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DAZL is essential for stress granule formation implicated in germ cell survival upon heat stress

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SUMMARY

Mammalian male germ cells should be maintained below body temperature for proper development. Here, we investigated how male germ cells respond to heat stress. A short exposure of mouse testes to core body temperature induced phosphorylation of eIF2α and the formation of stress granules (SGs) in male germ cells. We observed that DAZL, a germ cell-specific translational regulator, was translocated to SGs upon heat stress. Furthermore, SG assembly activity was significantly diminished in the early male germ cells of *Dazl*-knockout mice. The DAZL-containing SGs played a protective role against heat stress-induced apoptosis by the sequestration of specific signaling molecules, such as RACK1, and the subsequent blockage of the apoptotic MAPK pathway. Based on these results, we propose that DAZL is an essential component of the SGs, which prevent male germ cells from undergoing apoptosis upon heat stress.

KEY WORDS: Heat stress, Male germ cell apoptosis, Stress granules, Mouse

INTRODUCTION

In most mammals, the testes are located in the scrotum, outside the body cavity, and are maintained at a lower temperature than the core body. Developmental defects in testicular descent often lead to germ-cell loss in the affected testis (Hutson et al., 1997). Artificially induced cryptorchidism, in which a testis is forced to stay in the body cavity, causes apoptosis of the germ cells (Shikone et al., 1994; Yin et al., 1997). Experimentally, forced heat stress on the testis also causes apoptosis of the germ cells, leading to subfertility or infertility (Lue et al., 1999; Rockett et al., 2001). However, the mechanism of cellular responses to heat stress remains largely unknown in male germ cells.

Stresses such as heat, hypoxia and oxidative conditions often trigger the inhibition of general translation and the formation of stress granules (SGs) in eukaryotic cells (reviewed by Anderson and Kedersha, 2002; Buchan and Parker, 2009). SGs are non-membranous cytoplasmic particles at which translationally inert messenger ribonucleoproteins (mRNPs) accumulate in response to stress (Anderson and Kedersha, 2002). The known components of SGs include polyadenylated mRNAs [poly-$(A)^+$ RNAs], 40S ribosomal subunits, proteins involved in translation, such as the eukaryotic translation initiation factors eIF3, eIF4E, eIF4G and poly(A)-binding protein (PABP; PABPC1 – Mouse Genome Informatics), and several RNA-binding proteins including TIA1, HuR (ELAVL1 – Mouse Genome Informatics) and G3BP (Kedersha et al., 1999; Kedersha et al., 2002; Gallouzi et al., 2000; Tourrière et al., 2003). It is known that the phosphorylation of eIF2α (EIF2S1 – Mouse Genome Informatics) prevents the assembly of the translation initiation complex and induces the assembly of SGs (Kedersha et al., 1999). It is also known that the spontaneous assembly of SGs occurs when some of the SG components are overexpressed (Anderson and Kedersha, 2008). The mRNPs of SGs are in a dynamic equilibrium with polysomes (Kedersha et al., 1999; Kedersha et al., 2000). In this regard, SGs have been proposed to function as a transient place of mRNP remodeling for storage, degradation or reinitiation of translation during stress and recovery from stress (Kedersha and Anderson, 2002; Kedersha et al., 2005; Anderson and Kedersha, 2008). SGs have also been shown to regulate cell survival during stress by the sequestration of the signaling molecules implicated in apoptosis, such as TRAF2 and RACK1 (GNB2L1 – Mouse Genome Informatics) (Kim et al., 2005; Arimoto et al., 2008).

The human *DAZ* ('deleted in azoospermia') gene is located on the Y chromosome in a region in which microdeletions are frequently observed in infertile men (Reijo et al., 1995). The DAZ family proteins are crucial for germ cell development in *Caenorhabditis elegans* (Karashima et al., 2000), *Drosophila melanogaster* (Eberhart et al., 1996), *Xenopus laevis* (Houston and King, 2000) and mice (*Mus musculus*) (Ruggiu et al., 1997). Moreover, rescue experiments, in which an original gene was substituted with another gene family member, revealed evolutionarily conserved functions of the *DAZ* family genes in germ cell development (Houston et al., 1998; Slee et al., 1999; Xu et al., 2003). The DAZ family proteins are detected in male and female germ cells as well as in the germ plasm of early amphibian embryos (Ruggiu et al., 1997; Houston and King, 2000; Karashima et al., 2000; Reijo et al., 2000; Xu et al., 2001). DAZL is an RNA-binding protein and interacts with a specific set of mRNAs, such as the mouse *vasa* homolog, *Mvh* (*Ddx4* – Mouse Genome Informatics), and the synaptonemal complex component *Sycp3* (Reynolds et al., 2005; Reynolds et al., 2007). DAZL was proposed to function as an adaptor for mRNA transport and as a translational activator (Collier et al., 2005; Lee et al., 2006; Reynolds et al., 2005; Reynolds et al., 2007). In mouse oocytes and early embryos, DAZL is involved in translational activation of the preserved mRNAs to promote oocyte maturation and early embryonic development (Chen et al., 2011). DAZL is required for the formation of primordial germ cells from human embryonic stem cells (Kee et al., 2009). It was recently reported that DAZL plays a crucial role in licensing fetal germ cells to undergo gametogenesis (Gill et al., 2011).

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We observed previously that ectopic DAZL colocalizes with TIA1, an SG marker, in HeLa cells under oxidative stress (Lee et al., 2006). This finding led us to speculate about a novel function for DAZL upon heat stress. Here, we reveal that SGs are assembled in mouse male germ cells upon heat stress and that DAZL is an essential component of SGs. Furthermore, we provide evidence that the SGs in male germ cells have a protective function against apoptosis upon heat stress.

MATERIALS AND METHODS

Animals and heat treatment

All mice were housed and treated under approval of Institutional Animal Care and Use Committee at Seoul National University (SNU-090320-3). Adult (8-10 weeks) or one-week-old [7 days post-partum (dpp)] mice were used for heat-treatment experiment. Male mice were anesthetized and the lower third of the body was placed in a water bath at 32, 37, 40 or 42°C for the indicated time intervals. Unless otherwise indicated, 'heat stress' means a treatment at 42°C for 20 minutes. Control mice were anesthetized and left at room temperature. Following treatment, the mice were sacrificed and the testes were isolated for further analyses.

C57BL/6 inbred *Dazl*$^{Tm1Hgu/Tm1Hgu}$ mice were previously described (Ruggiu et al., 1997; Reynolds et al., 2007). To generate outbred C57BL/6 × CD1 lines, C57BL/6 *Dazl*$^{+/-}$ male mice were mated with CD1 females and the resulting *Dazl*$^{+/-}$ mice were intercrossed to produce *Dazl*$^{-/-}$ male mice. Male *Dazl*$^{-/-}$ and their littermates at 7 dpp were control- or heat-treated for further analyses. Genotypes were screened by PCR using DNAs isolated from tail tips as described previously (Ruggiu et al., 1997).

Some of outbred *Dazl*$^{-/-}$ male mice lack the whole male germ cells even at 7 dpp, owing to individual variations (Reynolds et al., 2007). The presence or absence of male germ cells was determined histologically in one of the testes pair of the 7 dpp outbred *Dazl*$^{-/-}$ mouse. Immunoblot analysis was performed with the other testis of the same animal.

