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RESEARCH ARTICLE

Deubiquitylases USP5 and USP13 are recruited to and regulate heat-induced stress granules through their deubiquitylating activities

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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, we show that the deubiquitylases 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, we 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 deubiquitylating protein-conjugated ubiquitin chains.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Ubiquitin, Deubiquitylase, Stress granule, USP5, USP13

INTRODUCTION

Stress granules (SGs) are non-membranous cytoplasmic compartments that are formed after cells are exposed to various stresses, including heat stress, oxidative stress, hypoxia and starvation. SGs contain non-translating mRNAs, translation initiation factors, 40S ribosomal subunits and many RNA-binding proteins, as well as non-RNA-binding proteins (Panas et al., 2016; Protter and Parker, 2016). The function of SGs is proposed to modulate mRNA translation and stress responses (Protter and Parker, 2016). Importantly, an abnormal formation of SGs is implicated in the pathogenesis of various diseases including neurodegenerative diseases (Li et al., 2013; Ramaswami et al., 2013). The mechanisms of SG assembly are becoming clear. Under stress, translation of most mRNAs is inhibited and polysomes are disassembled; released mRNAs are then associated with various RNA-binding proteins. The interactions between these RNA-binding proteins, some of which contain prion-like low-complexity domains, initiate the formation of SGs (Panas et al., 2016; Protter and Parker, 2016; Harrison and Shorter, 2017). On the other hand, when stress is relieved, SGs are disassembled. The mechanisms of SG disassembly are largely unknown, although recent reports indicate the involvement of molecular chaperones and autophagy (Buchan et al., 2013; Mateju et al., 2017).

SG components seem to be heterogeneous in many aspects. Firstly, stress-specific differences in the composition of SGs have been shown (Aulas et al., 2017). For example, the small heat-shock protein HSP27 (also known as HSPB1) was found in SGs induced by heat stress, but not in arsenite-induced SGs (Kedersha et al., 1999). Secondly, SGs have an average diameter of 1–2 µm, and are not uniform in structure (Souquere et al., 2009; Jain et al., 2016; Wheeler et al., 2016). PABP1 and G3BP (also known as PABPC1 and G3BP1, respectively), two major component proteins of SGs, are non-uniform in SGs and show clusters of higher concentration (Jain et al., 2016). These clusters have an average diameter of 200 nm, and are referred to as SG cores. The surrounding areas where these proteins are less concentrated are referred to as the SG shell. Thirdly, most of SG components show dynamic behavior and rapid exchange between cytosol and SGs, some components are immobile and seem to form aggregates in SGs (Mateju et al., 2017).

It has been reported that ubiquitin is present in SGs induced by some stressors (Kwon et al., 2007; Mateju et al., 2017). In general, ubiquitin is conjugated through an isopeptide bond between its C-terminal carboxyl group and the ε-amino group of lysine (K) residues in target proteins. Additional ubiquitin can be ligated to one of seven lysine residues in the ubiquitin already conjugated to target proteins, leading to the formation of ubiquitin chains. Most of 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, linkage types and the roles of ubiquitin chains in SGs are unknown.

Ubiquitylation is reversible, and deubiquitylases (DUBs) hydrolyze the isopeptide bond between ubiquitin and target

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proteins as well as between ubiquitin molecules in ubiquitin chains. The human genome encodes ∼90 DUBs (Komander et al., 2009; Eletr and Wilkinson, 2014), and some of them are localized to specific intracellular regions where they locally regulate ubiquitylation levels of target proteins (Coyne and Wing, 2016). Ubiquitin-specific protease 5 (USP5), also known as isopeptidase T, is a DUB in the USP family. USP5 harbors a catalytic core composed of the Cys- and His-boxes, as well as a ZnF-UBP domain in the N-terminal region (Bonnet et al., 2008) and two tandem UBA domains between the Cys- and His-boxes in the primary sequence (Buchberger, 2002). USP5 specifically recognizes the free C-terminal glycine-glycine sequence of ubiquitin through the ZnF-UBP domain and exhibits isopeptidase specificity toward ubiquitin chains that are not attached to any substrate (that is, unanchored ubiquitin chains), thereby regulating the cellular level of unanchored ubiquitin chains (Wilkinson et al., 1995; Amerik et al., 1997; Reyes-Turcu et al., 2006; Dayal et al., 2009). USP13 is a paralog of USP5, with the same domain structure as USP5. However, the ZnF-UBP domain of USP13 lacks the ability to bind to the free C-terminus of ubiquitin and therefore isopeptidase activity toward unanchored ubiquitin chains (Zhang et al., 2011). Accordingly, despite having the same domain organization, USP13 is reported to exert different cellular functions from USP5 by hydrolyzing ubiquitin chains attached to target proteins (Zhang et al., 2011, 2013; Fang et al., 2017).

In this study, we found the recruitment of USP5 and USP13 to heat-induced SGs, and provide evidence suggesting that the stability of SGs is regulated by ubiquitin chains, which is effected by hydrolysis mediated by these DUBs.

RESULTS

USP5 and USP13 are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008

In the process of studying the function of USP5 and USP13, two structural paralogs of DUBs, we found that they mostly exhibit a nuclear localization in human cervical cancer HeLa cells under normal conditions (Fig. 1A), but 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 a major SG component (PABP1 or G3BP), a fraction of USP5 as well as USP13 colocalized with PABP1 and G3BP on the puncta (Fig. 1B). This 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 upon a variety of cellular stresses, we 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. 1C–E). However, co-staining of the cells with anti-USP5 or anti-USP13 antibody revealed that both DUBs colocalize with PABP1 on SGs in these cells at much lower levels, if at all, than in heat-stressed cells (Fig. 1C–E). Puromycin, which dissociates polysomes and generates polysome-free mRNA, can efficiently induce SGs in the presence of VER-155008, an inhibitor of the HSP70 family, that increases the levels of misfolded proteins (Bounedjah et al., 2014; Fig. 1F). A fraction of the USP5 and USP13 was localized to SGs treated in this way (Fig. 1F). These results suggested that USP5 and USP13 are preferentially recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008.

Ubiquitin chains are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008

Recruitment of USP5 and USP13 to heat-induced SGs suggests that their substrates (i.e. unanchored ubiquitin chains for USP5 and protein-conjugated ubiquitin chains for USP13) are present in the SGs. We therefore double-stained heat-stressed cells with the FK2 anti-ubiquitin antibody together with the 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. 2A, 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, we next stained heat-stressed cells with linkage-specific anti-ubiquitin chain antibodies. Although antibodies against K48- and K63-linked ubiquitin chains clearly exhibited different staining patterns in cells under normal conditions (Fig. 2B), both antibodies stained SGs in heat-stressed cells, showing that K48- and K63-linked ubiquitin chains are present in the SGs (Fig. 2C). We were not able to determine whether the ubiquitin chains were 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, but stained SGs induced by puromycin and VER-155008 (Fig. 2D–G).

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

In confocal microscopy analyses, USP5 and USP13 did not appear to colocalize completely with PABP1, G3BP or ubiquitylated proteins in heat-induced SGs (Figs 1 and 2). To determine the more precise localization pattern of these DUBs in the SGs, we performed super-resolution microscopy analysis using structured illumination microscopy. We stained heat-stressed cells with anti-USP5 or anti-USP13 antibody together with anti-PABP1, anti-G3BP or the FK2 anti-ubiquitin antibody. In Fig. 3, each panel shows a single SG, clearly indicating that PABP1 and G3BP are non-uniform, and confirming the core–shell structure of heat-induced SGs. Ubiquitin is also non-uniform in SGs, and most of the USP5 (Fig. 3A) and USP13 (Fig. 3B) within SGs was detected at regions with less concentration of PABP1, G3BP and ubiquitin. These results demonstrated that USP5 and USP13 are localized to the SG shell. We also found that small portions of USP5 and USP13 within SGs are colocalized with ubiquitin (Fig. 3).

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

We 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. 1B; Fig. S1), indicating that a fraction of the USP5 and USP13 in the SGs are translocated from the nucleus. A similar translocation of a nuclear protein to SGs under stress conditions has been previously reported (Gao et al., 2015). Most of the K48-linked ubiquitin chains also appeared to translocate from the nucleus in response to heat stress (Fig. 2B,C). However, LMB treatment had

