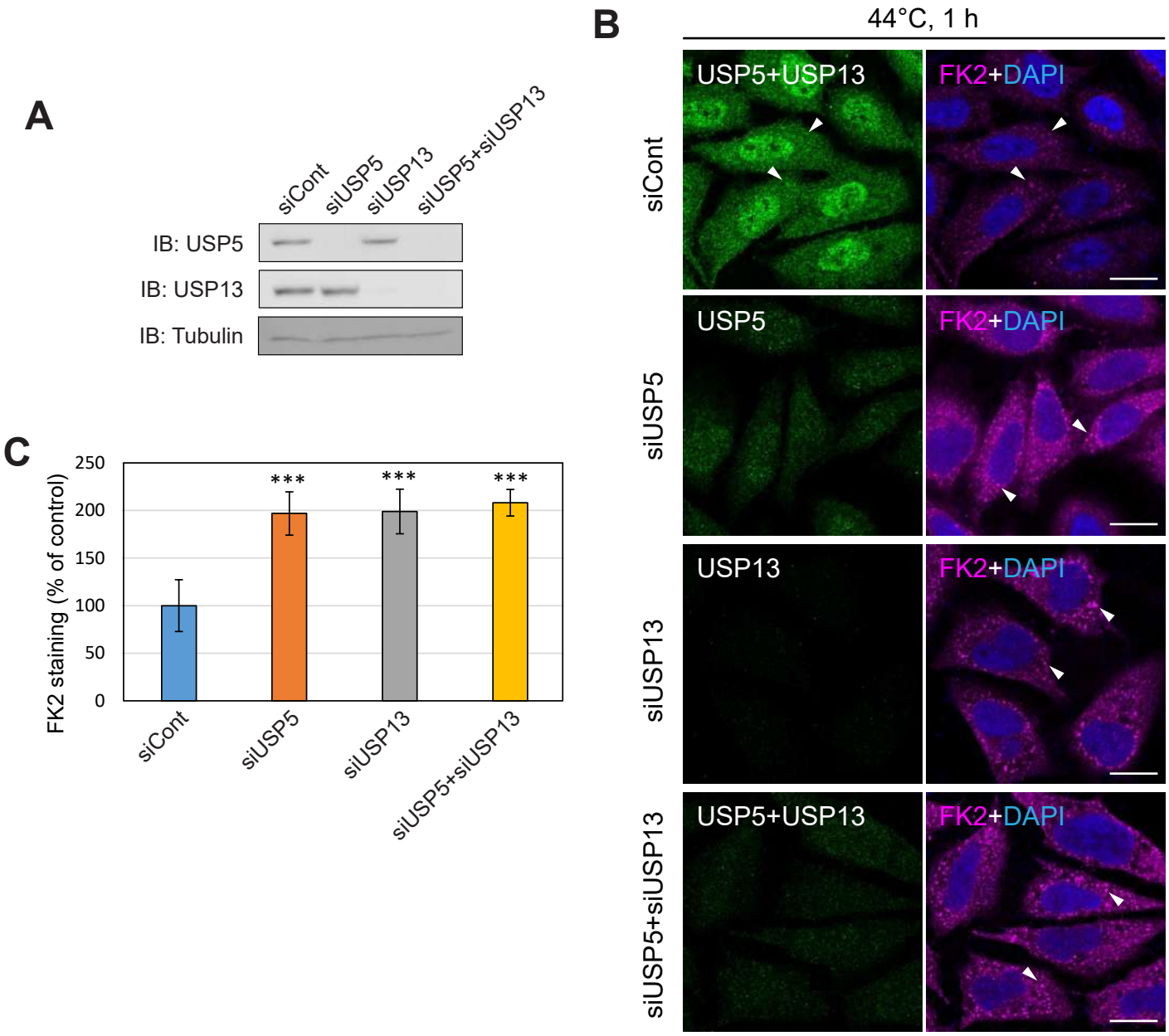

Fig. 10. Depletion of USP5 or USP13 elevates the level of conjugated ubiquitin in heat-induced SGs
Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-1), or siRNAs for USP13 (siUSP13-1) individually or in combination. (A) Lysates of the cells were subjected to immunoblotting with indicated antibodies. (B) The cells were subjected to heat stress (44°C for 1 h), and stained with anti-USP5 antibody and/or anti-USP13 antibody together with FK2 anti-ubiquitin antibody and DAPI. Arrowheads indicate typical ubiquitin-positive foci. Bars, 20 μm. (C) FK2 staining intensities in each SGs were measured, and shown as mean ± s.d. of 30 SGs. ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. 11

