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Importance of eIF2α Phosphorylation as a Protective Mechanism Against Heat Stress in Mouse Male Germ Cells

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SUMMARY

Mammalian male germ cells are exceptionally labile to heat stress. A temporal arrest of translation is one immediate response to heat, which involves heat-induced phosphorylation of eukaryotic initiation factor 2α (eIF2α) to block the formation of the translational initiation complex. Here, we investigated the protective mechanisms against heat stress in mouse male germ cells. All known eIF2α kinases were expressed in lineage- and developmental stage-specific manners in the testis; noteworthy was the presence of Gcn2 (General control nonderepressible 2 kinase) in spermatocytes of all seminiferous tubules. Multiple eIF2α kinases are likely activated upon heat stress in male germ cells. ISRIB (Integrated stress response inhibitor) was then used to determine the events downstream of eIF2α phosphorylation. ISRIB significantly reduced the rate of stress granule formation in spermatocytes at early-stage (III-IV) seminiferous tubules, and induced a number of apoptotic germ cells at late-stage (XI-XII) seminiferous tubules near the onset of meiosis. Thus, stress granule formation is a downstream event of eIF2α phosphorylation that may not directly protect cells from apoptosis, at least in spermatocytes of seminiferous tubules in early stages.

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INTRODUCTION

Mammalian male germ cells must be maintained at 2−3°C below core body temperature for proper spermatogenesis (Moore, 1922). Severe or repetitive heat exposure often induces male subfertility or infertility due to reduced sperm output (Thonneau et al., 1998). Varicocele and cryptorchidism are two examples of human diseases in which the testes are exposed to an elevated temperature, leading to fertility defects. Cryptorchidic patients are also known to have a higher incidence of developing testicular cancer (Lip et al., 2013; Sadov et al., 2016). Therefore, investigation of heat stress on male germ cells has clinical impact for male infertility and testicular cancer.

A temporal arrest of translation is one immediate response to heat stress in mouse male germ cells (Kim et al., 2013). eIF2α (Eukaryotic initiation factor 2α) becomes phosphorylated at temperatures as low as 37°C (Kim et al., 2012), thus inhibiting the eIF2-GTP-Met-tRNA ternary complex and causing global attenuation of translation in mouse male germ cells (Rowlands et al., 1988; Krishnamoorthy et al., 2001). Four kinases can phosphorylate eIF2α in response to diverse environmental stresses (Donnelly et al., 2013): Perk (Pkr-like endoplasmic reticulum kinase) is sensitive to

Abbreviations: Dazl, deleted in azoospermia-like; eIF2α, eukaryotic initiation factor 2α; Gcn2, general control nonderepressible 2; Hri, heme-regulated inhibitor; ISRIB, integrated stress response inhibitor; Perk, pkr-like endoplasmic reticulum kinase; Pkr, protein kinase R; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling.

endoplasmic reticulum stress (Schroder and Kaufman, 2005); Hri (Heme-regulated inhibitor kinase) is activated by heat-induced heme-deprivation in erythrocytes (Han et al., 2001); Pkr (Protein kinase R, a double-stranded RNA-dependent protein kinase) is switched on by viral infection (Barber, 2005); and Gcn2 (General control nonderepressible 2 kinase) is stimulated by amino acid depletion and ultra-violet irradiation (Kimball, 1999). Which kinase is activated in response to heat stress in mouse male germ cells remains to be determined.

Stress granule formation is another mechanism that leads to the global attenuation of translation following cellular stress. A stress granule is a transient subcellular structure containing stalled pre-initiation 48S complexes engaged with mRNAs, and may be detected in the cytoplasm with the TIA-1 antibody (Anderson and Kedersha, 2008). Stress granules may protect against heat stress by sequestering dormant mRNAs and proteins, including cellular signaling components (Kedersha et al., 2013). Heat-stressed mouse male germ cells accumulate stress granules mostly in spermatocytes of early-stage seminiferous tubules (I–VII) (Kim et al., 2012). Dazl (Deleted in azoospermia-like), a germ cell-specific RNA binding protein, is also abundant in the stress granules of spermatocytes (Ruggiu et al., 1997; Kim et al., 2012); indeed, Dazl is considered an essential component of heat stress-induced granules as these structure are rare in male germ cells of *Dazl*-deleted mice (Kim et al., 2012). The exact function of Dazl within stress granules remains to be elucidated.

Germ cell apoptosis is a typical phenotype in heat-stressed testes (Rockett et al., 2001). An inverse correlation between stress granule formation and germ cell apoptosis appears to exist in heat-stressed spermatocytes. Heat stress is known to induce germ cell apoptosis of spermatocytes in seminiferous tubules at late stages (VIII–XII), whereas no significant germ cell apoptosis is observed in the spermatocytes of seminiferous tubules at early stages (I–VII), when stress granules form (Kim et al., 2012; Yuen et al., 2014). Furthermore, *Dazl* expression is limited to the spermatocytes in early-stage seminiferous tubules (Ruggiu et al., 1997). Therefore, spermatocytes in early-stage seminiferous tubules may form stress granules as a means of avoiding apoptosis that would otherwise result from heat stress—although these two events also may not be functionally linked at all.