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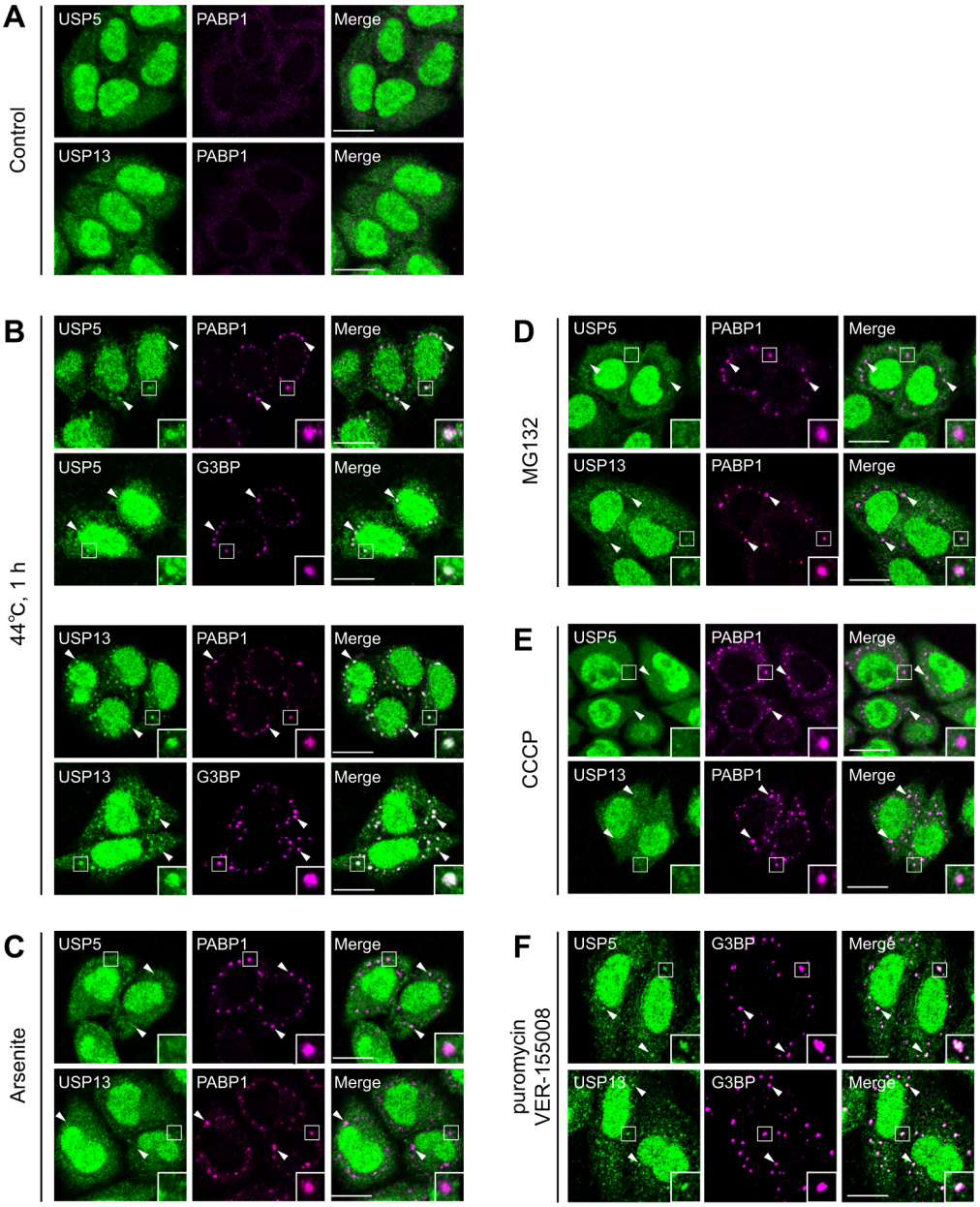

Fig. 1. USP5 and USP13 are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008. HeLa cells were cultured under normal conditions (A), subjected to heat stress (44°C for 1 h) (B), or treated with arsenite (0.5 mM for 45 min) (C), MG132 (0.2 mM for 3 h) (D), CCCP (20 µM for 1.5 h) (E), or both puromycin and VER-155008 (5 µg/ml and 100 µM, respectively, for 3 h) (F). 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. Scale bars: 20 µm. The experiments were repeated more than three times with similar results.

little effect on heat-induced translocation of ubiquitin chains (Fig. S1), indicating that ubiquitin chains are translocated in a CRM1-independent manner.

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

We tried to determine the domain or motif in the DUBs USP5 or USP13 that was responsible for their recruitment to heat-induced SGs (Fig. 4). When cells expressing Flag-tagged USP5 or USP13 were heat stressed, we confirmed that both Flag–USP5 and Flag–USP13 were localized to G3BP-positive SGs as assessed by staining with anti-Flag antibody (Fig. 4A). As observed for endogenous USP5 and USP13 (Fig. 1B), the SG localization was more clearly observed for Flag–USP13 than for Flag–USP5. We therefore chose USP13 and constructed mutants of this protein lacking the ZnF-UBP domain and/or the tandem UBA domains (ΔZnF, ΔUBA and ΔZnFΔUBA; Fig. 4B). When these mutants were expressed in cells, all of them were detected in heat-induced G3BP-positive SGs (Fig. 4C), suggesting that these domains are not necessary for the recruitment of USP13. We also constructed other USP13 mutants that lacked the USP domain or consisted of only the USP domain (ΔUSP and USP; Fig. 4B). 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. 4C), suggesting that USP domain itself has the ability to recruit USP13 to heat-induced SGs.

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

We next examined whether USP5 and USP13 hydrolyze ubiquitin chains in heat-induced SGs. We carried out knockdown experiments by transfecting small interfering RNAs (siRNAs; denoted siUSP5 and siUSP13 for USP5 and USP13, respectively). Immunoblotting analyses confirmed efficient knockdown of these DUBs (Fig. 5A). To evaluate the effect on the level of conjugated ubiquitin, regardless of whether they are unanchored chains or

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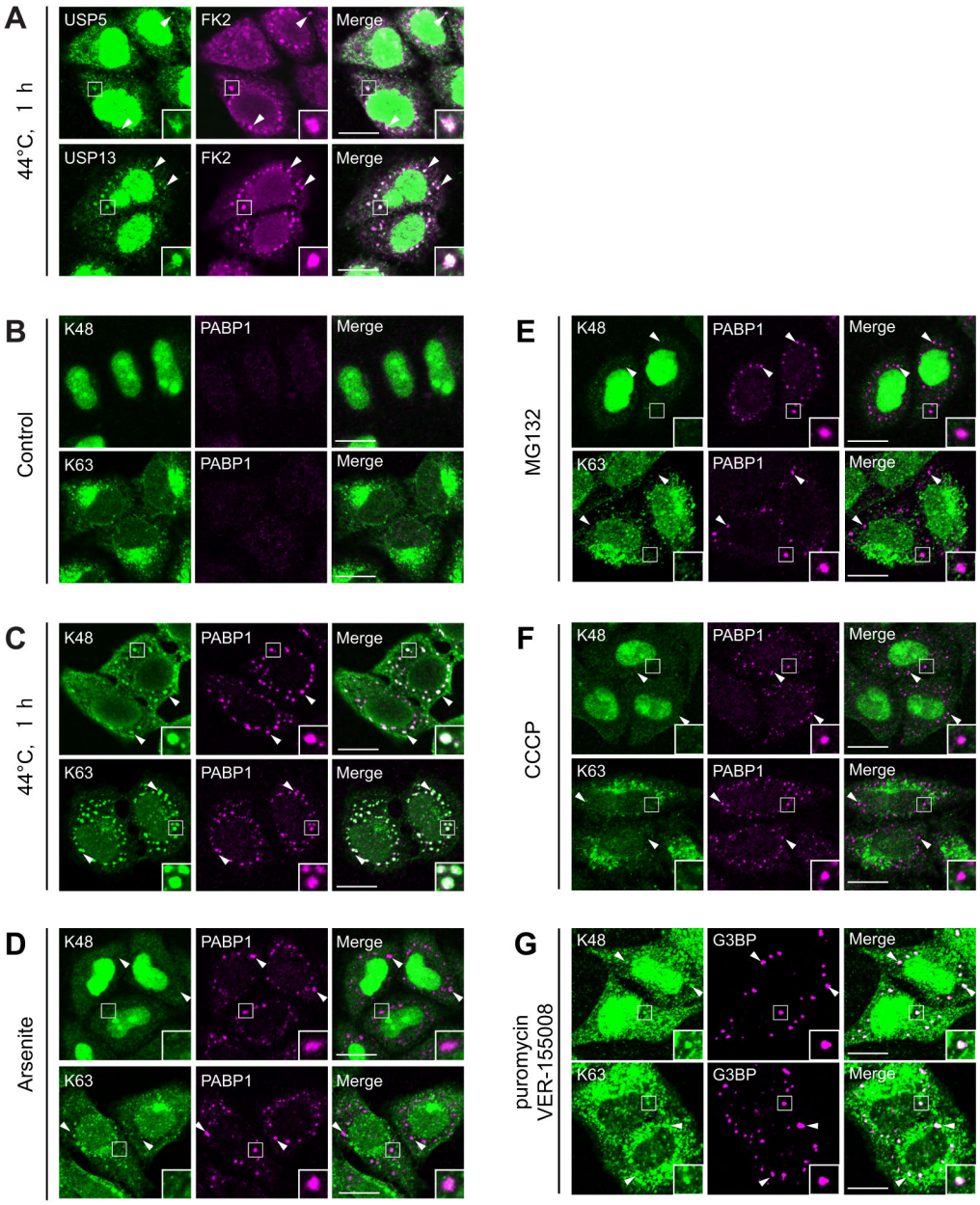

Fig. 2. Ubiquitin chains are recruited to heat-induced SGs, as well as SGs induced by puromycin and VER-155008. (A) Cells were subjected to heat stress (44°C for 1 h), and stained with anti-USP5 or anti-USP13 antibody together with the FK2 anti-ubiquitin antibody. (B–G) Cells were cultured under normal conditions (B), subjected to heat stress (44°C for 1 h) (C), or treated 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 both puromycin and VER-155008 (5 µg/ml and 100 µM, respectively, for 3 h) (G). 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. Scale bars: 20 µm. The experiments were repeated more than three times with similar results.

conjugated to target proteins, we stained cells with the 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. 5B,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. 5B). These results suggest that USP5 and USP13 exhibit DUB activity and regulate the ubiquitin chain levels in heat-induced SGs.

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

We examined the roles of the DUBs USP5 or USP13 in the assembly of heat-induced SGs. To evaluate the effects of their knockdown on SG formation rate, we 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. 6A). Importantly, quantitative image analyses revealed that the percentage of cells with SGs, the number of SGs per cell and the SG size were increased in cells transfected with siUSP5, siUSP13 or both (Fig. 6B–D), showing that depletion of USP5 or USP13 accelerates assembly of heat-induced SGs. Depletion of USP5 or USP13 by use of alternative siRNAs also accelerated the assembly of heat-induced SGs (Fig. S2), excluding the possibility of off-target siRNA effects.