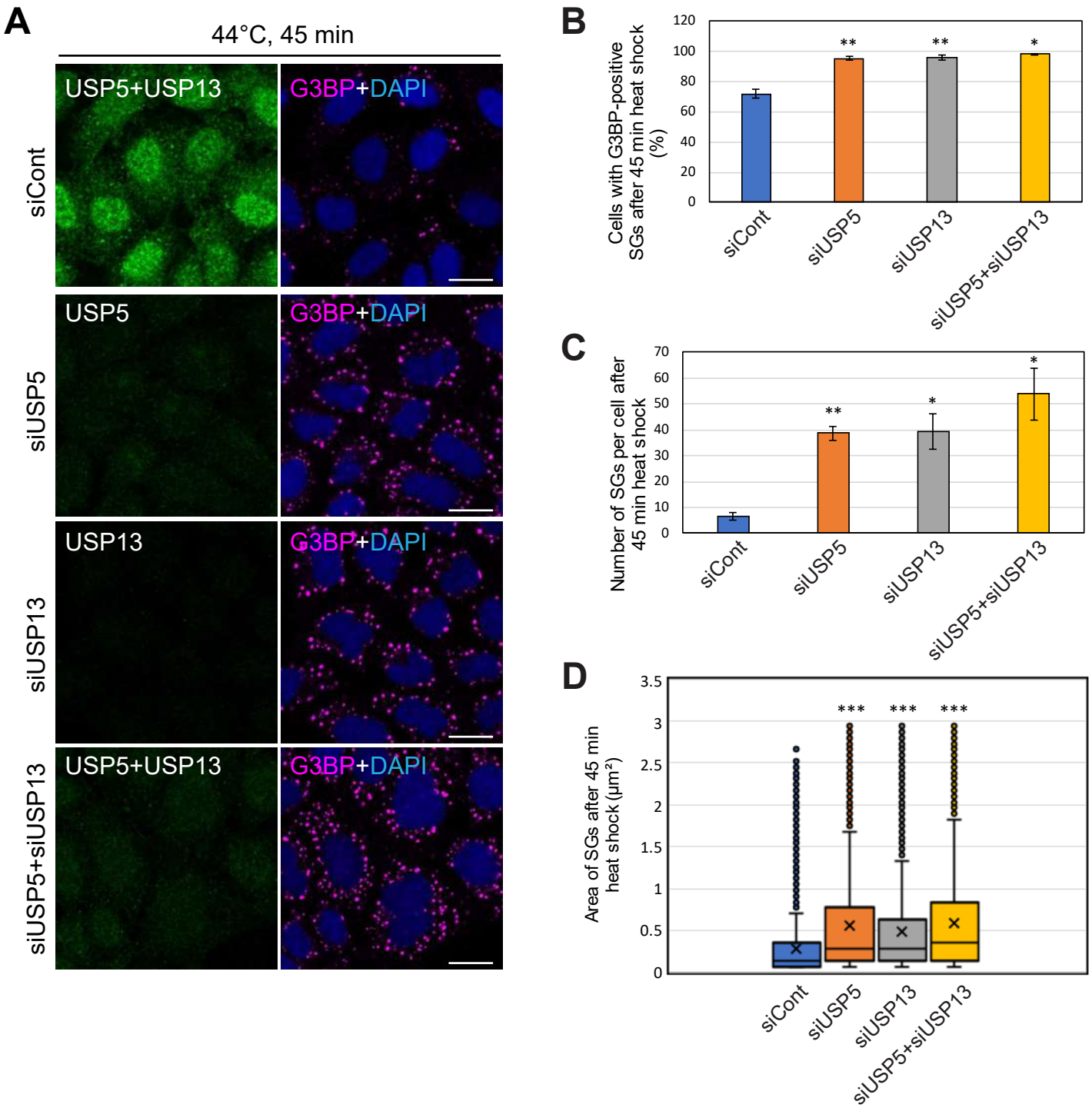

Fig. 11. Depletion of USP5 or USP13 accelerates assembly of heat-induced SGs
Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-1), or siRNAs for USP13 (siUSP13-1) individually or in combination. (A) The cells were subjected to heat stress (44°C for 45 min), and stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Bars, 20 μm. (B) The numbers of SG-bearing cells in B were counted and shown as a proportion to the total number of cells (mean ± s.e.m. of 3 independent experiments). *P<0.05, **P<0.01 versus control (two-tailed Student's *t*-test). (C) The numbers of SGs per cell were shown as mean ± s.e.m. of 3 independent experiments. *P<0.05, **P<0.01 versus control (two-tailed Student's *t*-test). (D) The sizes of all SGs observed in 3 independent experiments were measured and shown as the box and whisker plots, with the mean (x in the box), median (line in the box), 1st and 3rd quartiles (bottom and top of the box), maximum and minimum (bars extending outside of the box), and outlier values more than 1.5 times of 3rd quartile or less than 1.5 times of 1st quartile (dots). ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. 12

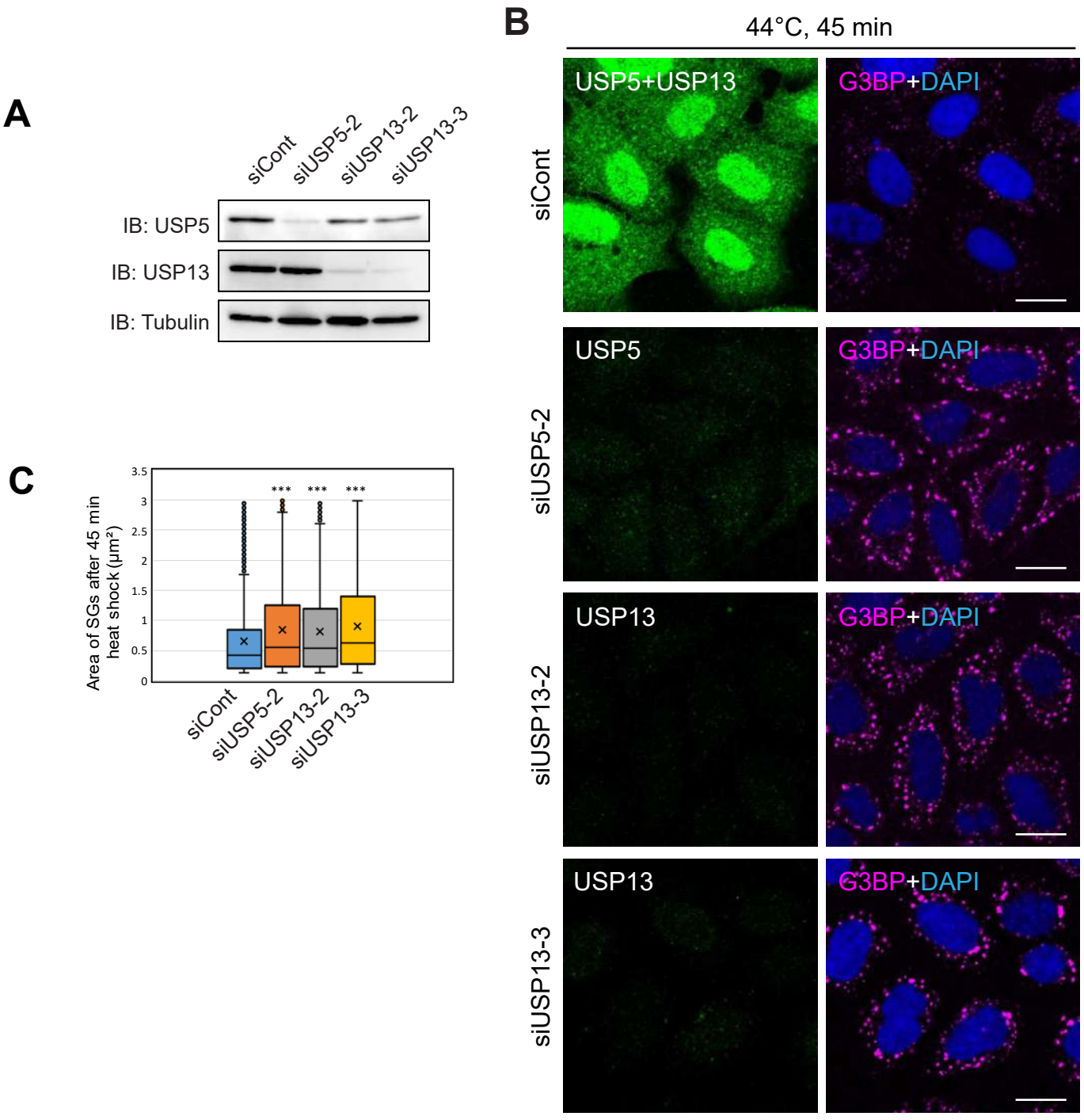

Fig. 12. Depletion of USP5 or USP13 by alternative siRNAs also accelerates assembly of heat-induced SGs

Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-2) or siRNAs for USP13 (siUSP13-2 or siUSP13-3). (A) Lysates of the cells were subjected to immunoblotting with indicated antibodies. (B) The cells were subjected to heat stress (44°C for 45 min), and stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Bars, 20 μm. (C) The sizes of all SGs observed in 3 independent experiments were measured and shown as the box and whisker plots in the same format as Fig. 11D. ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. 13

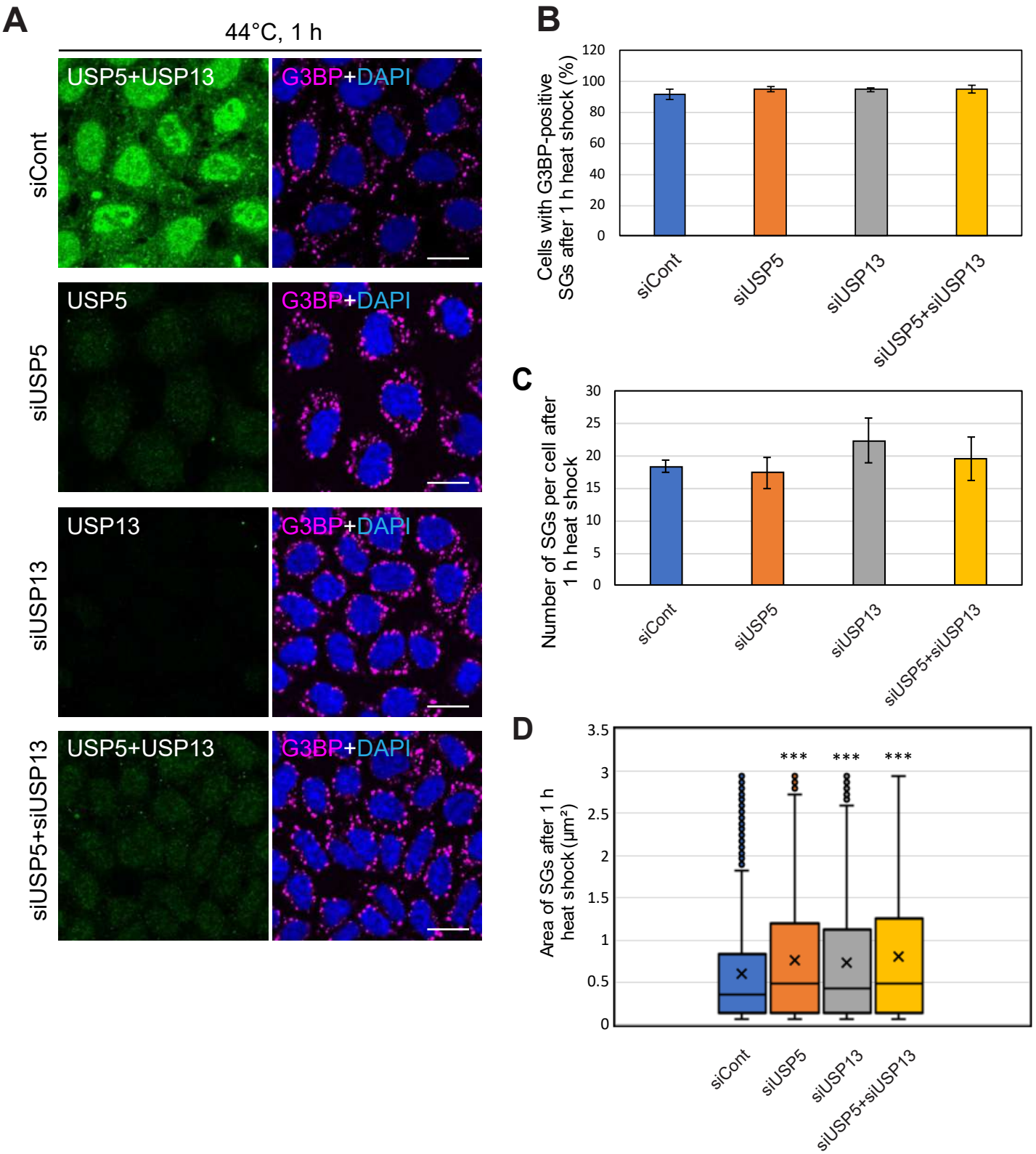

Fig. 13. Depletion of USP5 or USP13 does not affect the percentage of cells with SGs after 1h heat shock
(A) Cells were transfected with siRNAs for USP5 (siUSP5-1) and USP13 (siUSP13-1) individually or in combination, and subjected to heat stress (44°C for 1 h). Cells were then stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Bars, 20 μm. (B) The numbers of SG-bearing cells in A were counted and shown as a proportion to the total number of cells (mean ± s.e.m. of 3 independent experiments). There was no statistically significant difference between control and knockdown samples (two-tailed Student's *t*-test). (C) The numbers of SGs in A were shown as mean ± s.e.m. of 3 independent experiments. There was no statistically significant difference between control and knockdown samples (two-tailed Student's *t*-test). (D) The sizes of all SGs observed in 3 independent experiments were measured and shown as the box and whisker plots in the same format as Fig. 11D. ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. 14