In this study, we investigated the protective mechanisms against heat stress in male germ cells. We initially asked which testicular eIF2α kinases might be activated upon heat stress, and then determined the sequential downstream events of eIF2α phosphorylation in male germ cells using ISRIB (Integrated stress response inhibitor), an inhibitor of downstream signaling (Sidrauski et al., 2013). ISRIB blunted the effects of eIF2α phosphorylation in cells, and thus represents the first bona fide inhibitor that can act downstream of all eIF2α kinases (Sidrauski et al., 2015). Our results further suggest that stress granule formation and germ cell apoptosis are downstream of eIF2α phosphorylation following heat stress.

RESULTS

Expression of eIF2α Kinases in the Mouse Testis

We began our study by performing quantitative reverse-transcription PCR to determine the testicular expression of four eIF2α kinase genes: *Hri*, *Perk*, *Pkr*, and *Gcn2*. Specific transcripts of all four eIF2α kinase genes were detected in the mouse testis, each with a distinct pattern in the immature and adult testes. *Hri* transcripts were more abundant in adult testes, whereas the *Perk* transcripts were enriched in immature testes, which lacked post-meiotic germ cells (Fig. 1A). *Pkr* transcripts were abundant in 7-day-old and adult testes. Finally, *Gcn2* transcripts were relatively abundant in 17-day-old testis, which included pre-meiotic germ cells and Sertoli cells (Fig. 1A). A dramatic increase in the transcription of the control, *Prm1* (Protamine 1), was seen in the adult testes (Fig. 1A).

Immunoblot analysis was used to assess the testicular expression of eIF2α kinases at the protein level following heat stress at 42°C for 20 min. First, protein of all eIF2α kinases is present in testicular lysates of immature and adult mice (Fig. 1B), at levels comparable to their transcript (Fig. 1). As expected, intensities of the phospho-eIF2α-specific band were enhanced in the heat-treated testes of immature and adult mice (Fig. 1B). Heat stress only affected the total protein abundance of Perk in the adult testis, which was significantly reduced following heat stress (Fig. 1B and C).

We also performed immunohistochemical analysis to determine cell type-specific expression of the eIF2α kinases in immature and adult testes (Fig. 2). Unfortunately, we were not able to determine a specific expression pattern for Hri, Perk, or Pkr in the testis samples, probably due to their ubiquity in diverse testicular cells. By contrast, Hri and Gcn2 were enriched in male germ cells (Fig. 2). Gcn2 in particular was detected in spermatocytes of all seminiferous tubules, starting in 17-day-old testes, but not in spermatids of the adult testis (Fig. 2). We therefore focused on the importance of Gcn2 in stress granule formation in primary spermatocytes.

Heat Stress Response in the Testis of the *Gcn2*-Knockout Mouse

We used *Gcn2*-knockout mice to address the involvement of Gcn2 in the heat-stress response in mouse male germ cells. Immunoblot analysis confirmed that Gcn2 protein abundance is reduced in the testes of *Gcn2* heterozygotes and absent in *Gcn2* homozygotes (Fig. 3A), and further revealed that eIF2α is phosphorylated upon heat stress in wild-type and *Gcn2*-mutant testes (Fig. 3A). We next evaluated stress granule formation following heat stress, using a Dazl antibody to detect the stress granules in spermatocytes (Kim et al.,