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

We found that when cells were incubated at 44°C for 1 h, the percentage of cells with SGs was elevated to 90% or more in control cells, which is similar to the levels found in cells transfected with siUSP5, siUSP13 or both (Fig. 7A,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. S3A), whereas the SG sizes were still increased in cells transfected with siUSP5,

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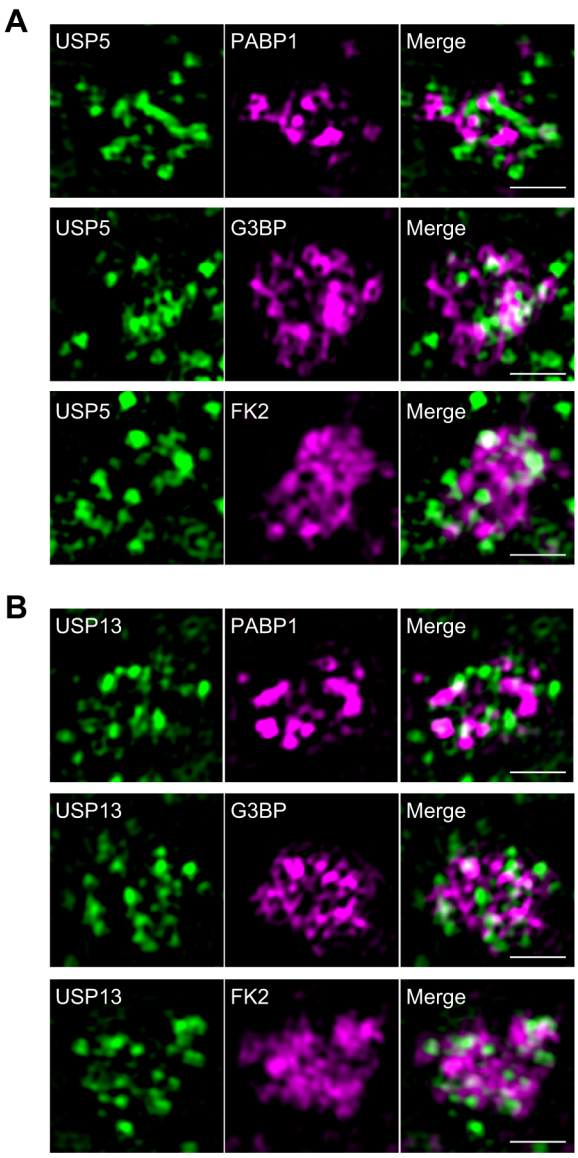

Fig. 3. USP5 and USP13 are adjacent to PABP1-, G3BP- or ubiquitin-positive regions within heat-induced SGs. Cells were subjected to heat stress (44°C for 1 h), stained with anti-USP5 antibody (A) or anti-USP13 antibody (B) together with anti-PABP1, anti-G3BP or FK2 anti-ubiquitin antibody, and observed by using structured illumination microscopy. Each panel shows a single SG. Scale bars: 1 µm. The experiments were repeated three times with similar results.

siUSP13 or both (Fig. S3B). We 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. 7C,D), suggesting that depletion of USP5 or USP13 represses disassembly of heat-induced SGs. Depletion of USP5 or USP13 by means of alternative siRNAs also repressed disassembly of heat-induced SGs (Fig. S4), excluding the possibility of siRNA off-target effects. In addition, we performed rescue experiments, and found that re-expression of these DUBs in the knockdown cells partially restored the SG disassembly (Fig. S5). 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 the increased cellular stress caused 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. S5), showing the importance of their deubiquitylating activities in the regulation of SG disassembly. We 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. S6).

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), we 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 the lack of the C-terminal diglycine motif in the proximal ubiquitin, which is required for the substrate recognition by USP5 (Wilkinson et al., 1995). We found that, in cells overexpressing N-terminally Flag-tagged ubiquitin$^{G75A/G76A}$, immunoblotting with anti-ubiquitin antibody (clone P4G7) or anti-Flag antibody detected three bands (Fig. 8A, 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 wild-type (WT) ubiquitin, we observed anti-Flag antibody-positive signals in the high-molecular-mass region (Fig. 8A, 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. 8A, top panel, asterisk).

We 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, the level of the nuclear signal of WT ubiquitin was reduced but that of ubiquitin$^{G75A/G76A}$ was not obviously affected. The reason for this difference was unclear. G3BP-positive SGs were observed at similar levels in both cells, and a fraction of WT ubiquitin as well as ubiquitin$^{G75A/G76A}$ was detected within the SGs (Fig. 8B). 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. 8B,C), indicating that accumulation of unanchored ubiquitin chains represses disassembly of heat-induced SGs.

DISCUSSION

The roles of ubiquitin chains in the regulation of SGs were unclear. Here, we provide evidence indicating that structural but not functional paralogs of DUBs, USP5 and USP13, are recruited to heat-induced SGs and play roles in the destabilization of SGs most likely through hydrolyzing ubiquitin chains in SGs.

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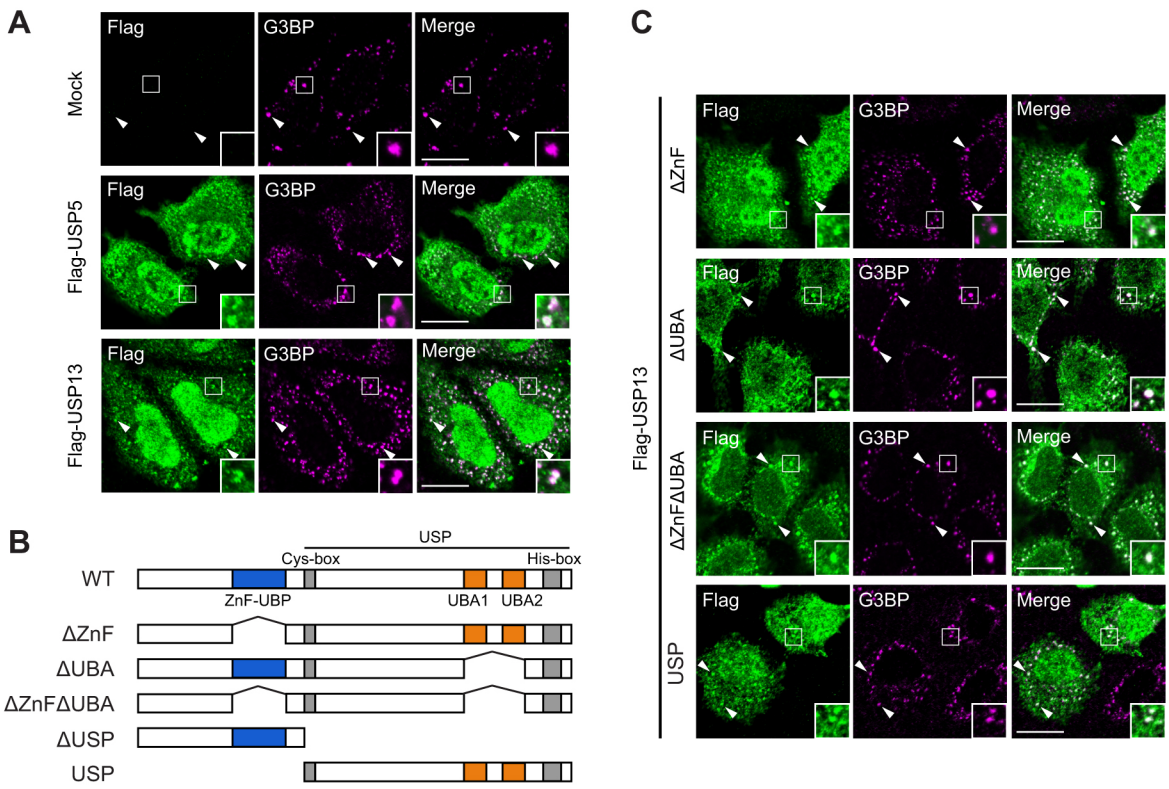

Fig. 4. The USP domain of USP13 has the ability to recruit USP13 to heat-induced SGs. (A) Cells were transfected with Flag-tagged USP5 or USP13, subjected to heat stress (44°C for 1 h), and then double-stained with anti-Flag and anti-G3BP antibodies. (B) The conserved domain structure of USP5 and USP13, and USP13 mutants used in C. ΔZnF, a deletion of amino acids 210–265; ΔUBA, a deletion of amino acids 654–766; ΔUSP, amino acids 1–336; USP, amino acids 337–863. (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 magnification images of regions indicated by squares. Scale bars: 20 µm. The experiments were repeated more than three times with similar results.

Recruitment of USP5, USP13 and ubiquitin chains to SGs

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), we found that USP5 and USP13 are recruited preferentially to heat-induced SGs as well as SGs induced by puromycin and VER-155008, rather than other stresses (Fig. 1). 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 (Fig. 2), suggesting a specific role for ubiquitin chains and their hydrolysis in the regulation of SGs induced by these stresses.

Our super-resolution microscopy analysis demonstrated that USP5 and USP13 are localized to the SG shell (Fig. 3). 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. Our analysis also revealed that a small portion of USP5 and USP13 within the SGs colocalized with ubiquitin (Fig. 3), suggesting that these DUBs transiently associate with and hydrolyze ubiquitin chains in SGs.

Mechanisms underlying the accumulation of ubiquitin chains in heat-induced SGs are obscure. Heat-induced translocation of K48-linked ubiquitin chains from the nucleus is likely to 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 our observation. Interestingly, this Mateju et al. (2017) also showed that concomitant treatment with HSP70 inhibitor triggers the accumulation of ubiquitin in SGs induced by treatment with arsenate or proteasome inhibitors. Thus, we speculate that stressors such as arsenate and proteasome inhibitor may induce the recruitment of ubiquitylated proteins to the SGs to a lesser extent than heat stress, but their accumulation in SGs is prevented by the action of HSP70.