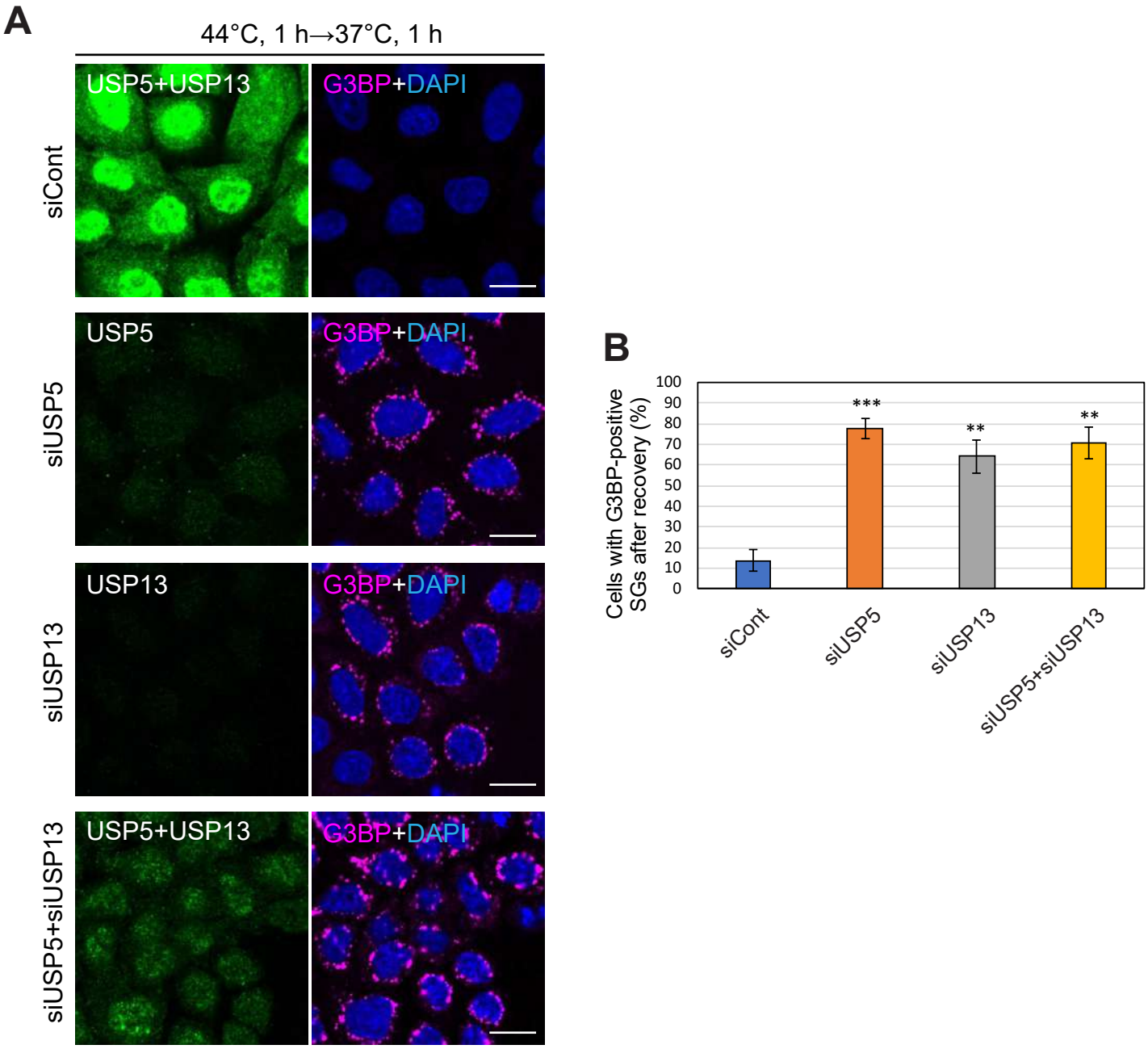

Fig. 14. Depletion of USP5 or USP13 represses disassembly of heat-induced SGs
(A) Cells treated as in Fig. 13A were then returned to normal conditions (37°C for 1 h), and stained as in Fig. 13A. Bars, 20 μm. (B) The numbers of SG-bearing cells in C were shown (mean ± s.e.m. of 3 independent experiments). **P<0.01, ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. 15