Immunostaining and in situ hybridization

Mouse testes were fixed in Bouin's solution (Sigma-Aldrich) and paraffin embedded. All tissues were sectioned at 5 μm, and were deparaffinized and hydrated for further analyses. Antigen retrieval was conducted by microwave boiling for 15 minutes in one of three buffers: 10 mM citrate (pH 6.0), 10 mM Tris-HCl (pH 9.0) or 1 mM EDTA (pH 8.0). After cooling at room temperature, the testicular sections were blocked with 3% BSA (bovine serum albumin) in PBST (phosphate-buffered saline with 0.1% Tween 20) for 30 minutes and then incubated with the following antibodies for 1 hour: affinity-purified anti-DAZL (1:10) (Lee et al., 2006), anti-TIA-1 (1:25, Santa Cruz), affinity-purified anti-DAZAP2 (1:10) (see below), anti-phospho-eIF2α (1:50, Stressgen), anti-DCP2 (1:50) (Wang et al., 2002), anti-S6 kinase (1:50, Santa Cruz) that cross-reacts with HEDLS (Stoecklin et al., 2006), anti-eIF3 (1:25, Santa Cruz), anti-TDRD1 (1:250) (Chuma et al., 2003), anti-γ-H2AX (1:500, Millipore), anti-MVH (1:250) (Toyooka et al., 2000), anti-RACK1 (1:50, BD Biosciences or Abcam), anti-MTK1 (1:40, Sigma-Aldrich) and anti-phospho-p38 (1:50, Cell Signaling). After three washes with PBST, the sections were incubated with either biotinylated (Vector) or fluorophore-conjugated secondary antibodies (Invitrogen) for 30 minutes. Sections treated with the biotinylated antibody were then incubated with avidin:biotinylated peroxidase complex (Vector) after PBST washing and the sections were developed with 3,3′-diaminobenzidin tetrachloride (DAB) (Sigma-Aldrich). The sections were counterstained with Hematoxylin and DAPI (Sigma-Aldrich) for DAB and fluorescence staining, respectively.

For in situ hybridization, deparaffinized and hydrated testicular sections were placed in buffer containing 2×SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) with 15% formamide for 5 minutes. Slides were then incubated with 50 ng of oligo-dT(45)-3′-TAMRA (5-caboxytetramethylrhodamin) (Cosmo) diluted in hybridization buffer (2×SSC, 15% formamide, 50 μg/ml heparin, 250 μg/ml tRNA, 0.1% Tween 20) at 37°C for 1 hour. After hybridization, slides were washed twice with 2×SSC, 15% formamide at 37°C for 30 minutes and were counterstained with DAPI. As a negative control, a slide was incubated in 1 mg/ml RNase at 37°C for 30 minutes before probe hybridization. For colocalization analysis, slides were processed for antigen retrieval, in situ hybridization, immunostaining with antibody and then DAPI staining.

The slides were observed with a light or fluorescence microscope (Olympus IX51), and images were acquired using a CCD camera (Qicam fast 1394, Qimaging) and processed using ImagePro 5.0 (Media Cybernetics). For measurement of p38 phosphorylation signal intensity, immunofluorescent images were processed using Adobe Photoshop (Adobe Systems). Statistical data were analyzed by a *t*-test using SigmaPlot (Systat Software).

Production of a polyclonal antibody

The coding region of the human *DAZAP2* cDNAs (Kim et al., 2008) was subcloned into *pGEX-4T* vector (GE Healthcare) for the generation of GST-DAZAP2 fusion protein. A rabbit polyclonal anti-DAZAP2 antibody was raised against bacterially expressed GST-DAZAP2 and affinity-purified using a method described elsewhere (Kim et al., 2009). Briefly, the antisera were incubated with a strip of nitrocellulose membrane blotted with GST-DAZAP2 fusion protein, and eluted with 100 mM glycine (pH 2.5).

Truncated mutant analysis

HeLa cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Transfection was carried out using FuGENE (Roche) for the prepared truncated DAZL mutants. HeLa cells cultured on a coverslip were either control or heat (44°C for 1 hour) treated and followed by immunocytochemistry.

For immunocytochemistry, the coverslips were briefly washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. After a wash with 0.3% PBST, the coverslips were blocked with 3% BSA in PBST for 30 minutes and then incubated with anti-HA (1:500, Sigma) and anti-TIA-1 (1:100) for 1 hour. After three washes with PBST, cells were incubated with secondary antibodies for 30 minutes, washed with PBST and counterstained with DAPI. Observation and image acquisition were described above.

Immunoblot analysis

Mouse testes were decapsulated and homogenized in 1×SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol). About 20-50 μg of protein was resolved by SDS-PAGE and transferred into nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) for 30 minutes and then incubated overnight at 4°C with the following antibodies: anti-phospho-eIF2α (1:500), anti-eIF2α (1:150) (Santa Cruz), anti-DAZL (1:100), anti-β-tubulin (1:1000) (Sigma) and anti-MVH (1:2000). After three washes with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies for 30 minutes. After a further three washes with TBST, peroxidase activity was detected using ECL reagent.

For measurement of eIF2α phosphorylation levels, immunoblot images were processed using Adobe Photoshop. The fold increase was determined by the signal intensity of eIF2α phosphorylation level normalized to total eIF2α.

Full scan images of the immunoblots shown in the figures are shown in supplementary material Fig. S4.

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling) assay was conducted using a FragEL DNA Fragmentation Detection Kit (Calbiochem) according to the manufacturer's instruction. For double-staining with antibody, slides were processed with antigen retrieval, TUNEL assay, immunostaining with antibody and then DAPI staining.

RESULTS

DAZL is recruited to SGs in heat-stressed male germ cells

DAZL was detected primarily in the cytoplasm of spermatogonia and spermatocytes (Fig. 1A) (Ruggiu et al., 1997). DAZL expression is developmental stage-specific and its expression is

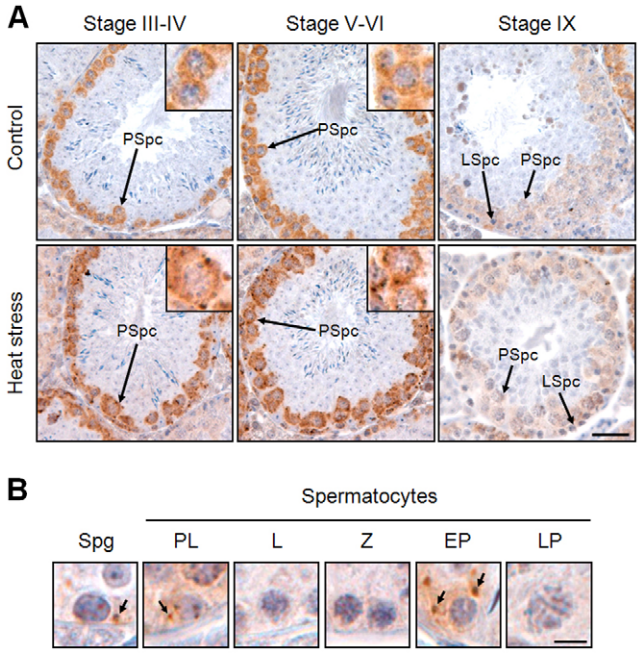

Fig. 1. DAZL is recruited to the cytoplasmic granules in heat-stressed mouse male germ cells. (**A**) Adult mice were placed in a warm bath (42°C) for 20 minutes, sacrificed and used for immunohistochemistry. The control and heat-stressed testes were immunostained with a DAZL antibody. The stages of the seminiferous epithelial cycle were determined according to Russell et al. (Russell et al., 1990). Magnified views of pachytene spermatocytes are shown in the insets. PSpc, pachytene spermatocyte; LSpc, leptotene spermatocyte. (**B**) Representative DAZL stainings at specific developmental stages of the mouse male germ cells in heat-stressed testes. The DAZL-positive granules are marked with arrows. Spg, spermatogonium; PL, preleptotene; L, leptotene; Z, zygotene; EP, early pachytene; LP, late pachytene. Scale bars: 50 μm in A; 10 μm in B.

significantly reduced in spermatocytes near meiosis at tubular stage IX (Fig. 1A). No DAZL signal was detected in postmeiotic spermatids of all tubular stages (Fig. 1A). We examined the subcellular localization of DAZL in heat-stressed mouse testes. When adult mice were placed in a warm water bath at 42°C for 20 minutes, DAZL formed aggregates at the perinuclear region of the male germ cells at tubular stages III-VI (Fig. 1A). The DAZL granules were detected in spermatogonia and in preleptotene and early pachytene spermatocytes, but not in leptotene, zygotene and late pachytene spermatocytes, in which DAZL expression was minimal (Fig. 1B).