What is the mechanism of recruitment of USP5 and USP13 to heat-induced SGs? USP5 harbors four ubiquitin-binding domains: a USP domain that contains the enzyme active site, a ZnF-UBP domain, and two UBA domains (Reyes-Turcu et al., 2008). USP13 also has these domains although its ZnF-UBP domain lacks the ubiquitin-binding ability (Zhang et al., 2011). Our 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. 4). Therefore, it is possible that the USP domain recruits these DUBs to

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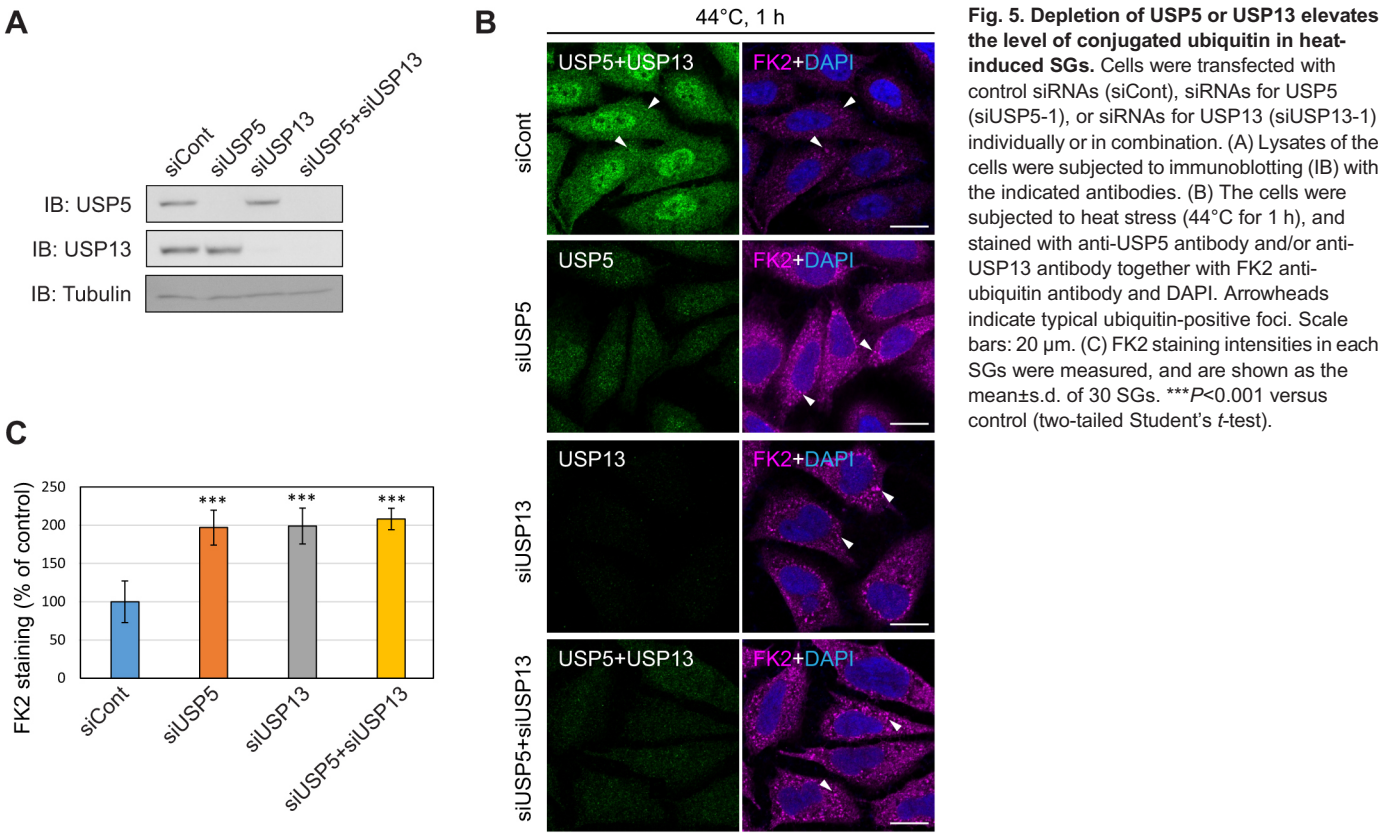

Fig. 5. Depletion of USP5 or USP13 elevates the level of conjugated ubiquitin in heat-induced SGs. Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-1), or siRNAs for USP13 (siUSP13-1) individually or in combination. (A) Lysates of the cells were subjected to immunoblotting (IB) with the 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. Scale bars: 20 µm. (C) FK2 staining intensities in each SGs were measured, and are shown as the mean±s.d. of 30 SGs. ***P<0.001 versus control (two-tailed Student's t-test).

the SGs through an 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 (Figs 1F and 2G).

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

Depletion of USP5 or USP13 elevates ubiquitylated protein levels in heat-induced SGs (Fig. 5), accelerates the SG assembly (Fig. 6) and represses SG disassembly (Fig. 7). We believe that, during heat stress, the supply of ubiquitylated proteins to SGs may exceed the deubiquitylating capacity of these DUBs, causing the accumulation of ubiquitin chains in SGs. When cells are returned to a normal temperature, the supply of ubiquitylated proteins diminishes and ubiquitylated protein levels in SGs are reduced by these DUBs, contributing to the SG disassembly.

USP10, known as Ubp3 in yeast, is another DUB recruited to SGs upon various stresses, 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 budding yeast (Nostramo et al., 2015), while this DUB is dispensable for SG assembly and disassembly in fission yeast (Wang et al., 2012). Mammalian USP10 is essential for SG assembly, independently of its DUB activity but dependent on 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.

Although both USP5 and USP13 are recruited to heat-induced SGs, our results indicate that these DUBs have non-redundant roles in the SGs because depletion of USP5 or USP13 individually had significant effects on the assembly and disassembly of heat-induced SGs (Figs 6 and 7). 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, we 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. Owing to the lack of anti-ubiquitin antibodies that can specifically stain unanchored chains for immunofluorescence visualization, we were not able to examine whether unanchored ubiquitin chains are indeed present in heat-induced SGs and whether their level is elevated upon USP5 depletion. We therefore took another approach using a C-terminal mutant of ubiquitin, and provided evidence that elevating the unanchored ubiquitin chain levels in heat-induced SGs represses their disassembly (Fig. 8), supporting our proposal that hydrolysis of unanchored ubiquitin chains is required for the SG disassembly. On the other hand, it is important to determine what kinds of proteins are deubiquitylated by USP13 in the SGs.

What are possible mechanisms by which the hydrolysis of ubiquitin chains by USP5 and USP13 can promote the SG disassembly? One possible mechanism is that USP5-induced hydrolysis of unanchored ubiquitin chains that associate with unfolded proteins in SGs may inhibit the accumulation of unfolded proteins at SGs, leading to the SG disassembly. In cells, most of the unfolded proteins are ubiquitylated, and the ubiquitin chains are often released from misfolded proteins by the 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 the

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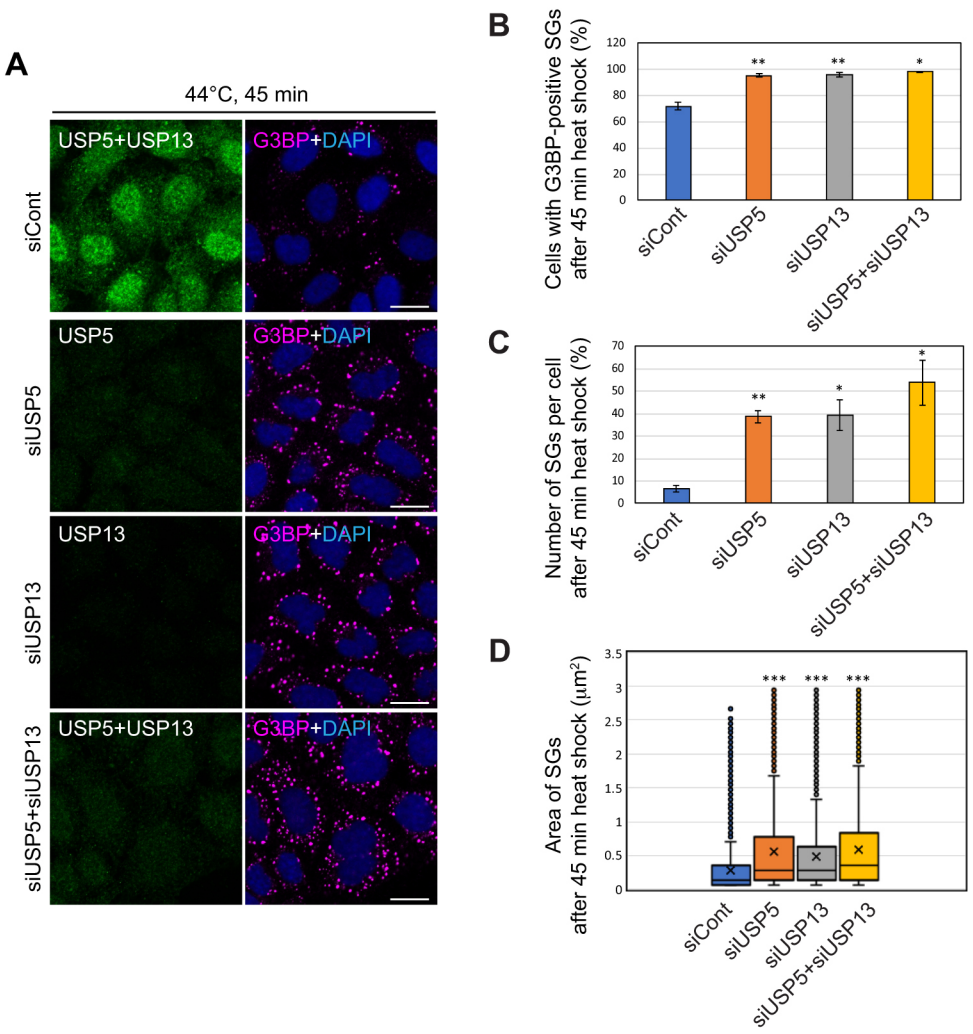