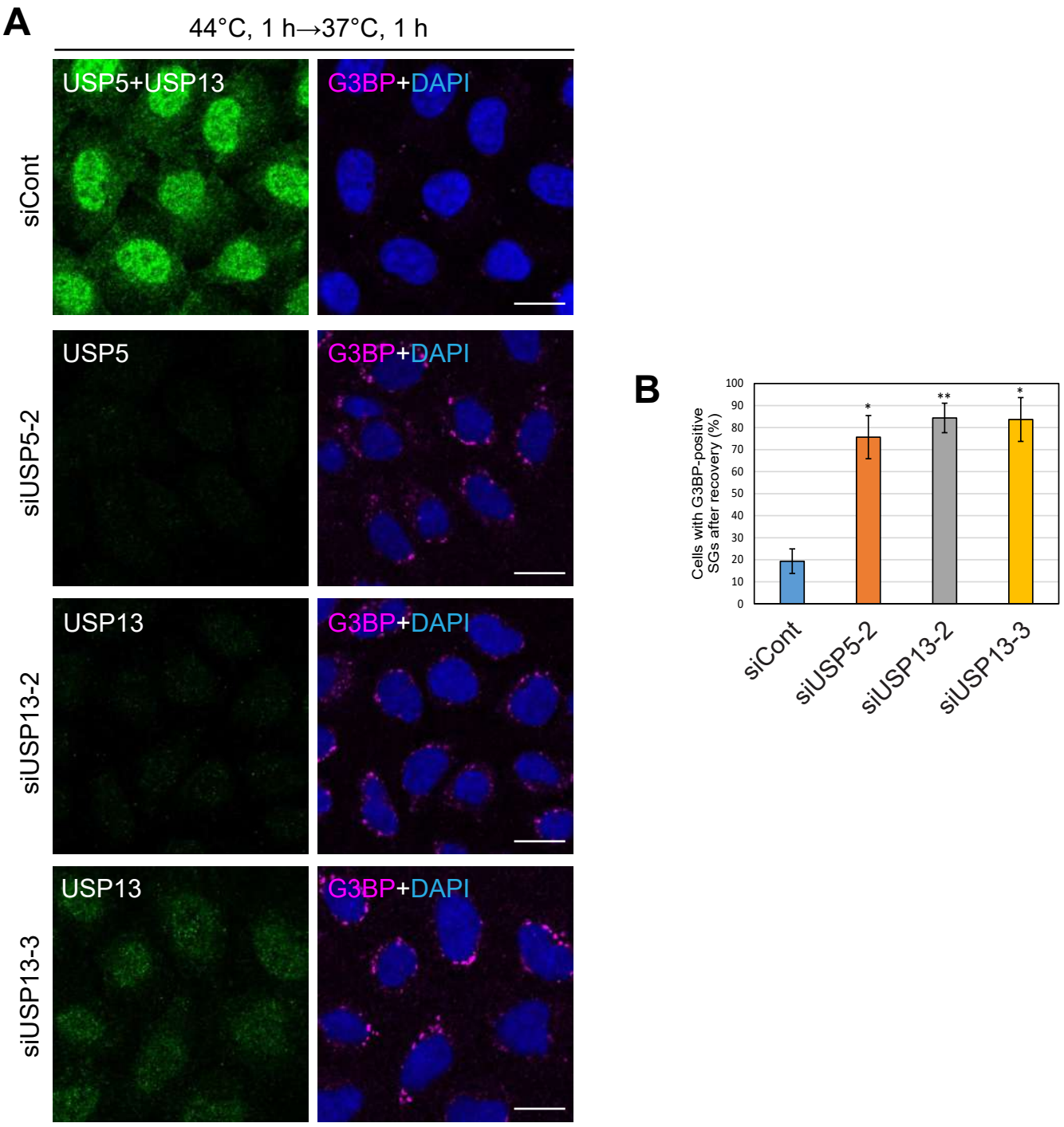

Fig. 15. Depletion of USP5 or USP13 by alternative siRNAs also represses disassembly of heat-induced SGs

(A) Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-2) or siRNAs for USP13 (siUSP13-2 or siUSP13-3), and subjected to heat stress (44°C for 1 h). Cells were then returned to normal conditions (37°C for 1 h), and stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Bars, 20 μm. (B) The numbers of SG-bearing cells in A were shown (mean ± s.e.m. of 3 independent experiments). *P<0.05, **P<0.01 versus control (two-tailed Student's *t*-test).

Fig. 16

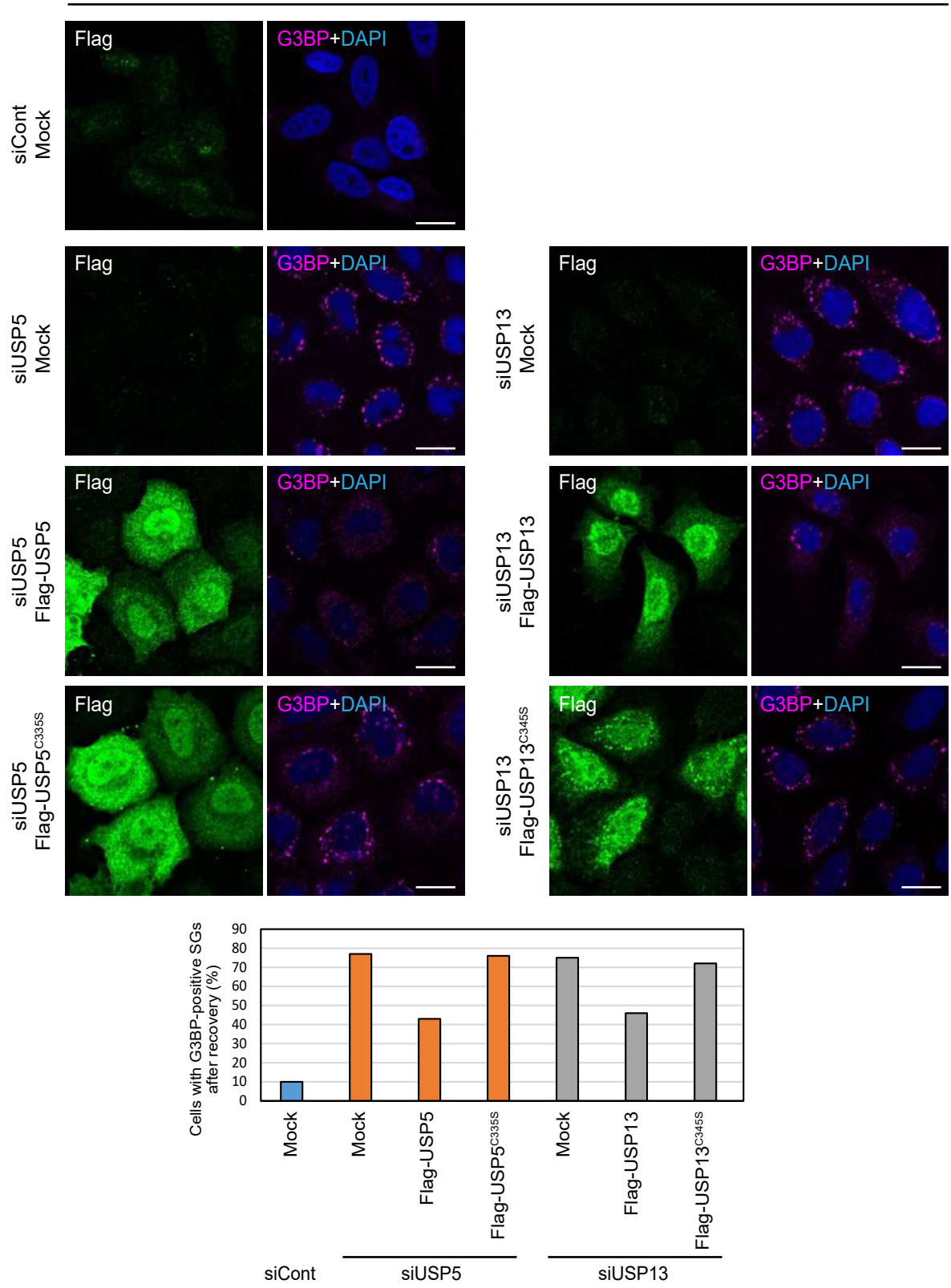

Fig. 16. Re-expression of USP5 or USP13 partially restore the repression of SG disassembly by knock-down of USP5 or USP13

Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-1), or siRNAs for USP13 (siUSP13-2). After 24 h, cells were transfected with an empty vector or siRNA-resistant plasmids encoding Flag-tagged USP5, USP13 or their mutants in which a cysteine residue in the catalytic site was substituted [USP5, Cys335 to Ser (C335S); USP13, Cys345 to Ser (C345S)]. At 48 h after the plasmid transfection, cells were subjected to heat stress (44°C for 1 h) and then returned to normal conditions (37°C for 1.5 h). Cells were stained with anti-Flag antibody, anti-G3BP antibody and DAPI. Bars, 20 μm. In mock-transfected sample, the numbers of cells with G3BP-positive SGs in 100 cells were counted. In the other samples, the numbers of cells with G3BP-positive SGs in 100 Flag-positive cells were counted. The graph shows the representative data of 3 independent experiments.