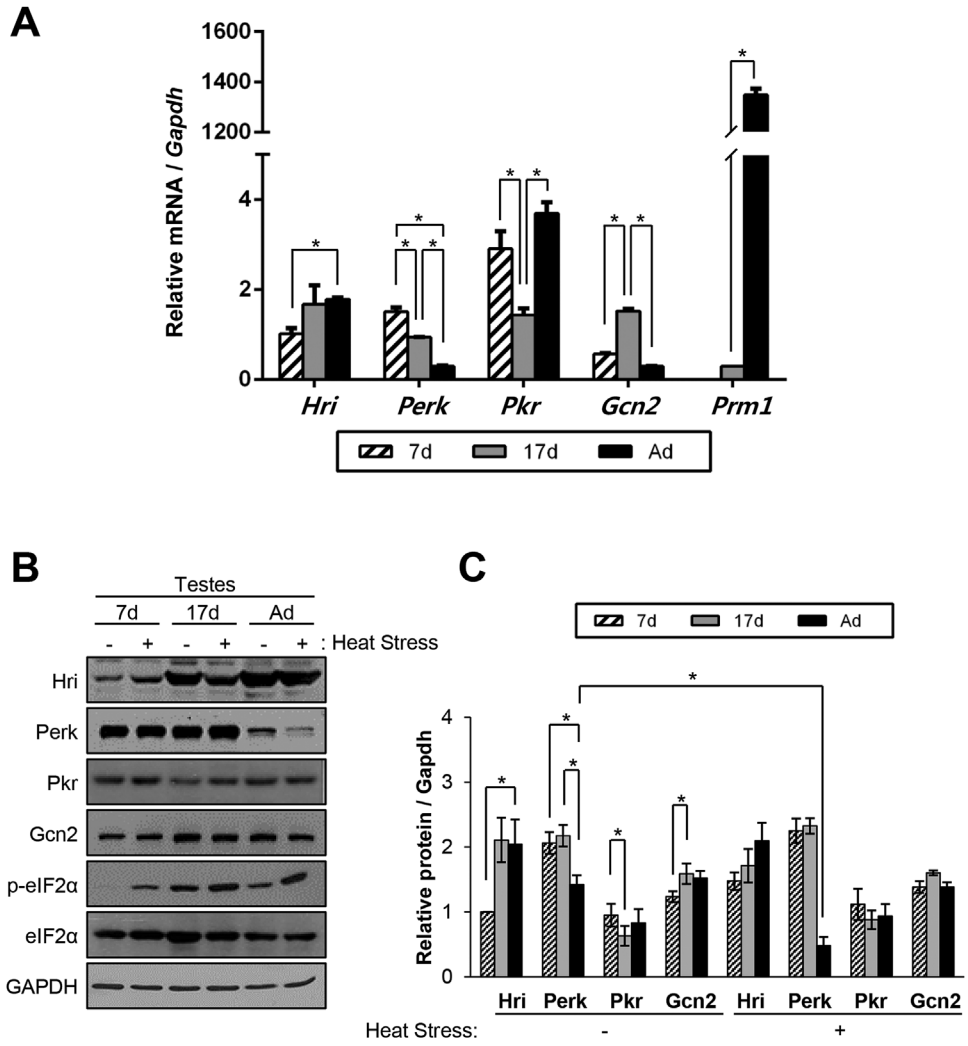

Figure 1. Expression of eIF2α kinases in the mouse testis. **A**: Quantitative reverse-transcription PCR for eIF2α kinase mRNAs (*Hri*, *Perk*, *Pkr*, and *Gcn2*) in the testes of immature (7- and 17-day-old) and adult (42-day-old) mice. Protamine-1 (*Prm1*) was used as a positive control. Values are shown as the means ± standard errors from three independent experiments. **B**: Immunoblot analysis of eIF2α kinases in testes of immature and adult mice exposed to heat stress at 42°C for 20 min. Phospho-eIF2α and eIF2α abundance was also measured. GAPDH was used as the loading control. **C**: The relative protein abundance of eIF2α kinases in control and heat-treated testes (immature and mature testes). The levels of each eIF2a kinase were normalized to GAPDH. $^{*}P < 0.01$.

2012). Dazl-positive stress granules were detected following heat stress in most spermatocytes in both wild-type and *Gcn2*-mutant mice (Fig. 3B and C). Together, these results indicate that eIF2α is phosphorylated and stress granules are formed even in the absence of *Gcn2* in mouse male germ cells.

Effects of ISRIB on the Heat Stress Response

Dazl is an essential component of stress granules in mouse male germ cells and, based on the observation that stress granule formation following heat stress was significantly reduced in *Dazl*-knockout mice, we further proposed that Dazl plays a role in the formation of stress granules (Kim et al., 2012). A HeLa cell line in which doxycycline-inducible ectopic FLAG-Dazl expression was generated to examine if ectopic expression of Dazl could trigger stress granule formation in other cell types (Fig. 4A). Heat treatment of the HeLa cells (1 hr at 42°C) was sufficient to induce phosphorylation of eIF2α (Fig. 4B). Immunostaining analysis revealed that ectopic expression of FLAG-Dazl itself did not induce stress granule formation, although this recombinant protein does localize to the TIA-1-positive stress granules following heat stress (Fig. 4C). Addition of ISRIB did not affect eIF2α phosphorylation in the HeLa cells (Fig. 4B), but did significantly reduced stress granule formation, irrespective of FLAG-Dazl expression (Fig. 4C and D). Taken together, these results indicate that FLAG-Dazl is a constituent of stress granules, but does not induce stress