Fig. 6. Depletion of USP5 or USP13 accelerates assembly of heat-induced SGs. Cells were transfected with control siRNAs (siCont), siRNAs for USP5 (siUSP5-1), or siRNAs for USP13 (siUSP13-1) individually or in combination. (A) The cells were subjected to heat stress (44°C for 45 min), and stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Scale bars: 20 µm. (B) The numbers of SG-bearing cells in B were counted and are shown as a proportion (%) of the total number of cells (mean±s.e.m. of three independent experiments). **P*<0.05, ***P*<0.01 versus control (two-tailed Student's *t*-test). (C) The numbers of SGs per cell are shown as mean±s.e.m. of three independent experiments. **P*<0.05, ***P*<0.01 versus control (two-tailed Student's *t*-test). (D) The sizes of all SGs observed in three independent experiments were measured and are shown in box-and-whisker plots, with the mean (× in the box), median (line in the box), 1st and 3rd quartiles (bottom and top of the box), maximum and minimum excluding outliers (whiskers), 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).

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 results suggest that the complexes of unanchored ubiquitin chains and unfolded proteins may accumulate at SGs through the action of the 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 level of ubiquitin chain aggregates, resulting in the destabilization 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 precise molecular mechanisms by which USP5 and USP13 facilitate SG destabilization.

MATERIALS AND METHODS

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.

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,

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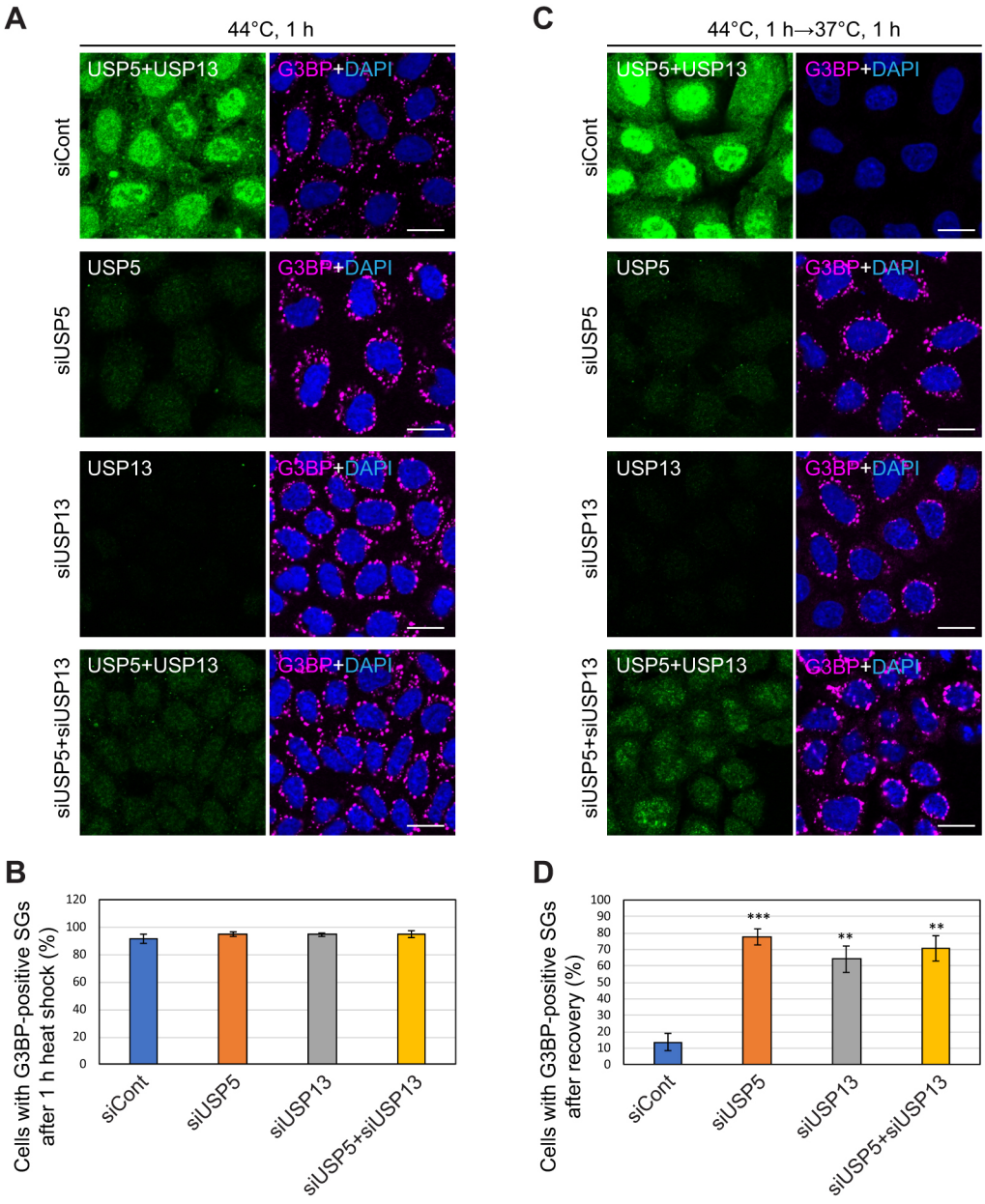

Fig. 7. Depletion of USP5 or USP13 represses disassembly of heat-induced SGs. (A) Cells were transfected with siRNAs for USP5 (siUSP5-1) and USP13 (siUSP13-1) individually or in combination, and subjected to heat stress (44°C for 1 h). Cells were then stained with anti-USP5 antibody and/or anti-USP13 antibody together with anti-G3BP antibody and DAPI. Scale bars: 20 µm. (B) The number of SG-bearing cells in A were counted and is shown as a proportion (%) of the total number of cells (mean±s.e.m. of three independent experiments). There was no statistically significant difference between control and knockdown samples (two-tailed Student's *t*-test). (C) Cells treated as in A were then returned to normal conditions (37°C for 1 h), and stained as in A. (D) The numbers of SG-bearing cells in C are shown (mean±s.e.m. of three independent experiments). $^{**}P<0.01$, $^{***}P<0.001$ versus control (two-tailed Student's *t*-test).

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, or (5) with 5 µg/ml puromycin (Wako) and 100 µM VER-155008 (Sigma-Aldrich) for 3 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.

DNA and siRNA transfection

Plasmid DNA transfection was performed using the FuGene6 transfection reagent (Promega, Madison, WI) and polyethylenimine (catalog no. #24765, batch #644175, Polysciences, Warrington, PA) 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.

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) and rabbit anti-Flag (0.8 µg/ml;

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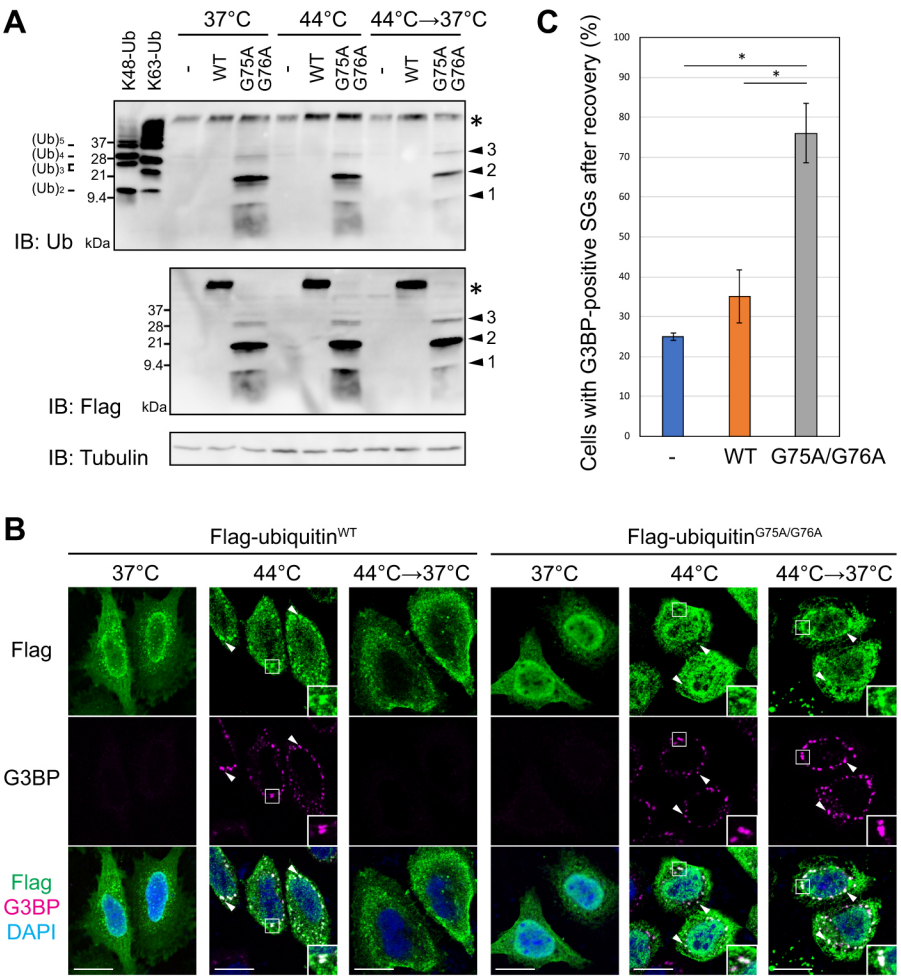

Fig. 8. Accumulation of unanchored ubiquitin chains represses disassembly of heat-induced SGs. Cells were transfected with N-terminally Flag-tagged WT ubiquitin (Ub) or its G75A/G76A mutant, subjected to heat stress (44°C for 55 min), and returned to normal conditions (37°C for 3 h). (A) The cell lysates were subjected to immunoblotting (IB) 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. Scale bars: 20 µm. (C) The number of SG-bearing cells in B was counted and is shown as a proportion (%) of the total number of cells (mean± s.e.m. of three independent experiments). *P<0.05 (two-tailed Student's t-test).

catalog no. #F7425, batch #093M4798, Sigma-Aldrich) antibodies. Secondary antibodies were Alexa Fluor 488- and 594-conjugated anti-mouse 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. The images were analyzed by means of ImageJ (NIH, Bethesda, MA). The following quantitative analyses were performed using wide-field view of G3BP staining (~100 cells in each image in Figs 6, 7, 8, Figs S2, S3, S4, S5 and S6). 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 in Fig. 5B 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.