We performed co-staining analyses to examine the possibility that the DAZL-positive granules are SGs. TIA1 is known to form a prion-like aggregation for SG assembly in a stressed cell (Kedersha et al., 1999; Gilks et al., 2004). In normal testis, TIA1 was detected in the nuclei of spermatocytes and spermatids as well as somatic cells, such as Sertoli and Leydig cells (Fig. 2A). However, no specific TIA1 signal was detected in spermatogonia (Fig. 2A). In the heat-stressed testis, TIA1 assembled into cytoplasmic granules in spermatocytes and colocalized with the DAZL-positive granules (Fig. 2A). Poly-$(A)^+$ RNAs, a major component of SGs, were found in large granules that were identified as chromatoid bodies in round spermatids (Fig. 2A) (Kotaja et al., 2006). The Poly-$(A)^+$ RNAs were also detected in the cytoplasm of spermatocytes and spermatogonia (Fig. 2A). Upon heat stress, however, poly-$(A)^+$ RNAs colocalized with the DAZL-positive granules in spermatocytes and spermatogonia (Fig. 2A). Another SG component, DAZAP2, is known to interact with eIF4G and to induce SG formation (Kim et al., 2008). In normal testis, DAZAP2 was detected in the nucleus and cytoplasm of male germ cells of all developmental stages except for elongated spermatids (Fig. 2A). In the heat-stressed testis, DAZAP2 colocalized with the DAZL-positive granules in spermatocytes and spermatogonia (Fig. 2A). Phospho-eIF2α, an SG component, also migrated to and colocalized with the DAZL-positive cytoplasmic granules upon heat stress (Fig. 2A) (Kedersha et al., 2005). Taken together, these data reveal that DAZL localizes to the SGs in spermatogonia as well as in pachytene spermatocytes of tubular stages III-VI. SGs were detected neither in spermatocytes at tubular stages I-II and VII-XII nor in spermatids of all stages, in which DAZL is absent.

In mammalian cells, SGs are dynamically linked to processing bodies (PBs) where mRNA degradation takes place (Kedersha et al., 2005). We performed co-immunostaining analysis to determine whether DAZL localizes to PBs or not. PBs, which were immunostained with antibodies specific to a decapping enzyme (DCP2) and a decapping factor (HEDLS; EDC4 – Mouse Genome Informatics), were detected as cytoplasmic foci of pachytene spermatocytes in control- and heat-treated testes (Fig. 2B; supplementary material Fig. S1A). The co-immunostaining analyses revealed that the DAZL-positive granules in heat-stressed spermatocytes did not overlap with the PB markers (Fig. 2B; supplementary material Fig. S1A). To confirm an exclusive localization of DAZL into SGs, we co-immunostained DAZL with eIF3, a protein found in SGs but not in PBs (Kedersha et al., 2005). The results showed that DAZL-positive granules did colocalize with eIF3 in the pachytene spermatocytes of tubular stages III-VI (supplementary material Fig. S1B). These results confirm that the DAZL-positive granules in heat-stressed animals are indeed SGs.

Mammalian male germ cells are known to contain an RNA granule termed nuage. Tudor domain-containing 1 (TDRD1) proteins are characteristically located at the nuage structure (Chuma et al., 2003; Chuma et al., 2006). To determine whether nuages and SGs are the same structures in heat-stressed mice, we performed co-immunostaining analysis with antibodies specific to DAZL and TDRD1. The results showed that heat stress did not affect the subcellular distribution of the TDRD1-positive granules (Fig. 2C). Furthermore, the DAZL-containing SGs did not colocalize with the TDRD1-containing nuages (Fig. 2C). These results reveal that DAZL-containing SGs are distinct from nuages in male germ cells.

We examined which domain of DAZL is responsible for the SG recruitment. DAZL, an RNA-binding protein, is 298 amino acids long with an RNA recognition motif and a conserved DAZ repeat (Fig. 2D). A series of deletion mutants were transiently transfected into HeLa cells and the subcellular distribution of the truncated DAZL mutant proteins was examined in control and heat-stressed cells. The full length and N-terminal truncated DAZL variants were recruited into SGs, whereas the C-terminal and short N-terminal truncated mutants appeared diffused throughout the cytoplasm in the heat-stressed condition (Fig. 2D). The truncated mutant with a deletion between RRM and the DAZ repeats (Δ116-166) also failed to localize in SGs, suggesting that the 116-166 region is crucial for the recruitment of DAZL to SGs (Fig. 2D).