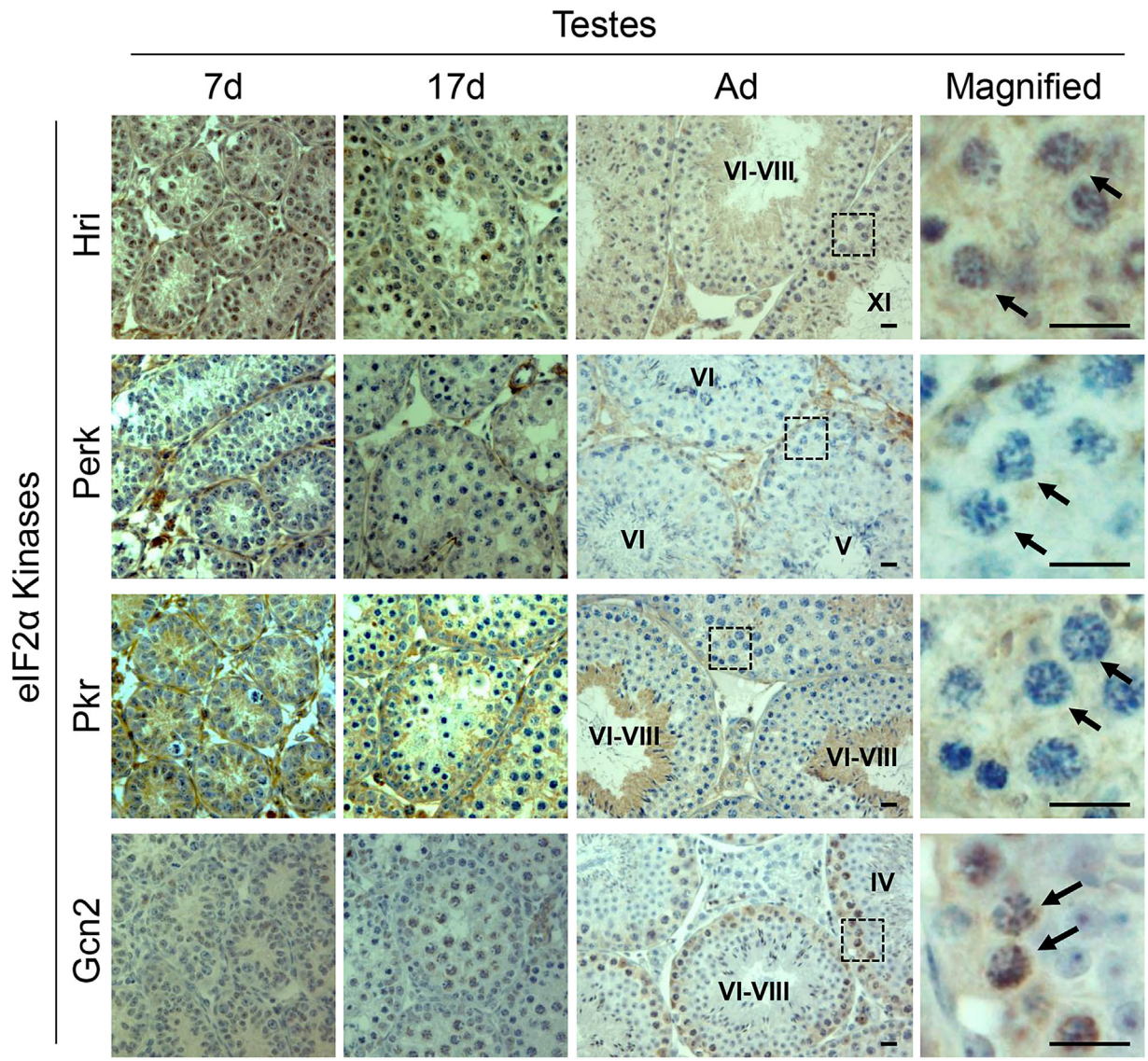

Figure 2. Immunohistochemical analysis of the eIF2α kinases in testes of immature (7- and 17-day-old) and adult (2-month-old) mice. Brown staining indicates specific signal for the indicated eIF2α kinases. Nuclei are shown in blue. The panels on the right are magnified images of the insets within the adult testes. Black arrows indicate primary spermatocytes. Scale bars, 20 μm.

granule formation by itself in HeLa cells. We also confirmed that stress granule formation is blocked by ISRIB, even in cells in which eIF2α phosphorylation was induced with heat stress.

We next examined the effects of ISRIB on stress granule formation upon heat stress in mouse male germ cells. As expected, eIF2α was phosphorylated following heat stress for 20 min at 42°C, and dephosphorylated by 6 hr after recovery; ISRIB did not affect phosphorylation or de-phosphorylation kinetics of eIF2α (Fig. 5A). Using the Dazl antibody (Kim et al., 2012), we observed the formation of stress granules following heat stress in spermatocytes in early-stage seminiferous tubules (III-IV), and their disassembly 6 hr after recovery (Fig. 5B). ISRIB effectively inhibited stress granule formation in spermatocytes in a dose-dependent manner (Fig. 5B and C). Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling (TUNEL) assays were also performed to determine the level of germ cell apoptosis. As shown previously (Kim et al., 2012), germ cell apoptosis was more frequent in late stage (XI-XII) than in early stage (III-IV) seminiferous tubules, and heat stress induced more germ cell apoptosis at late stages of seminiferous tubules (Fig. 5D and E). Furthermore, germ cell apoptosis was significantly elevated with ISRIB treatment (Fig. 5D and E). Thus, eIF2α phosphorylation appears to be a protective mechanism against germ cell apoptosis following heat stress.