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), and anti-α-tubulin (1:2500; catalog no. #ab15246, batch #GR121903-2, Abcam) 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).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: X.X., S.M., A.E., T.F., M.K.; Methodology: X.X., S.M., A.E., T.F., M.K.; Validation: X.X., S.M., A.E., T.F., M.K.; Investigation: X.X., S.M., A.E., M.K.; Resources: H.K.; Writing - original draft: X.X., M.K.; Writing - review & editing: A.E., T.F., Y.S., M.K.; Visualization: X.X., M.K.; Supervision: T.F., M.K.; Funding acquisition: M.K.

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Supplementary information

Supplementary information available online at
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References

Amerik, A. Y., Swaminathan, S., Krantz, B. A., Wilkinson, K. D. and Hochstrasser, M. (1997). *In vivo* disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *EMBO J.* **16**, 4826-4838.

Aulas, A., Fay, M. M., Lyons, S. M., Achorn, C. A., Kedersha, N., Anderson, P. and Ivanov, P. (2017). Stress-specific differences in assembly and composition of stress granules and related foci. *J. Cell Sci.* **130**, 927-937.

Bonnet, J., Romier, C., Tora, L. and Devys, D. (2008). Zinc-finger UBPs: regulators of deubiquitylation. *Trends Biochem. Sci.* **33**, 369-375.

Bounedjah, O., Desforges, B., Wu, T.-D., Pioche-Durieu, C., Marco, S., Hamon, L., Curmi, P. A., Guerquin-Kern, J.-L., Piétrement, O. and Pastré, D. (2014). Free mRNA in excess upon polysome dissociation is a scaffold for protein multimerization to form stress granules. *Nucleic Acids Res.* **42**, 8678-8691.

Buchan, J. R., Kolaitis, R.-M., Taylor, J. P. and Parker, R. (2013). Eukaryotic stress granules are cleared by autophagy and Cdc48/VCP function. *Cell* **153**, 1461-1474.

Buchberger, A. (2002). From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol.* **12**, 216-221.

Coyne, E. S. and Wing, S. S. (2016). The business of deubiquitination - location, location, location. *F1000Res.* **5**, F1000 Faculty Rev-163.

Dayal, S., Sparks, A., Jacob, J., Allende-Vega, N., Lane, D. P. and Saville, M. K. (2009). Suppression of the deubiquitinating enzyme USP5 causes the accumulation of unanchored polyubiquitin and the activation of p53. *J. Biol. Chem.* **284**, 5030-5041.

Dormann, D., Rodde, R., Edbauer, D., Bentmann, E., Fischer, I., Hruscha, A., Than, M. E., Mackenzie, I. R. A., Capell, A., Schmid, B. et al. (2010). ALS-associated fused in sarcoma (FUS) mutations disrupt Transportin-mediated nuclear import. *EMBO J.* **29**, 2841-2857.

Elden, A. C., Kim, H.-J., Hart, M. P., Chen-Plotkin, A. S., Johnson, B. S., Fang, X., Armakola, M., Geser, F., Greene, R., Lu, M. M. et al. (2010). Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. *Nature* **466**, 1069-1075.

Eletr, Z. M. and Wilkinson, K. D. (2014). Regulation of proteolysis by human deubiquitinating enzymes. *Biochim. Biophys. Acta* **1843**, 114-128.

Fang, X., Zhou, W., Wu, Q., Huang, Z., Shi, Y., Yang, K., Chen, C., Xie, Q., Mack, S. C., Wang, X. et al. (2017). Deubiquitinase USP13 maintains glioblastoma stem cells by antagonizing FBXL14-mediated Myc ubiquitination. *J. Exp. Med.* **214**, 245-267.

Fujimuro, M. and Yokosawa, H. (2005). Production of antipolyubiquitin monoclonal antibodies and their use for characterization and isolation of polyubiquitinated proteins. *Methods Enzymol.* **399**, 75-86.

Gao, X., Fu, X., Song, J., Zhang, Y., Cui, X., Su, C., Ge, L., Shao, J., Xin, L., Saarikettu, J. et al. (2015). Poly(A)(+) mRNA-binding protein Tudor-SN regulates stress granules aggregation dynamics. *FEBS J.* **282**, 874-890.

Hao, R., Nanduri, P., Rao, Y., Panichelli, R. S., Ito, A., Yoshida, M. and Yao, T.-P. (2013). Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. *Mol. Cell* **51**, 819-828.

Harrison, A. F. and Shorter, J. (2017). RNA-binding proteins with prion-like domains in health and disease. *Biochem. J.* **474**, 1417-1438.

Hodgins, R. R., Ellison, K. S. and Ellison, M. J. (1992). Expression of a ubiquitin derivative that conjugates to protein irreversibly produces phenotypes consistent with a ubiquitin deficiency. *J. Biol. Chem.* **267**, 8807-8812.

Jain, S., Wheeler, J. R., Walters, R. W., Agrawal, A., Barsic, A. and Parker, R. (2016). ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**, 487-498.

Kato, M., Miyazawa, K. and Kitamura, N. (2000). A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP. *J. Biol. Chem.* **275**, 37481-37487.

Kedersha, N. L., Gupta, M., Li, W., Miller, I. and Anderson, P. (1999). RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2α to the assembly of mammalian stress granules. *J. Cell Biol.* **147**, 1431-1442.

Kedersha, N., Panas, M. D., Achorn, C. A., Lyons, S., Tisdale, S., Hickman, T., Thomas, M., Lieberman, J., McInerney, G. M., Ivanov, P. et al. (2016). G3BP-Caprin1-USP10 complexes mediate stress granule condensation and associate with 40S subunits. *J. Cell Biol.* **212**, 845-860.

Kim, H. J., Kim, N. C., Wang, Y.-D., Scarborough, E. A., Moore, J., Diaz, Z., MacLea, K. S., Freibaum, B., Li, S., Molliex, A. et al. (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* **495**, 467-473.

Komander, D., Clague, M. J. and Urbé, S. (2009). Breaking the chains: structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol.* **10**, 550-563.

Kulathu, Y. and Komander, D. (2012). Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat. Rev. Mol. Cell Biol.* **13**, 508-523.

Kwon, S., Zhang, Y. and Matthias, P. (2007). The deacetylase HDAC6 is a novel critical component of stress granules involved in the stress response. *Genes Dev.* **21**, 3381-3394.

Li, Y. R., King, O. D., Shorter, J. and Gitler, A. D. (2013). Stress granules as crucibles of ALS pathogenesis. *J. Cell Biol.* **201**, 361-372.

Liu-Yesucevitz, L., Bilgutay, A., Zhang, Y.-J., Vanderweyde, T., Citro, A., Mehta, T., Zaarur, N., McKee, A., Bowser, R., Sherman, M. et al. (2010). Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue. *PLoS ONE* **5**, e13250.

Mateju, D., Franzmann, T. M., Patel, A., Kopach, A., Boczek, E. E., Maharana, S., Lee, H. O., Carra, S., Hyman, A. A. and Alberti, S. (2017). An aberrant phase transition of stress granules triggered by misfolded protein and prevented by chaperone function. *EMBO J.* **36**, 1669-1687.

Morimoto, D., Walinda, E., Fukada, H., Sou, Y.-S., Kageyama, S., Hoshino, M., Fujii, T., Tsuchiya, H., Saeki, Y., Arita, K. et al. (2015). The unexpected role of polyubiquitin chains in the formation of fibrillar aggregates. *Nat. Commun.* **6**, 6116.

Nostramo, R., Varia, S. N., Zhang, B., Emerson, M. M. and Herman, P. K. (2015). The catalytic activity of the Ubp3 deubiquitinating protease is required for efficient stress granule assembly in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **36**, 173-183.

Ohn, T., Kedersha, N., Hickman, T., Tisdale, S. and Anderson, P. (2008). A functional RNAi screen links O-GlcNAc modification of ribosomal proteins to stress granule and processing body assembly. *Nat. Cell Biol.* **10**, 1224-1231.

Ouyang, H., Ali, Y. O., Ravichandran, M., Dong, A., Qiu, W., MacKenzie, F., Dhe-Paganon, S., Arrowsmith, C. H. and Zhai, R. G. (2012). Protein aggregates are recruited to aggresome by histone deacetylase 6 via unanchored ubiquitin C termini. *J. Biol. Chem.* **287**, 2317-2327.

Panas, M. D., Ivanov, P. and Anderson, P. (2016). Mechanistic insights into mammalian stress granule dynamics. *J. Cell Biol.* **215**, 313-323.