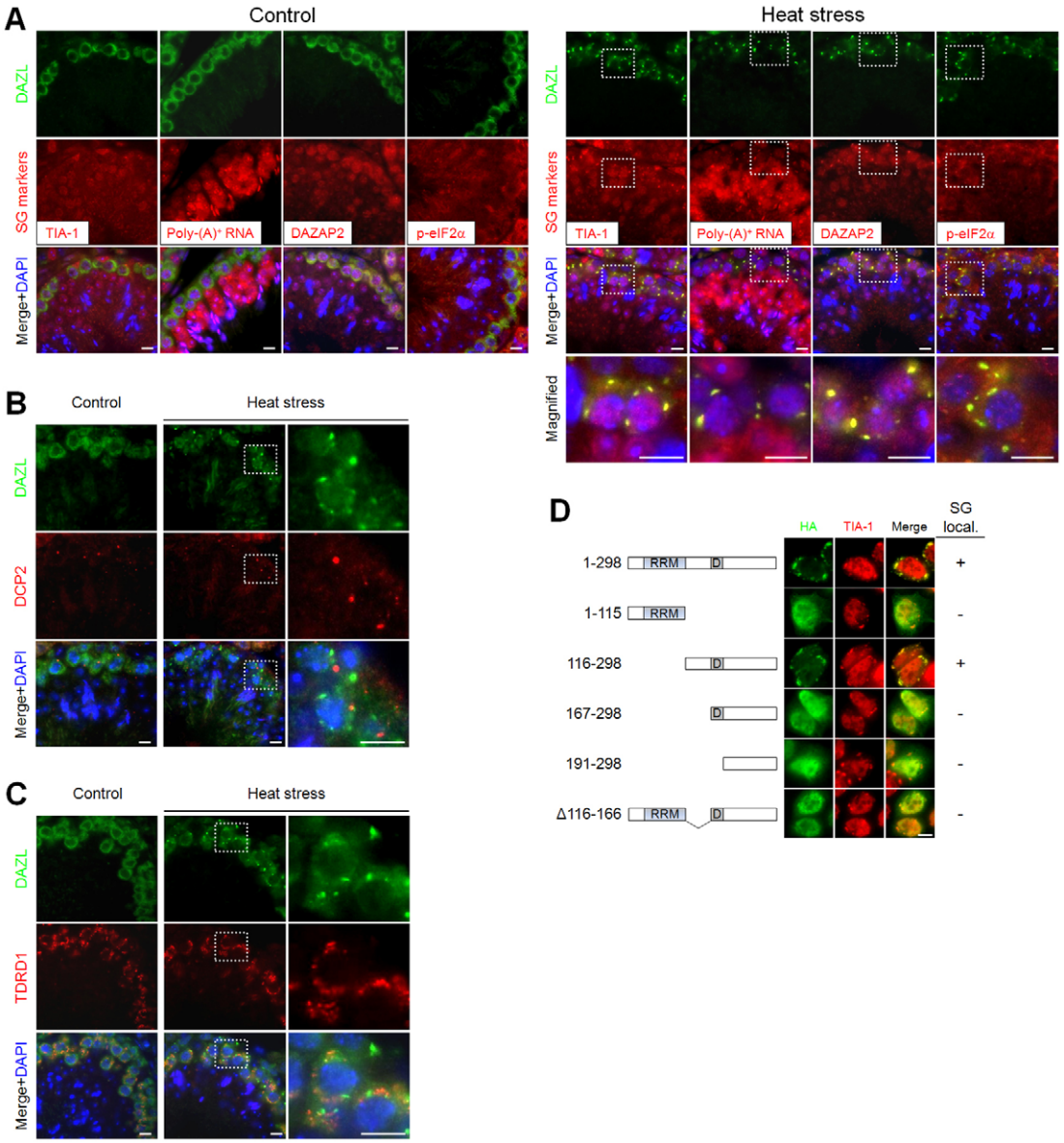

Fig. 2. The DAZL-positive granules in heat-stressed testes are SGs. (**A**) Adult testes from control and heat-treated mice were co-stained for DAZL (green), along with SG markers, such as TIA1, polyadenylated RNAs, DAZAP2 and phospho-eIF2α (red). DNA was stained with DAPI (blue). Magnified images are shown at the bottom on the right. (**B**,**C**) Adult testes from control and heat-treated mice were co-immunostained with the DAZL (green) and DCP2 (red, PBs; B) or TDRD1 (red, nuages; C) antibodies. DNA was stained with DAPI (blue). Magnified images are shown on the right. All data were taken from the seminiferous epithelial stages III-IV. (**D**) A truncated mutant analysis for the SG localization of DAZL. HeLa cells were transfected with a series of N-terminal HA-tagged DAZL deletion mutants. 24 hours after transfection, the cells were subjected to heat stress (44°C) for 1 hour and then immunostained for HA (green) and TIA1 (red). Schematic of the truncated mutants, representative images showing SG localization and summary of the results are shown. RRM, RNA recognition motif; D, DAZ repeat. Scale bars: 10 μm.

The SGs of male germ cells are assembled in hyperthermic testes

The mammalian core body temperature (37°C) is hyperthermic for male germ cells (Hutson et al., 1997). We carefully examined SG formation in male germ cells under hyperthermic conditions. First, we performed immunoblot analysis to determine the phosphorylation status of eIF2α, a molecular signature of translational inhibition and SG assembly, in heat-stressed testes (Kedersha et al., 1999). The results showed that eIF2α began to be phosphorylated even after treatment at 37°C for 20 minutes and that the phospho-eIF2α levels increased further at higher temperatures (Fig. 3A). Next, immunohistochemical analysis was performed to confirm SG formation at 37°C. The results showed that SGs, which were immunostained with antibodies specific to DAZL and TIA1, started to be assembled at 37°C and were conspicuous at 40°C in early pachytene spermatocytes (Fig. 3B). These results reveal that the core body temperature is sufficient to induce SG formation in pre-meiotic male germ cells.

We examined further the dynamics of eIF2α phosphorylation and SG formation at 37°C. Upon prolonged exposure to heat stress at 37°C, the mouse testis showed an increase in eIF2α phosphorylation and SG formation; phosphorylation of eIF2α was induced within half an hour and it continued to increase slightly up to 2 hours (Fig. 3C). Similarly, SG started to

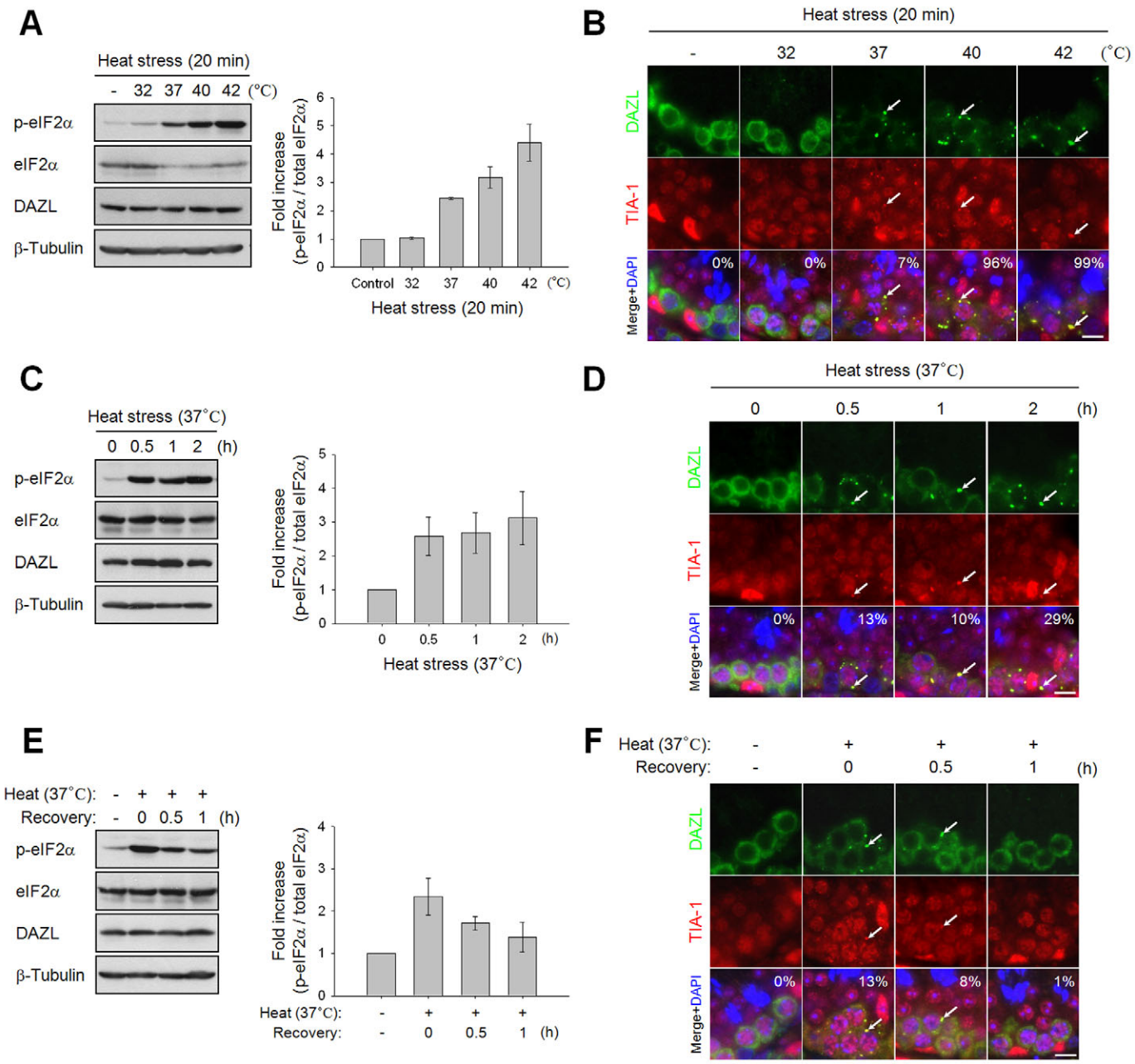

Fig. 3. Dynamics of SG assembly in heat-stressed mouse testes. (**A**,**C**,**E**) Adult mice were placed in a warm bath (32, 37, 40 or 42°C) for the indicated time periods and the testicular lysates were immunoblotted with antibodies specific to phospho-eIF2α, eIF2α, DAZL and β-tubulin. The phospho-eIF2α levels in testicular lysates that are shown on the graphs have been normalized to total eIF2α. Error bars represent s.e.m. from three different animals. (**B**,**D**,**F**) Testis sections from the heat-treated animals were co-immunostained with antibodies against DAZL (green) and TIA1 (red). DNA was stained with DAPI (blue). SGs are marked with arrows. Percentages of the SG-containing pachytene spermatocytes at tubular stages III-IV were determined. The experiments were performed with three different animals and over 500 germ cells in ten different tubules per animal were counted. In E and F, adult mice subjected to heat stress (37°C for half an hour) were recovered at room temperature for 0, 0.5 or 1 hours. Scale bars: 10 μm.

assemble in 10% of early pachytene spermatocytes at tubular stages III-IV within half an hour and gradually increased to 29% after 2 hours (Fig. 3D). It is known that mammalian SGs are reversibly assembled and disassembled in accordance with heat stress and recovery (Kedersha et al., 1999). When the heat-stressed mice were placed at room temperature for 1 hour, eIF2α was rapidly dephosphorylated and the SGs were disassembled (Fig. 3E,F). DAZL protein levels were unchanged for either the stress or recovery conditions (Fig. 3A,C,E). Taken together, these results reveal that SG formation is an immediate and reversible response to hyperthermic stress in pre-meiotic spermatocytes.