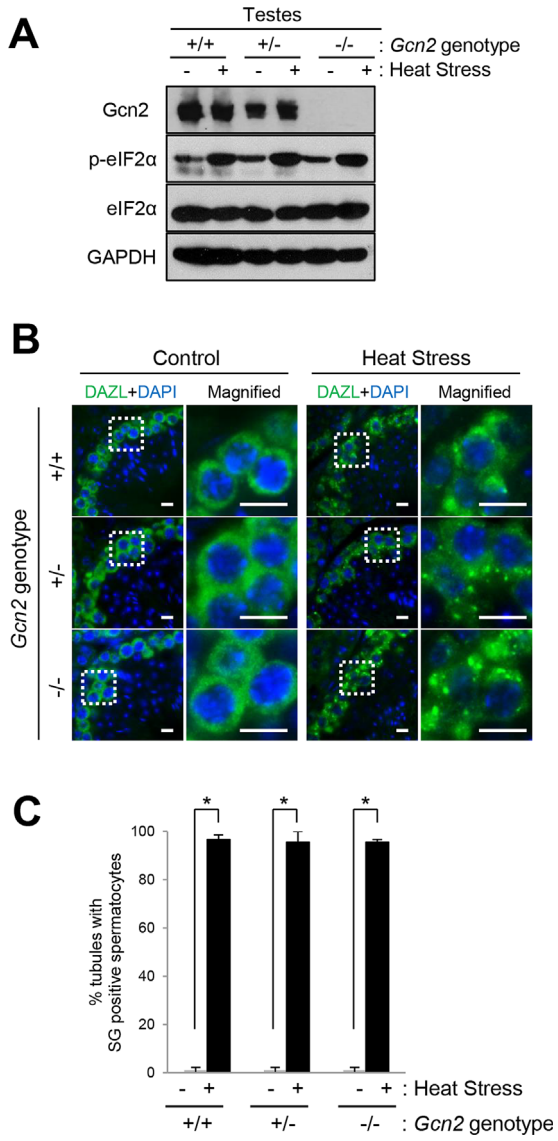

Figure 3. Stress granule formation in *Gcn2*-knockout mice. **A**: Immunoblot analysis for Gcn2, phospho-eIF2α, and eIF2α in heat-stressed testes of *Gcn2* homozygotes (−/−), heterozygotes (+/−), and wild-type (+/+) adult mice. **B**: Stress granule formation upon heat stress in the spermatocytes of *Gcn2*-mutant mice. Dazl (green) is a marker for stress granules. Scale bars, 10 μm. **C**: The number of seminiferous tubules with stress granule-positive spermatocytes. A total of 60 seminiferous tubules were counted in three independent experiments. Values are shown as the means ± standard errors of the percent of tubules with stress granule-positive spermatocytes. $^{*}P < 0.01$.

DISCUSSION

In this study, we investigated how translational activity is regulated upon heat stress in mouse male germ cells. We initially searched for an eIF2α kinase whose activity was enhanced upon heat stress in mouse male germ cells, but our results revealed that all known eIF2α kinases are expressed in lineage- and developmental stage-specific manners in the testis. Furthermore, elimination of one kinase, Gcn2, did not affect the stress response as eIF2α was still phosphorylated and stress granules were formed following heat stress in the testes of *Gcn2*-deleted mice. Thus, Gcn2 is not the only eIF2α kinase whose activity responds to heat stress; indeed, another single or multiple eIF2α kinase(s) may be activated by heat stress in male germ cells.

The involvement of multiple eIF2α kinases under stress conditions has been illustrated in other systems. For example, Gcn2 plays a compensatory role for Hri following stresses such as heat shock, cadmium exposure, and arsenite stress in *Schizosaccharomyces pombe* (Zhan et al., 2004). Another example was reported in a mouse epilepsy model in which Pkr is the primary eIF2α kinase, whereas Perk is activated in *Pkr*-deficient animals (Carnevalli et al., 2006). Therefore, compensatory mechanisms may also function in mouse male germ cells following heat stress, particularly given the independent means of detecting versus reacting to the stress. Indeed, heat itself may be detected by a temperature-sensing receptor (Xu et al., 2002; Mizrak et al., 2008), whereas reaction to heat stress, such as protein deformation, may be sensed by another mechanism. Thus, multiple molecular mechanisms may be activated upon heat stress in male germ cells.

Stress granule formation and apoptosis are the main outcomes of heat stress in male germ cells. Dazl is essential for stress granule formation in mouse male germ cells, as their assembly was dramatically reduced in the testes of *Dazl*-knockout mice (Kim et al., 2012). Yet, ectopic Dazl itself did not induce or facilitate stress granule formation in HeLa cells (data not shown). Therefore, the role of Dazl in stress granule formation may be specific to male germ cells.

We also determined the functional linkage between eIF2α phosphorylation and downstream events using ISRIB, a downstream inhibitor of eIF2α phosphorylation (Sidrauski et al., 2013). ISRIB did not affect eIF2α phosphorylation upon heat stress in mouse male germ cells or in HeLa cells, whereas stress granule formation was significantly reduced by ISRIB treatment, indicating that their formation is downstream of eIF2α phosphorylation. In contrast, the number of apoptotic germ cells significantly increased with ISRIB treatment, suggesting that eIF2α phosphorylation and stress granule formation may be protective against germ cell apoptosis following heat stress.

Interestingly, spermatocytes with stress granules are only detected in early stages of seminiferous tubules, in which few apoptotic cells are observed under heat stress (Kim et al., 2012). Conversely, apoptotic germ cells are generally detected among male germ cells at the onset of meiosis in late stages of seminiferous tubules (Fig. 5). ISRIB treatment significantly increased the number of apoptotic germ cells at late stages of seminiferous tubules (Fig. 5), suggesting that germ cells avoid apoptosis via translational inhibition, as led by eIF2α phosphorylation. Yet ISRIB treatment did not increase the number of apoptotic cells in the early tubular stages of seminiferous tubules, indicating that stress granule formation may not directly protect germ cells from apoptosis, at least in spermatocytes in seminiferous tubules of early stages.