Protter, D. S. W. and Parker, R. (2016). Principles and properties of stress granules. *Trends Cell Biol.* **26**, 668-679.

Ramaswami, M., Taylor, J. P. and Parker, R. (2013). Altered ribostasis: RNA-protein granules in degenerative disorders. *Cell* **154**, 727-736.

Reyes-Turcu, F. E., Horton, J. R., Mullally, J. E., Heroux, A., Cheng, X. and Wilkinson, K. D. (2006). The ubiquitin binding domain ZnF UBP recognizes the C-terminal diglycine motif of unanchored ubiquitin. *Cell* **124**, 1197-1208.

Reyes-Turcu, F. E., Shanks, J. R., Komander, D. and Wilkinson, K. D. (2008). Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T. *J. Biol. Chem.* **283**, 19581-19592.

Souquere, S., Mollet, S., Kress, M., Dautry, F., Pierron, G. and Weil, D. (2009). Unravelling the ultrastructure of stress granules and associated P-bodies in human cells. *J. Cell Sci.* **122**, 3619-3626.

Takahashi, M., Higuchi, M., Matsuki, H., Yoshita, M., Ohsawa, T., Oie, M. and Fujii, M. (2013). Stress granules inhibit apoptosis by reducing reactive oxygen species production. *Mol. Cell. Biol.* **33**, 815-829.

Wang, C.-Y., Wen, W.-L., Nilsson, D., Sunnerhagen, P., Chang, T.-H. and Wang, S.-W. (2012). Analysis of stress granule assembly in *Schizosaccharomyces pombe*. *RNA* **18**, 694-703.

Wheeler, J. R., Matheny, T., Jain, S., Abrisch, R. and Parker, R. (2016). Distinct stages in stress granule assembly and disassembly. *eLife* **5**, e18413.

Wilkinson, K. D., Tashayev, V. L., O'Connor, L. B., Larsen, C. N., Kasperek, E. and Pickart, C. M. (1995). Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry* **34**, 14535-14546.

Zhang, Y.-H., Zhou, C.-J., Zhou, Z.-R., Song, A.-X. and Hu, H.-Y. (2011). Domain analysis reveals that a deubiquitinating enzyme USP13 performs non-activating catalysis for Lys63-linked polyubiquitin. *PLoS ONE* **6**, e29362.

Zhang, J., Zhang, P., Wei, Y., Piao, H.-L., Wang, W., Maddika, S., Wang, M., Chen, D., Sun, Y., Hung, M.-C. et al. (2013). Deubiquitylation and stabilization of PTEN by USP13. *Nat. Cell Biol.* **15**, 1486-1494.

Fig. S1

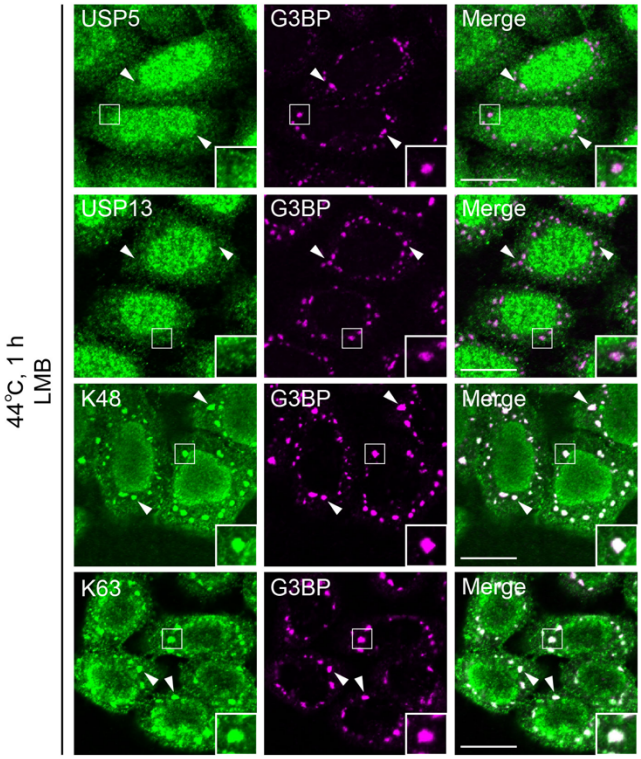

Fig. S1. A part of USP5 and USP13 in heat-induced SGs are translocated from the nucleus
Cells were cultured at 44°C for 1 h in the presence of 50 nM LMB, and stained with anti-USP5, anti-USP13, anti-K48-linked ubiquitin chain, or K63-linked ubiquitin chain antibody together with anti-G3BP antibody. Arrowheads 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. S2

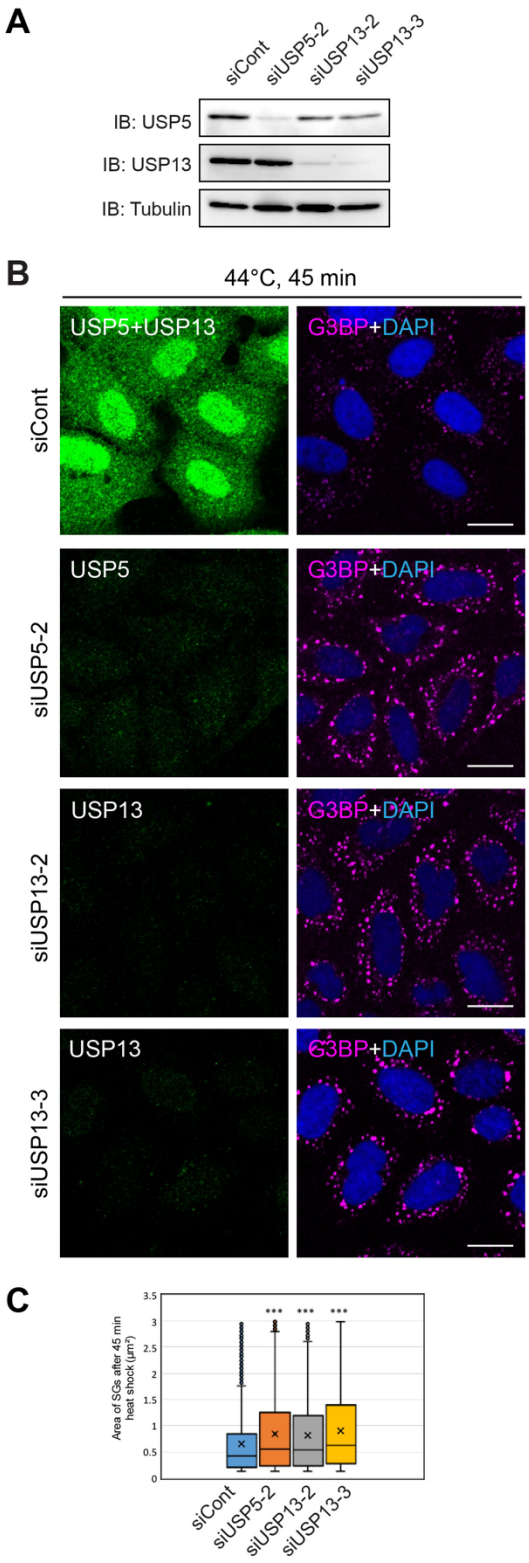

Fig. S2. Depletion of USP5 or USP13 by alternative siRNAs also accelerates assembly of heat-induced SGs (related to Fig. 6)

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. 6D. ***P<0.001 versus control (two-tailed Student's t-test).

Fig. S3

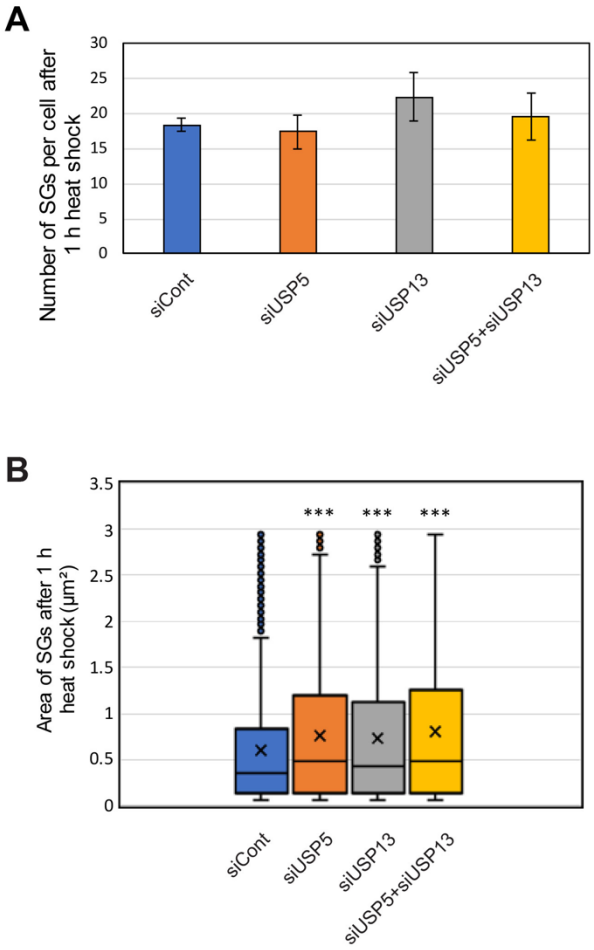

Fig. S3. The numbers and sizes of SGs in USP5/USP13-depleted cells under heat stress (44°C for 1 h) (related to Fig. 7A, B)

(A) The numbers of SGs in USP5/USP13-depleted cells under heat stress (44°C for 1 h) 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). (B) 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. 6D. ***P<0.001 versus control (two-tailed Student's *t*-test).