DAZL is essential for SG formation in heat-stressed testes

We decided to use *Dazl*-knockout (*Dazl*-KO) mice to explore the involvement of DAZL in SG formation. It is known that adult *Dazl*-KO mice lack germ cells in their testes (Ruggiu et al., 1997).

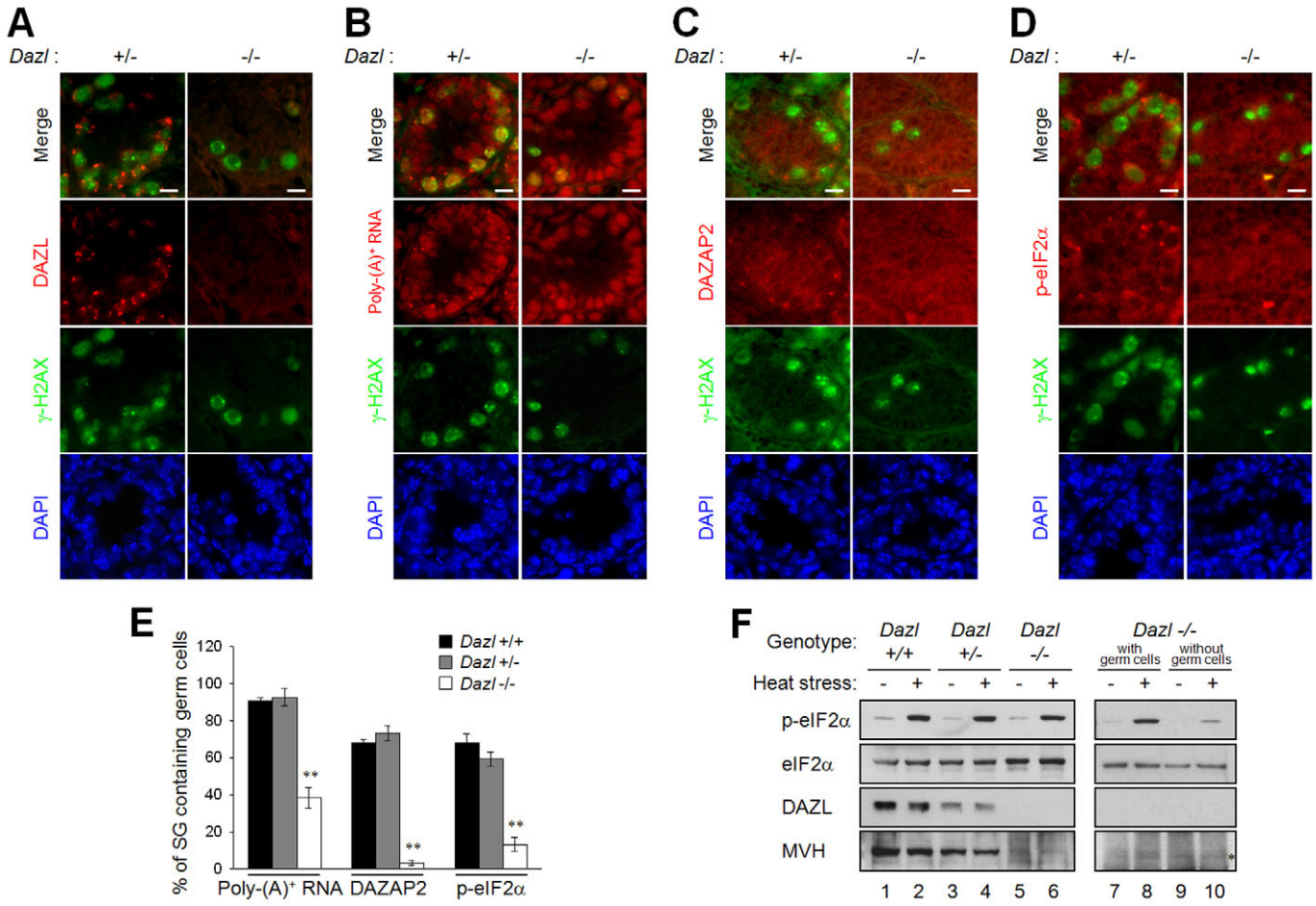

Fig. 4. DAZL is required for SG formation. (**A-D**) Seven-day-old (7 dpp) *Dazl*$^{-/-}$ mice and their littermates were placed in a warm bath at 42°C for 20 minutes. The testicular sections were co-stained for γ-H2AX (green), along with DAZL (A), oligo-dT (B), DAZAP2 (C) or phospho-eIF2α (D) (red). DNA was stained with DAPI (blue). Scale bars: 10 μm. (**E**) *Dazl*$^{-/-}$ mice and their littermates were subjected to heat stress and SG formation was determined by the localization of SG components (polyadenylated RNAs, DAZAP2 and phospho-eIF2α). The germ cells were immunostained with a γ-H2AX antibody and those with SGs were counted and statistically analyzed. At least 50 germ cells per testis were counted in three different animals. Error bars represent s.e.m. $^{**}P<0.001$ (by *t*-test). (**F**) *Dazl*$^{-/-}$ mice and their littermates were subjected to heat stress and the testicular lysates were immunoblotted with the antibodies specific to phospho-eIF2α, eIF2α, DAZL and MVH. The *Dazl*$^{-/-}$ testis with or without germ cells was analyzed further in order to assess the contribution of germ cells to the total tissue levels of eIF2α phosphorylation (right). An asterisk indicates a cross-reacting band that appeared when the film was overexposed.

Careful examination revealed that the *Dazl*-KO male mice with an inbred C57BL/6 background lose their germ cells starting at embryonic day 15.5, but those with an outbred genetic background, C57BL/6 × CD1, can maintain their germ cells until 7 dpp (Lin and Page, 2005; Reynolds et al., 2007). Some of the testes of *Dazl*-KO mice lacked male germ cells at 7 dpp owing to the individual variation within a mixed genetic background providing a valuable control (Reynolds et al., 2007). Therefore, we used the testes from 7 dpp *Dazl*-KO mice of a mixed genetic background for our experiments.

First, we analyzed the stress responses of wild-type testes at 7 dpp. Phosphorylation of eIF2α was observed following heat stress of 40°C or 42°C for 20 minutes (supplementary material Fig. S2A). SGs were assembled in most of the wild-type germ cells, which were identified with the γ-H2AX antibody (supplementary material Fig. S2B) (Mahadevaiah et al., 2001). We then evaluated SG formation in the testes of *Dazl*-KO mice by staining for SG markers in addition to γ-H2AX (H2AFX – Mouse Genome Informatics). As expected, DAZL was absent in the surviving germ cells of the *Dazl*$^{-/-}$ mice, whereas DAZL was expressed in the *Dazl*$^{+/-}$ testes and formed aggregates upon heat stress (Fig. 4A). The selected SG markers, namely poly-(A)$^+$ RNAs, DAZAP2 and phospho-eIF2α, relocated to the aggregates upon heat stress in *Dazl*$^{+/-}$ germ cells, but few such aggregates were formed in *Dazl*$^{-/-}$ germ cells (Fig. 4B-D). We counted the number of germ cells with SGs in the heat-stressed *Dazl*$^{-/-}$ animals and their littermates. For all three SG markers tested, SG formation was significantly reduced in the *Dazl*$^{-/-}$ testes compared with the wild-type and *Dazl*$^{+/-}$ testes (Fig. 4E). These results indicate that DAZL is essential for SG formation upon heat stress. TIA1 cannot be used as a SG marker because it is not expressed in the early germ cells at 7 dpp.