Fig. 17

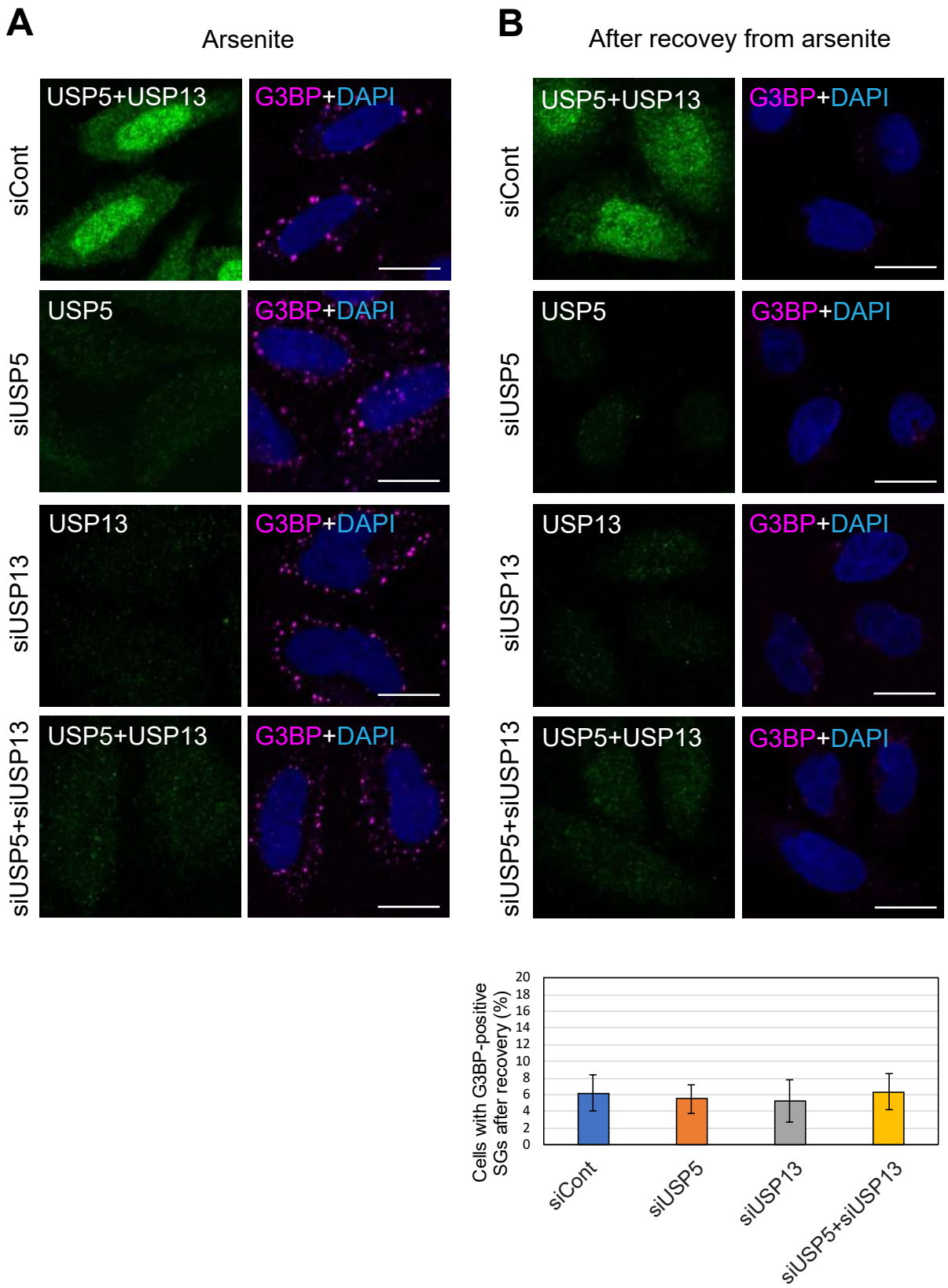

Fig. 17. Depletion of USP5 or USP13 does not affect assembly and disassembly of arsenite-induced SGs
Cells were transfected with siRNAs for USP5 (siUSP5-1) and siRNAs for USP13 (siUSP13-1) individually or in combination, subjected to treatment with arsenite (0.5 mM for 45 min) (A), and returned to normal conditions without arsenite (3 h) (B). Cells were then stained with anti-USP5 antibody and/or anti-USP13 antibody, together with anti-G3BP antibody and DAPI. Bars, 20 μm. The numbers of SG-bearing cells in B are shown as a proportion to the total number of cells (mean ± s.e.m. of 3 independent experiments) in the graph. There was no statistically significant difference between control and knockdown samples (two-tailed Student's *t*-test).