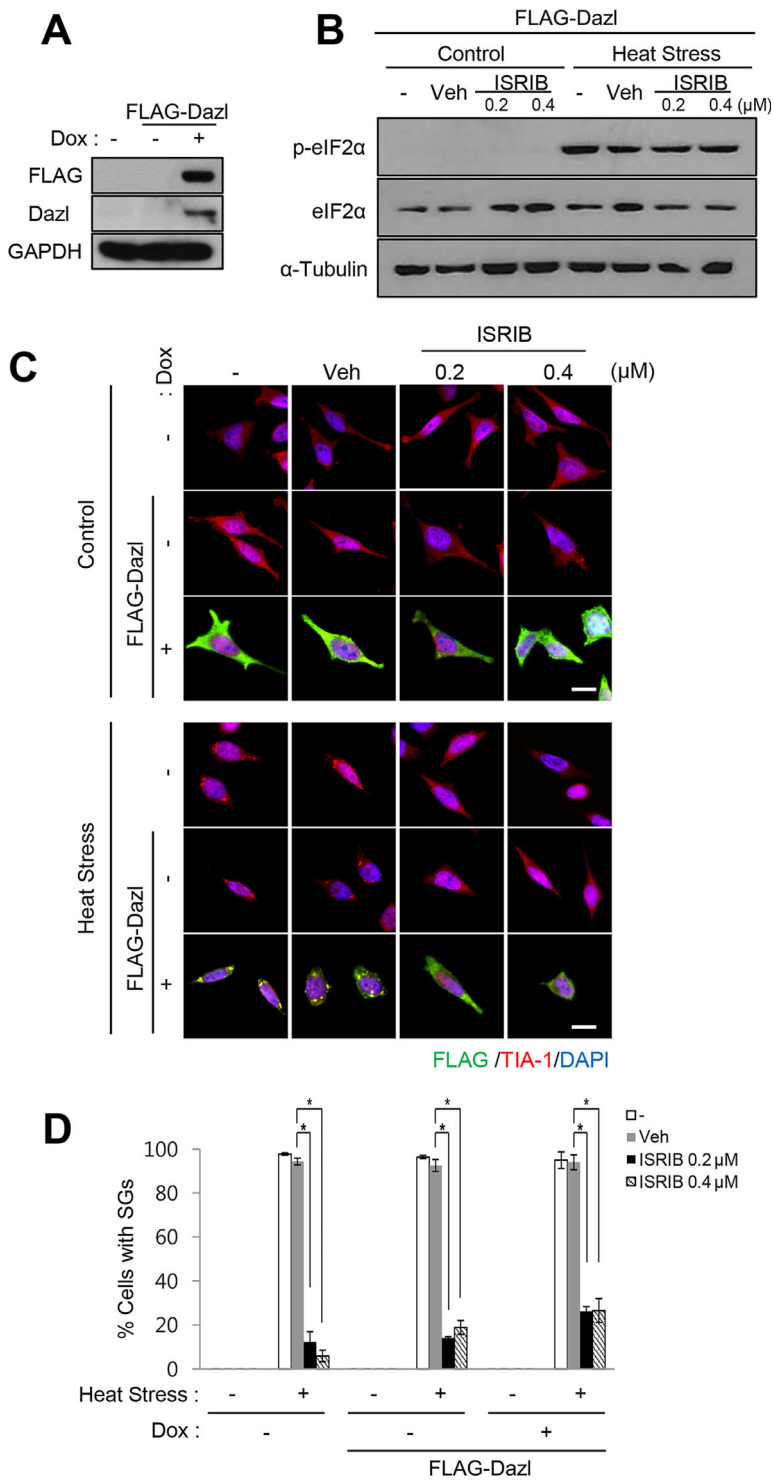

Figure 4. Effects of ISRIB on stress granule formation in HeLa cells. **A**: Establishment of HeLa cells in which FLAG-Dazl expression was induced with doxycycline (Dox) treatment. **B**: Effects of ISRIB on eIF2α phosphorylation under heat stress in HeLa cells expressing FLAG-Dazl. The untreated (−) and dimethyl sulfoxide-treated (Veh) groups were used as controls. **C**: Effects of ISRIB on stress granule formation following heat stress in HeLa cells expressing FLAG-Dazl. The cells were co-immunostained for FLAG (green) and TIA-1 (red); DNA is shown in blue. Scale bars, 10 μm. **D**: The number of cells with stress granules. Over 300 cells per experimental group were counted in three independent experiments. Values are shown as the means ± standard errors of percent cells with stress granules. $^{*}P < 0.01$.