We observed previously the presence of phospho-eIF2α in the SGs of male germ cells under heat stress (Fig. 2A, Fig. 4D). It is known that the phosphorylation of eIF2α often precedes SG assembly (Kedersha et al., 1999; Anderson and Kedersha, 2008). Therefore, we investigated whether eIF2α is phosphorylated in the male germ cells of *Dazl*-KO mice where SGs are not assembled

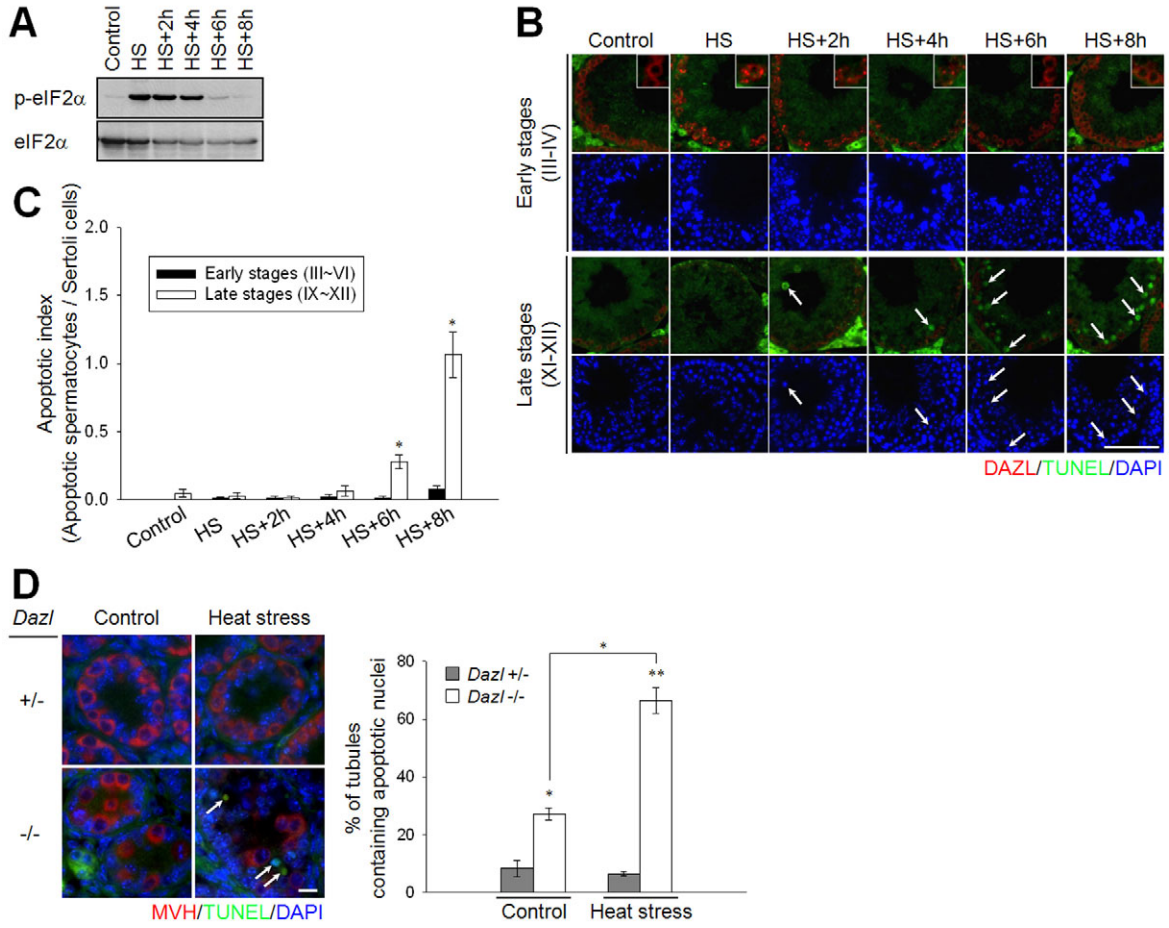

Fig. 5. Importance of DAZL for male germ cell survival under heat stress. (**A**) Adult mice were placed in a warm bath at 42°C for 20 minutes and recovered at room temperature for the indicated time periods. The testis lysates were subjected to immunoblot analysis with phospho-eIF2α and eIF2α antibodies. (**B**) TUNEL assays were carried out with the testis sections from heat-recovered mice (green). The green signals in the inter-tubular region are nonspecific. The same sections were also immunostained with a DAZL antibody (red). Representative seminiferous tubules at the early stages (III-IV) and the late stages (XI-XII) of development are shown. Magnified views of early pachytene spermatocytes are shown in the insets. Arrows indicate TUNEL-positive spermatocytes. (**C**) Apoptotic indices were calculated in the early and late tubular stages of the testis sections. Error bars indicate s.e.m. from ten tubules of the same stage group. (**D**) The 7 dpp *Dazl*$^{-/-}$ mice and their littermates were heat-treated at 42°C for 20 minutes. The testis sections were subjected to TUNEL assay (green), followed by immunostaining with an MVH antibody (red, germ cells). Arrows indicate the TUNEL-positive fragmented nuclei. The number of germ cell-containing seminiferous tubules with TUNEL-positive nuclei was counted in three different animals. Over 30 tubules per animal were analyzed and presented as the mean and s.e.m. In all staining procedures, DNA was stained with DAPI (blue). Scale bars: 100 μm in B; 10 μm in D. *P<0.01; **P<0.001 (by t-test).

under heat stress. Immunoblot analysis confirmed a complete depletion of DAZL expression in the *Dazl*$^{-/-}$ testes (Fig. 4F). Despite the absence of DAZL, eIF2α was still phosphorylated at a similar level in the heat-stressed testis (Fig. 4F, lanes 1-6). It is possible that a large portion of the phospho-eIF2α proteins in the *Dazl*-KO testes originates from cell types other than the germ cells. To test this possibility, we evaluated the phospho-eIF2α levels in *Dazl*-KO testes with and without germ cells under a heat-stress condition. The presence of male germ cells in the 7 dpp testes was determined with an MVH antibody (Fig. 4F, lanes 7-10). The results show that the phospho-eIF2α levels in *Dazl*-KO testes without germ cells were significantly lower than those with germ cells (Fig. 4F, lanes 8 and 10). These results suggest that the increase in phospho-eIF2α levels in heat-stressed testes is largely due to the germ cells, and also strongly suggest that eIF2α phosphorylation in germ cells upon heat stress is independent of DAZL.