Fig. 18

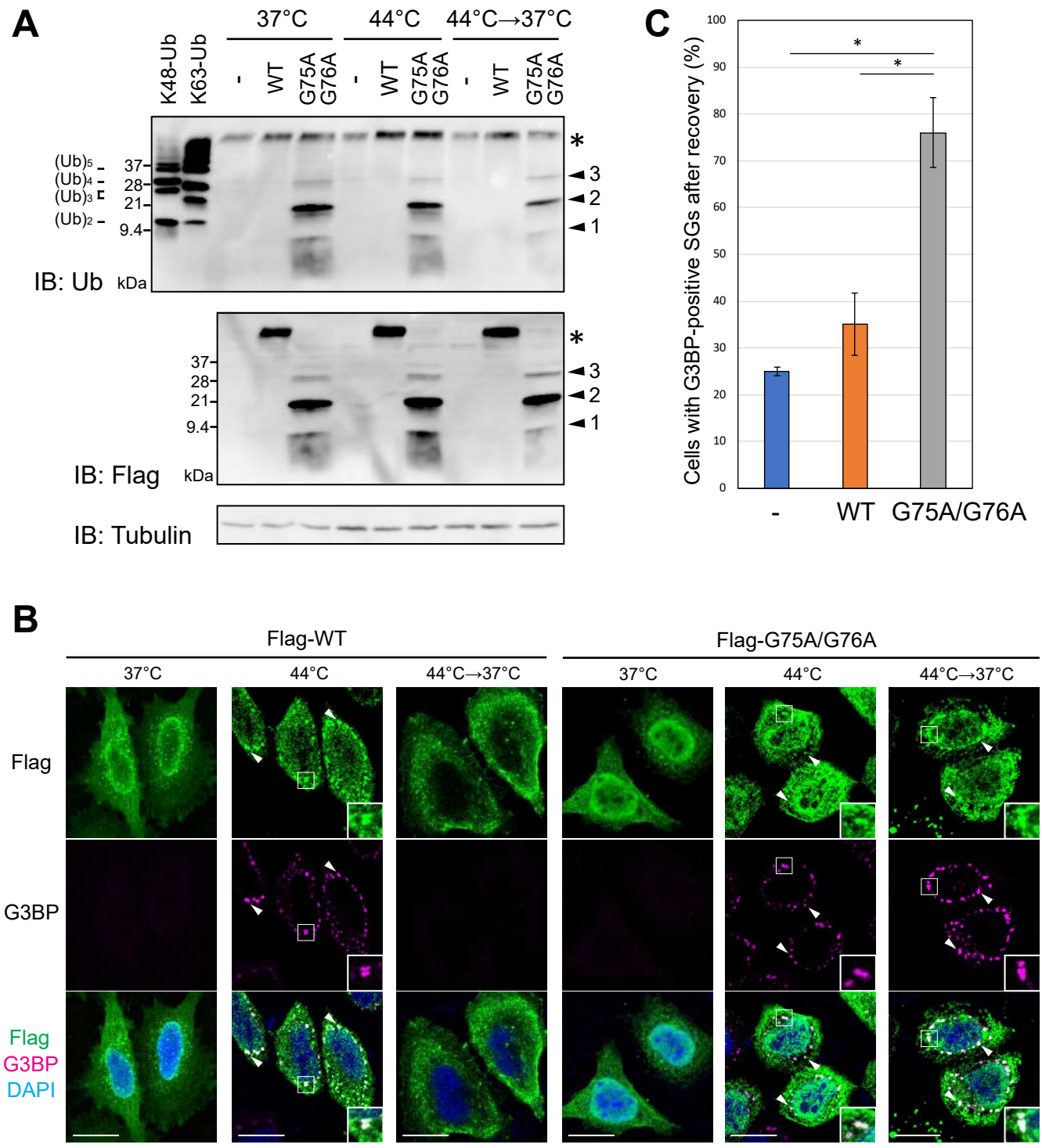

Fig. 18. Accumulation of unanchored ubiquitin chains represses disassembly of heat-induced SGs
Cells were transfected with N-terminally Flag-tagged WT ubiquitin (Ub) or its G75A/G76A mutant, subjected to heat stress (44°C for 55 min), and returned to normal conditions (37°C for 3 h). (A) The cell lysates were subjected to immunoblotting with the indicated antibodies. As size markers, purified K48- and K63-linked ubiquitin chains were loaded. (B) The cells were stained with anti-Flag and anti-G3BP antibodies and DAPI. Arrowheads indicate typical SGs. Insets show higher magnification images of regions indicated by squares. Bars, 20 μm. (C) The number of SG-bearing cells in B was counted and shown as a proportion to the total number of cells (mean ± s.e.m. of 3 independent experiments). *P<0.05 (two-tailed Student's *t*-test).