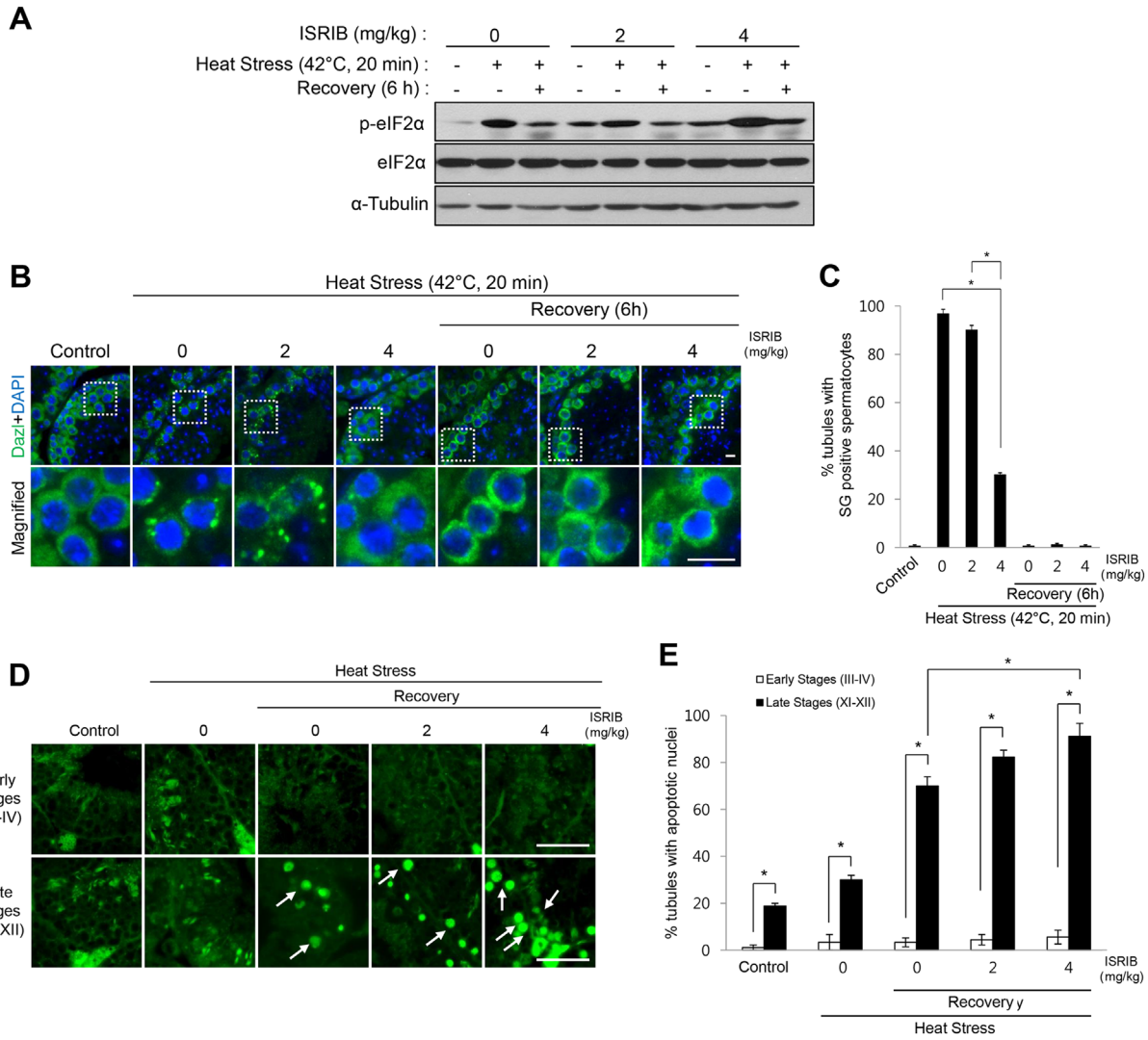

Figure 5. Effects of ISRIB on the heat-stress response in adult mouse testes. **A**: Adult mice were exposed to heat stress at 42°C for 20 min in the presence of ISRIB. Immunoblot analysis was performed with the eIF2α and phospho-eIF2α antibodies in the testes upon heat stress. **B**: Immunohistochemical analysis was performed using the Dazl antibody as a marker of stress granules in the spermatocytes (green); DNA is shown in blue. Scale bars, 10 μm. **C**: The number of seminiferous tubules with stress granule-positive spermatocytes. A total of 60 seminiferous tubules were counted in three independent experiments. Values are shown as the means ± standard errors of percent tubules with stress granule-positive spermatocytes. **D**: TUNEL assays performed to determine apoptosis in the testes of the ISRIB-treated mice exposed to heat stress. The stages of seminiferous tubules were categorized as early (III-IV) and late (XI-XII). Arrows indicate TUNEL-positive spermatocytes. Scale bars, 50 μm. **E**: The number of seminiferous tubules with TUNEL-positive nuclei. A total of 90 seminiferous tubules were analyzed in three independent experiments. Values are shown as the means ± standard errors. $^{*}P < 0.01$.

MATERIALS AND METHODS

Animals and Heat Treatment

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-140429-12-1). *Gcn2*-knockout mice were purchased from The Jackson Laboratory (Stock no. 008452). Genotypes of the *Gcn2* mutant mice ($Gcn2^{+/+}$, $Gcn2^{+/-}$, and $Gcn2^{-/-}$) were determined with genomic PCR analyses.

Immature (7- and 17-day-old) and adult (42-day-old or 2-month-old) C57BL/6 male mice were used for heat-treatment experiments. The mice were anesthetized with intraperitoneal injection (2.5% Avertin; 17 μL/g body weight), and immersed in a water bath for the indicated time periods.

ISRIB Treatment

ISRIB was obtained from Dr. P. Walter (Sidrauski et al., 2013). ISRIB was diluted to a final concentration of 2 mg/mL in 50% dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO) and 50% polyethylene glycol 400 (Sigma–Aldrich). In the mouse experiments, ISRIB was intra-peritoneally injected at a final concentration of 2 or 4 mg/kg. Two hours after

TABLE 1. Primers Sequences

Gene	Sequence
Hri	5′-GTGCTACGGGAAGTGAAGGT
	5′-AATCGGAACTCTGTCTTGTGG
Gapdh	5′-TCAAGAAGGTGGTGAAGCAG
	5′- AGGTGGAAGAGTGGGAGTTG
Gcn2	5′-CCTCCTGCTGTTGTCTCTCA
	5′-GGGCTCCATCTTTCCTTGTC
Perk	5′-CTCCTGTCTTGGTTGGGTCT
	5′-GTGCTCCGCTTATTCCTTTC
Pkr	5′-CCGAACAAGGAGAACAGGAA
	5′-CCCAAAGCAAAGATGTCCAC
Prm1	5′-CGCCGCTCATACACCATAAG
	5′-GGTGGCATTGTTCCTTAGCAG

ISRIB injection, the mice were exposed to heat stress for 20 min at 42°C. In cell culture experiments, the cells were treated with ISRIB at a final concentration of 0.2 or 0.4 μM for 2 hr prior to heat treatment.