A protective role of SG against germ cell apoptosis

It is known that mammalian SGs are implicated in cell survival during stress (Kwon et al., 2007; Arimoto et al., 2008; Eisinger-Mathason et al., 2008). It is also well known that male germ cells at specific developmental stages are sensitive to heat stress. The apoptotic rate increases in pre-meiotic spermatocytes at tubular stages IX-XII, and in early spermatids at tubular stages I-II (supplementary material Fig. S3) (Lue et al., 1999). We have observed that spermatocytes at tubular stages IX-XII are devoid of DAZL and do not assemble SGs under heat stress (Fig. 1). Therefore, we decided to test the hypothesis that the SGs of male germ cells have a protective role against heat stress. We first observed SG formation and apoptosis in the adult mouse testis after heat stress and recovery. The results showed that eIF2α was immediately phosphorylated upon heat stress and reversibly dephosphorylated 6 hours after the recovery (Fig. 5A). DAZL-containing SGs in

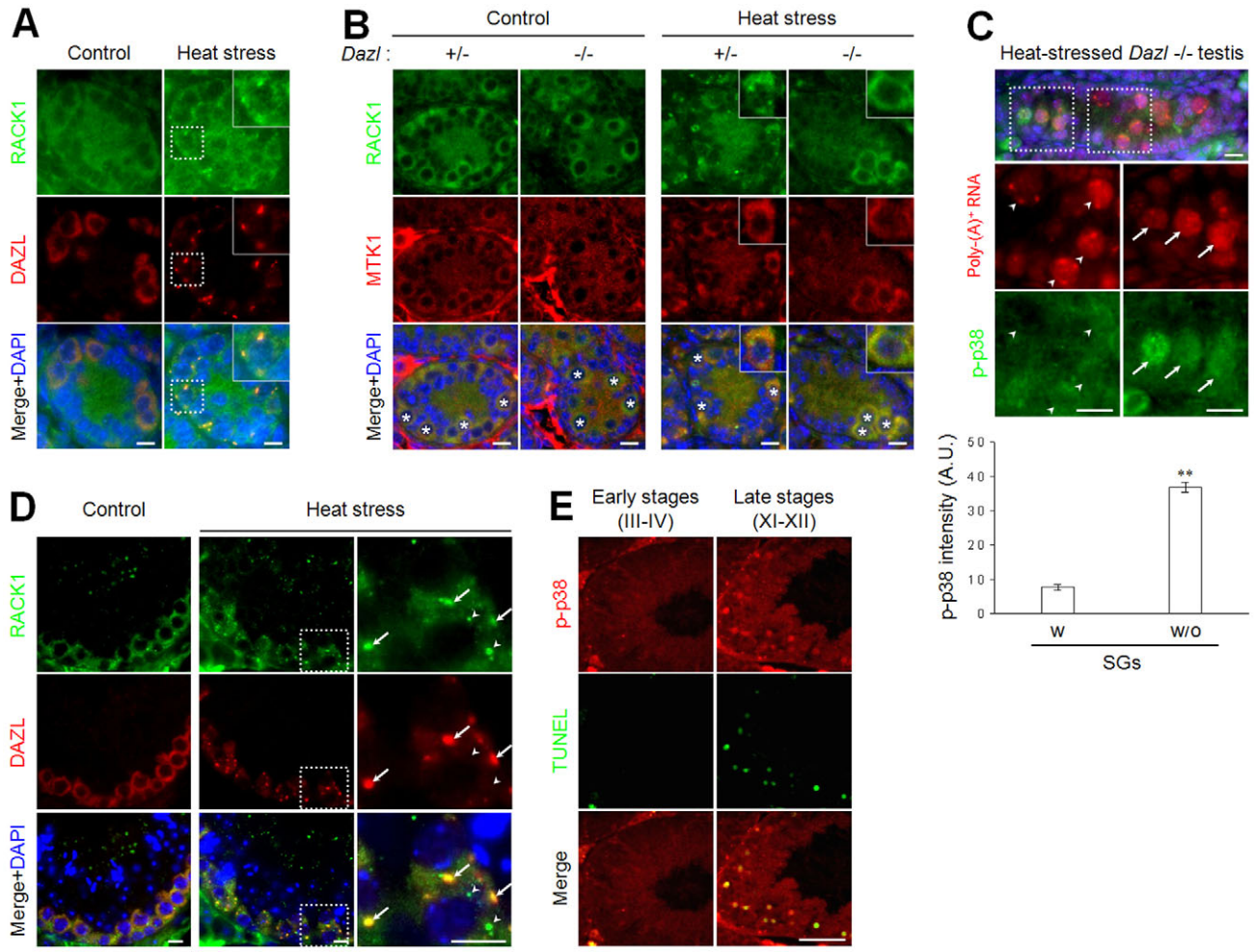

Fig. 6. Sequestration of RACK1 in SGs and inhibition of the apoptotic MAPK in heat-stressed male germ cells. (**A**) The 7 dpp mice were heat-stressed and the testis sections were co-immunostained with RACK1 (green) and DAZL (red) antibodies. A magnified view of germ cells with SGs is shown in the inset. (**B**) Testis sections from heat-treated 7 dpp *Dazl*$^{-/-}$ mice and their littermates were co-immunostained with RACK1 (green) and MTK1 (red) antibodies. Magnified views of germ cells are shown in the insets. Asterisks indicate germ cells. (**C**) Testis sections from the heat-treated 7 dpp *Dazl*$^{-/-}$ mice and their littermates were stained for polyadenylated RNAs (red) and phospho-p38 (green). Magnified views of the germ cells with SG (arrowheads) and those without SG (arrows) are shown. The intensities of phospho-p38 were determined and statistically analyzed. Over 120 germ cells were analyzed in three different animals and presented as the mean and s.e.m. $^{**}P<0.001$ (by *t*-test). (**D**) Adult testes from control and heat-treated animals were co-immunostained with RACK1 (green) and DAZL (red) antibodies. Magnified images are shown on the right. Arrows and arrowheads indicate SGs and RACK1-specific granules, respectively. The data were taken from the seminiferous epithelial stages III-IV. (**E**) Testicular sections from mice recovered for 6 hours after heat stress were subjected to TUNEL assay (green), followed by immunostaining with a phospho-p38 antibody (red). The seminiferous tubules from the early stages (III-IV) and the late stages (XI-XII) are shown. In all staining procedures, DNA was stained with DAPI (blue). Scale bars: 10 μm in A-D; 50 μm in E.

spermatocytes at tubular stages III-IV were assembled and disassembled under the same conditions of heat stress and a recovery period (Fig. 5B). However, no DAZL-containing SGs were formed in the spermatocytes at tubular stages XI-XII upon heat stress (Fig. 5B). We performed the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling) assay to identify any germ cell apoptosis. The results showed that germ cell apoptosis occurred 6 hours after heat stress and recovery in the late spermatocytes, but not in the early spermatocytes in which SGs had been assembled (Fig. 5B,C). These results suggest a functional association of the DAZL-containing SGs with germ cell survival.

The importance of DAZL in germ cell survival under a heat stress condition was examined in *Dazl*-KO mice. Cells undergoing apoptosis were detected more frequently in the testes of *Dazl*$^{-/-}$ mice than in those of *Dazl*$^{+/-}$ mice even under normal conditions (Fig. 5D). Furthermore, heat stress induced a significant increase in the number of apoptotic cells by 40% in *Dazl*$^{-/-}$ testes, but not in the control testes (Fig. 5D). These results support the notion that DAZL protects germ cells against apoptosis upon heat stress by inducing SG formation.

A protective function of SGs against apoptosis has been demonstrated in cultured somatic cells. The sequestration of RACK1 into SGs perturbs its interaction with MTK1, a mitogen-activated protein kinase kinase kinase (MAP3K4), and, consequentially, blocks the apoptotic MAPK signaling pathway (Arimoto et al., 2008). In fact, it was reported that the p38-mediated MAPK signaling pathway is involved in male germ cell apoptosis upon heat stress (Jia et al., 2009). Therefore, we hypothesized that SGs inhibit germ cell apoptosis by sequestering RACK1 in SGs, thus blocking the p38-MAPK signaling pathway in male germ cells.