Fig. S4

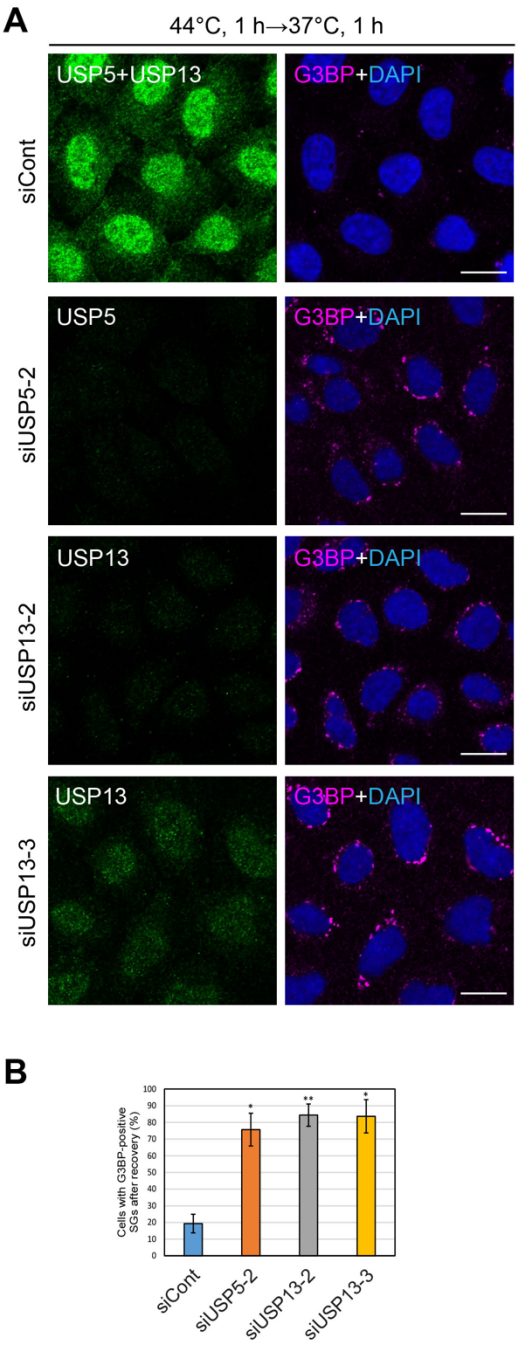

Fig. S4. Depletion of USP5 or USP13 by alternative siRNAs also represses disassembly of heat-induced SGs (related to Fig. 7C, D)

(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. S5

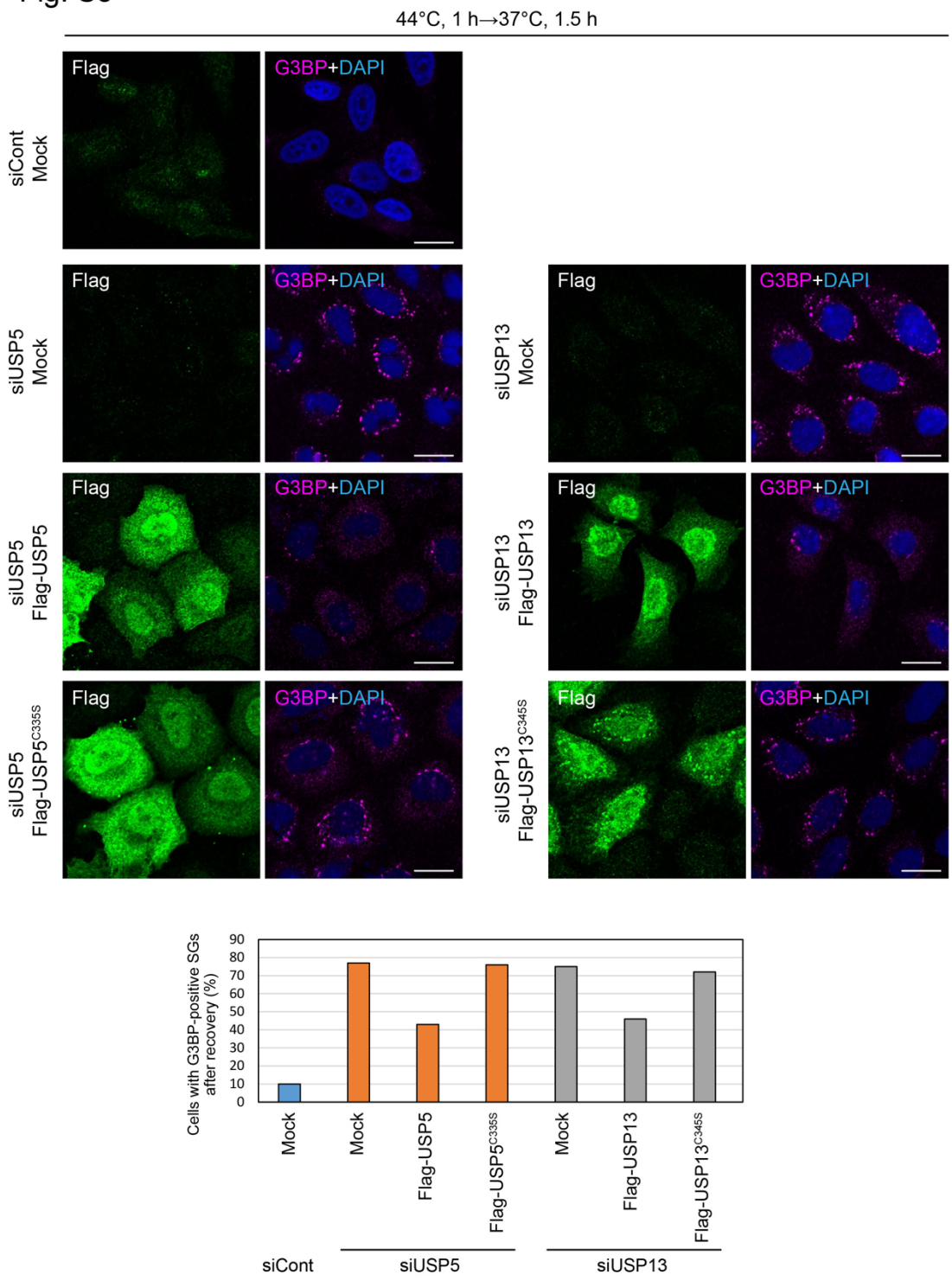

Fig. S5. Re-expression of USP5 or USP13 partially restore the repression of SG disassembly by knockdown of USP5 or USP13

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. S6

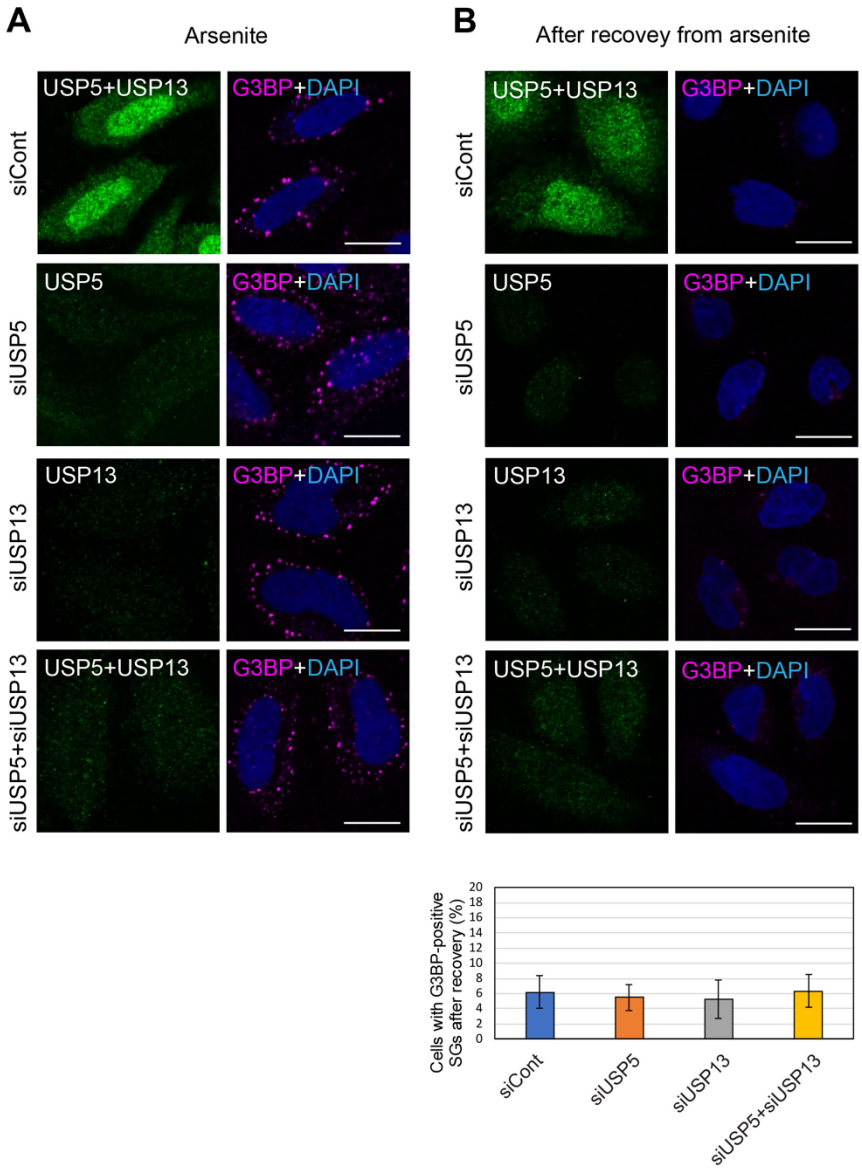

Fig. S6. Depletion of USP5 or USP13 does not affect assembly and disassembly of arsenite-induced SGs

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).