Fig. 19

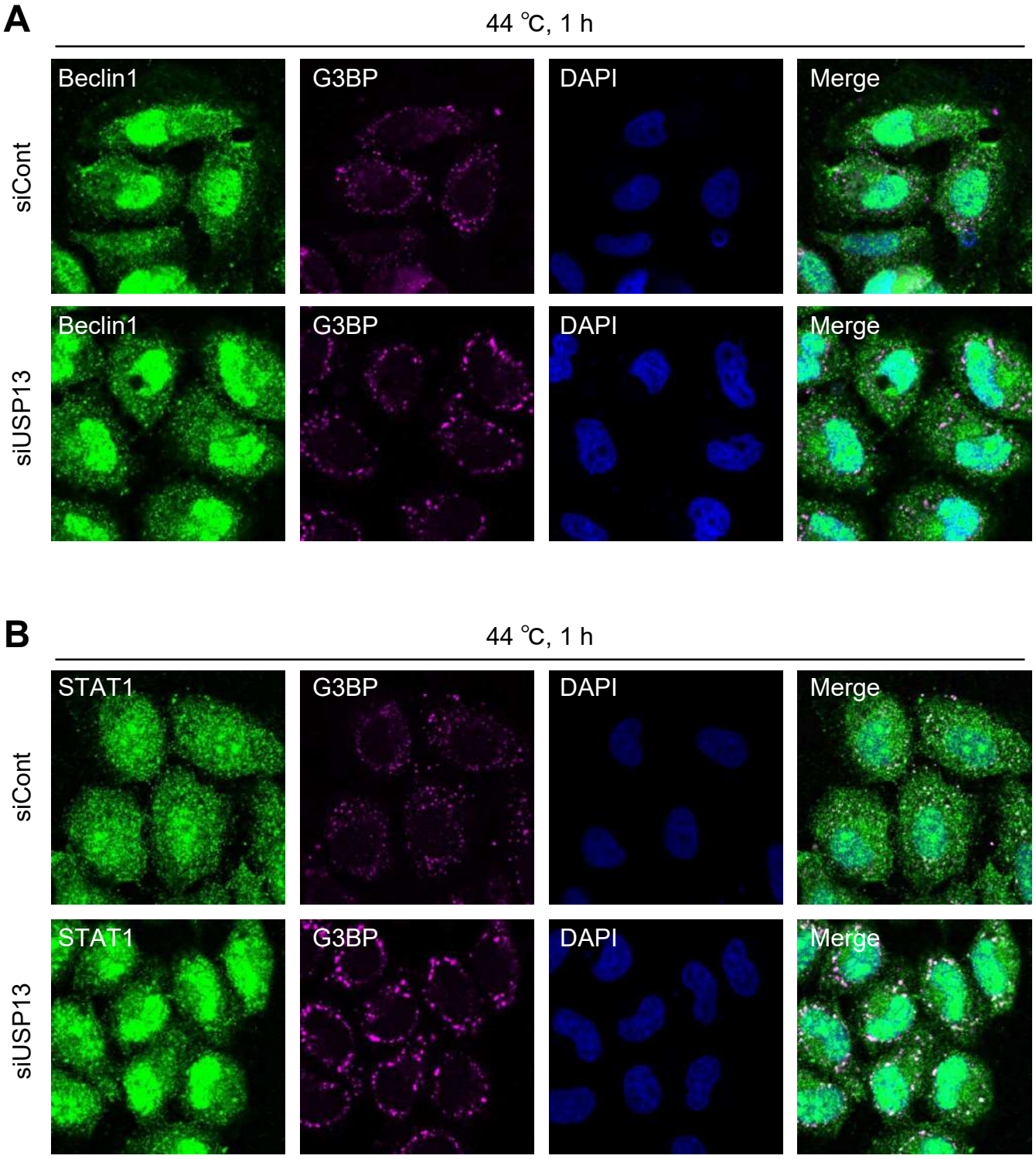

Fig. 19. Depletion of USP13 increases neither Beclin1 nor STAT1 in heat-induced SGs
HeLa cells were transfected with control siRNAs (siCont), or siRNAs for USP13. At 72 h after transfection, cells were subjected to heat stress (44°C for 1 h) and then stained with anti-Beclin1 antibody (A) or anti-STAT1 antibody (B) together with anti-G3BP antibody and DAPI.

Fig. 20

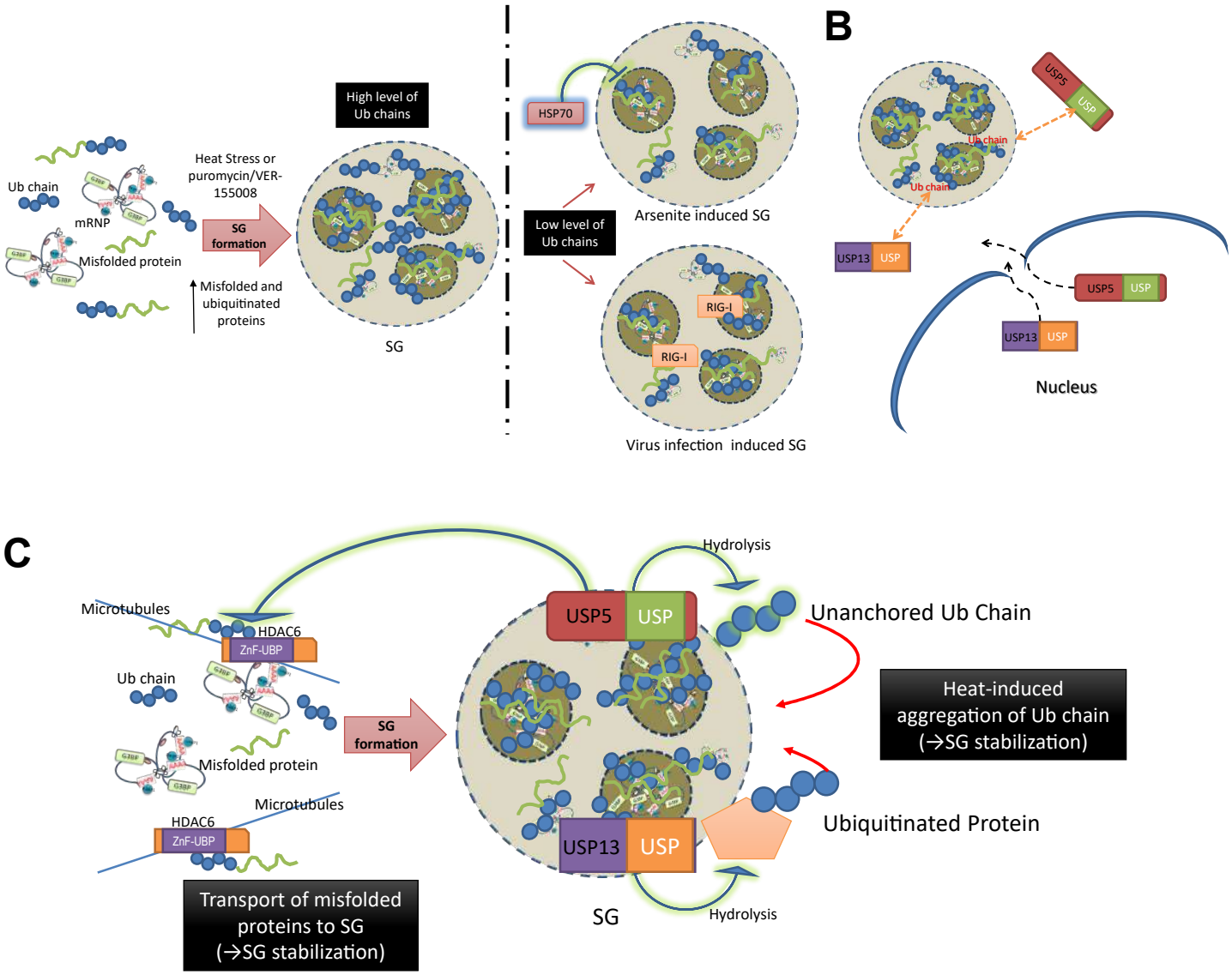

Fig. 20. Working hypothesis

(A) Mechanisms underlying the accumulation of ubiquitin chains in SGs under specific stress conditions. Heat stress generates large amounts of misfolded and ubiquitinated proteins, which are recruited to SGs by unknown mechanisms. Ubiquitin chains autonomously assemble under high temperature conditions, enhancing their accumulation in SGs. The treatment with puromycin and HSP70 inhibitor also generate large amounts of misfolded and ubiquitinated proteins, causing their accumulation in SGs. Other stressors such as arsenite may generate ubiquitinated proteins to a lesser extent than heat stress, and their accumulation in SGs is prevented by the action of HSP70. Virus infection induces SGs containing ubiquitin chains attached to specific anti-virus proteins (e.g. Rig-I).

(B) Mechanisms of the recruitment of USP5 and USP13 to SGs. USP5 and USP13 are in the cytoplasm and the nucleus under basal condition. They are preferentially recruited to SGs containing ubiquitin chains, possibly through the interaction between their USP domain and ubiquitin chains in SGs.

(C) In heat-induced SGs, USP5 hydrolyzes unanchored ubiquitin chains and USP13 hydrolyzes ubiquitin chains conjugated to target proteins. The ubiquitin chains may inhibit SG destabilization by forming heat-induced ubiquitin aggregates. Unanchored ubiquitin chains may also inhibit SG destabilization by enhancing HDAC6 activity to transport misfolded proteins to mature SGs.