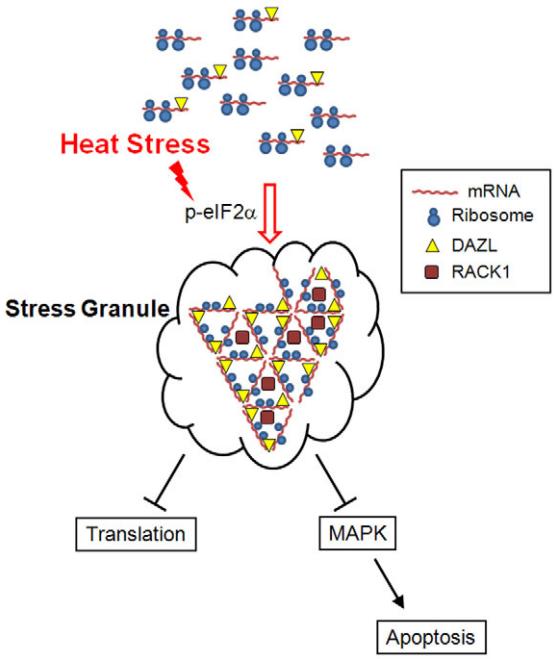

Fig. 7. Model of SG formation in male germ cells. DAZL is involved in transport and translation of specific mRNAs during male germ cell development. Once the germ cells are under heat stress, eIF2α is phosphorylated and SGs are formed. DAZL is essential for SG formation and might play a crucial role in the recruitment of the associated mRNAs into SGs. The SGs not only reduce global translation activity, but also prevent male germ cell apoptosis by the sequestration of RACK1 and subsequent suppression of the apoptotic MAPK pathway.

We examined initially the subcellular localization of RACK1 in heat-stressed mouse testes. RACK1 was distributed throughout the cytoplasm of male germ cells in a diffuse pattern, but was recruited to SGs upon heat stress (Fig. 6A). In *Dazl*$^{-/-}$ germ cells, RACK1 remained in the cytoplasm, probably because SGs were not assembled upon heat stress (Fig. 6B). By contrast, MTK1, a RACK1-binding partner, was localized to the cytoplasm in a diffuse pattern even under heat stress (Fig. 6B). These results raise the possibility that the apoptotic MAPK signaling could be blocked in the germ cells by sequestration of RACK1 in SGs, resulting in MTK1 inactivation. To test this possibility, we analyzed p38 activation in heat-stressed *Dazl*$^{-/-}$ male germ cells in which SG formation is impaired but not completely eliminated (Fig. 4E). As expected, p38 activation, as shown by the phospho-p38 signal, was higher in the germ cells without SGs than in those with SGs (Fig. 6C). Consistent with this finding, we also observed that RACK1 was localized to SGs along with DAZL in heat-stressed adult testes (Fig. 6D). The phospho-p38-positive cells were selectively detected in germ cells at tubular stages XI-XII, which are the developmental stages at which germ cell apoptosis occurs upon heat stress (Fig. 6E). These results support the hypothesis that SG formation inhibits germ cell apoptosis, at least partially, by blocking the p38-MAPK signaling pathway in male germ cells.

DISCUSSION

In this study, we report that phosphorylation of eIF2α is one of the immediate responses in male germ cells to heat stress. SGs are assembled at the same time. These changes might induce a global repression of translation, as shown by the reduction of the polysomes in heat-stressed testes (Cataldo et al., 1997). A proteomic study revealed that transient heat stress induces an immediate change in the protein profile of male germ cells (Zhu et al., 2006). Phosphorylation of eIF2α probably causes this swift inhibition of translation. SGs might store a specific set of mRNAs until the testis has recovered from heat stress.

We also observed that the cytoplasmic DAZL in male germ cells was relocated to the SGs upon heat stress. SGs can be formed only at specific developmental stages of male germ cells; SGs were detected in spermatogonia and early pachytene spermatocytes, in which DAZL is abundantly expressed, but not in late pachytene and diplotene spermatocytes, in which DAZL expression is reduced. Furthermore, SG assembly was significantly diminished in the early male germ cells of *Dazl*-KO mice. These results suggest that DAZL is involved in the heat-stress response as an essential component of SGs in male germ cells (Fig. 7).

One of the unresolved issues in this study is how DAZL is recruited to SGs and how it regulates their formation upon heat stress. It is known that protein-protein interactions are important for SG assembly (Anderson and Kedersha, 2008). Our truncated mutant analysis revealed that residues 116-166 of DAZL are required for SG localization in HeLa cells. This region is highly conserved among the DAZ family proteins. It is also responsible for the specific interaction with PABP, a major SG component, suggesting that DAZL is recruited to SGs through specific interaction with PABP (Kedersha et al., 1999; Collier et al., 2005). It is also noteworthy that DAZL interacts with DAZAP2, an SG component and an interacting partner of the translation initiation factor eIF4G (Tsui et al., 2000; Kim et al., 2008). At the same time, DAZL might function as a nucleator of SG assembly, as ectopic expression of DAZL induces SG formation in HeLa cells (Lee et al., 2006).

A protective role of SGs against apoptosis has been reported in cultured somatic cells (Arimoto et al., 2008). Here, we report evidence that the DAZL-containing SGs in male germ cells also have a protective role against apoptosis upon heat stress. The male germ cells in *Dazl*-KO mice did not assemble SGs properly upon heat stress and the number of apoptotic cells increased correspondingly. In adult mice, germ cell apoptosis occurred mostly at tubular stages IX-XII when the cellular DAZL level is reduced and SGs are absent. Our immunohistochemical results are consistent with the hypothesis that germ cell apoptosis is inhibited by the sequestration of specific signaling molecules, such as RACK1 in SGs, thereby causing the blockage of the apoptotic MAPK pathway (Arimoto et al., 2008). In fact, MAPK is known to be crucial for male germ cell apoptosis triggered by hormone deprivation or heat stress (Jia et al., 2009). We also do not rule out another possible protective role for SGs against apoptosis, specifically the salvaging of mRNAs that are crucial for developmental events, such as meiosis. Once the germ cells have recovered from the stress, these mRNAs might be released from the SGs and translated to resume germ cell development without interruption.

It was reported previously that DAZL functions as a translational regulator for a specific set of genes (Collier et al., 2005; Reynolds et al., 2005; Reynolds et al., 2007). It was also proposed that DAZL functions as an mRNA adaptor for the dynein motor complex for intracellular transport (Lee et al., 2006). Recently, DAZL was found to be an intrinsic factor that enables embryonic germ cells to initiate meiosis in response to retinoic acid (Lin et al., 2008). In addition, DAZL was shown to regulate the preserved mRNAs by translational activation to promote early embryonic development

(Chen et al., 2011). In this study, we propose that DAZL is an essential component of SGs and has a protective function against heat stress (Fig. 7). We speculate that the stress-protective function of DAZL is not mutually exclusive with the other known functions of DAZL under normal conditions. DAZL might be associated with a specific set of mRNAs for transport and translational regulation. Once cells are under heat stress, DAZL becomes an essential component of SGs and might actively bring the associated mRNAs into SGs (Fig. 7).

Our study suggests that SG formation is one of the immediate protective mechanisms against heat stress. However, it remains to be investigated whether SGs also play a protective role against prolonged stresses, such as cryptorchidism. It is unlikely that heat stress is the main cause of male germ cell death in *Dazl*-KO mice, which occurs as early as embryonic day 15.5 (Lin and Page, 2005). Nonetheless, it is possible that the heat-protective function of DAZ might be related to male fertility in humans. There are three *DAZ* family genes in humans; *DAZL* and *BOULE* (*BOLL* – Human Gene Nomenclature Database) are located in autosomal chromosomes and four copies of *DAZ* are located at the *AZFc* locus of the Y chromosome. Because microdeletions at the *AZFc* locus are frequently observed in infertile males, *DAZ* is suspected to be a male infertility gene. However, there are *AZFc*-deficient males with normal fertility, indicating that the phenotypic penetration of *DAZ* is incomplete (Chang et al., 1999; Kühnert et al., 2004). Genetic and biochemical evidence supports the idea that the DAZ family proteins share common biological functions at the different developmental stages of male germ cells (reviewed by Reynolds and Cooke, 2005). If DAZ family proteins are important for SG formation, then *DAZ*-deficient male patients might be more susceptible to heat stress than normal males, which would intensify male infertility. This possibility should be examined extensively for the purpose of clinical application.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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