Establishment of Stable Cell Lines and Cell Culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and maintained in a 5% CO_2 incubator at 37°C. For heat treatment, the HeLa cell plates were placed in a 5% CO_2 incubator at 42°C for 1 hr.

Mouse *Dazl* cDNA (897 bp) was subcloned into the *pcDNA5/FRT/TO/3 × Flag* vector with an inducible promoter. The Dazl expression vector was stably transfected into the FRT-HeLa cells. The ectopic Dazl expression was induced with doxycycline treatment (1 μg/mL).

Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted from the testes of 7-day-old, 17-day-old, and adult mice using TRIzol reagent (Invitrogen, Waltham, MA). Reverse transcription was conducted with random hexamers. Real-time PCR was carried out with the Applied Biosystems (Carlsbad, CA) 7300 Real Time PCR System. The primer sequences are listed in Table 1. Real-time PCR was performed in triplicate, and the experiment was repeated twice. Relative gene expression was determined with the comparative Ct method, and normalized to the *Gapdh* abundance.

Immunoblot Analysis

Mouse testicular samples were homogenized with RIPA buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1 mM EGTA, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM NaV, and a protease inhibitor cocktail), and centrifuged to remove debris. The supernatant lysates were mixed with 2 × SDS–PAGE sample buffer. HeLa cell lysates were prepared with 1 × SDS–PAGE sample buffer.

Lysates were loaded onto 6% SDS–polyacrylamide gels, and resolved by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The blots were blocked in 5% bovine serum albumin (BSA) or 5% skim milk in TBST (20 mM Tris pH 7.3, 150 mM NaCl, and 0.1% Tween 20); incubated overnight at 4°C with the indicated antibodies (Table 2); washed three times with TBST; incubated at room temperature for 30 min with the appropriate peroxidase-conjugated secondary antibodies; washed with TBST; and exposed to the enhanced chemiluminescence reagents.

Immunostaining

Mouse testes were fixed overnight at 4°C in Bouin's solution (Sigma–Aldrich), embedded in paraffin, and sectioned. The 5-μm-thick testis sections were boiled for the antigen retrieval process, blocked for 30 min with 0.1% PBST (phosphate-buffered saline [PBS] with 0.1% Triton X-100) containing 3% BSA, and incubated overnight at 4°C with primary antibodies (Table 2). After washing with PBST, the testis sections were incubated at room temperature for 30 min with an appropriate secondary antibody. Fluorophore-conjugated (Invitrogen) secondary antibodies were used for indirect immunofluorescence analysis, followed by a 30-sec incubation with 4′,6-diamidino-2-phenylindole (DAPI) before mounting with ProLong Gold (Thermo Fisher, Bartlesville, OK). Biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) were used for immunohistochemical development, followed by a 30-min incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC HRP Kit) (Vector Laboratories). The

TABLE 2. Antibodies Antibody Sources and Working Dilutions

Target	Source (catalog number)	Immunoblot dilution	Immunostaining dilution
Dazl	Kim et al. (2012)	1:100	1:10
phospho-eIF2α	Stressgen (KAP-CP131)	1:150	1:100
eIF2α	Santa Cruz (sc-11386)	1:150	n/a
Hri	Millipore (07-728)	1:1,000	1:150
Gcn2	Cell signaling (3302)	1:100	1:20
Perk	Cell signaling (3192)	1:500	1:100
Pkr	Santa Cruz (sc-708)	1:300	1:60
FLAG	Sigma (F3165)	1:20,000	1:2,000
TIA-1	Santa Cruz (sc-1751)	n/a	1:25

color was developed with 3, 3′-diaminobenzidine tetrachloride (Sigma–Aldrich), followed by hematoxylin counterstaining.

HeLa cells were cultured on a glass coverslip. The coverslips were washed with cold PBS, placed in 100% methanol for 10 min, and washed again with PBS. The cells were permeabilized with 0.1% PBST, blocked with 3% BSA for 30 min, incubated with the primary antibodies for 1 hr (Table 2), washed with 0.1% PBST three times, incubated at room temperature for 30 min with the Alexa Fluor 594-conjugated secondary antibodies (1:1,000 dilution) (Life Technologies, Camarillo, CA), washed with 0.1% PBST three times, incubated for 2 min with DAPI, and finally mounted with ProLong Gold (Thermo Fisher).

TUNEL Assay

The TUNEL assay was performed for detection of germ cell apoptosis, using the FragEL™ DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA) and strictly following the manufacturer's instruction. In brief, the rehydrated testicular sections were permeabilized with proteinase K and treated with 1 × TdT (terminal deoxynucleotidyl transferase) equilibrium buffer to tag the exposed 3′-OH ends of the DNA fragments.

Imaging and Statistical Analysis

Immunofluorescence images were obtained with an Olympus IX51 fluorescence microscope equipped with QImaging QICAM Fast 1394 (Olympus, Seoul, Korea). DAB staining images were acquired with an Olympus BX51. Acquired images were processed with ImagePro 5.0 (Media Cybernetics, Rockville, MD), ProgRes® CapturePro V2.8.8 (JENOPTIK, Brighton, MI), Image J 1.49 (National Institutes of Health, Bethesda, MD), and Photoshop CS6 (Adobe, San Jose, CA). The results were analyzed with a paired t-test.

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