Sqst1–GFP knock-in mice reveal dynamic actions of Sqst1 during autophagy and under stress conditions in living cells

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ABSTRACT

Sqst1 serves as a signaling hub and receptor for selective autophagy. Consequently, dysregulation of Sqst1 causes imbalances in signaling pathways and disrupts proteostasis, thereby contributing to the development of human diseases. Environmental stresses influence the level of Sqst1 by altering its expression and/or autophagic degradation, and also changes the localization of Sqst1, making it difficult to elucidate the actions and roles of this protein. In this study, we developed knock-in mice expressing Sqst1 fused to GFP (Sqst1–GFPK1). Using these Sqst1–GFPK1 mice, we revealed for the first time the dynamics of endogenous Sqst1 in living cells. Sqst1–GFP was translocated to a restricted area of LC3-positive structures, which primarily correspond to the inside of autophagosomes, and then degraded. Moreover, exposure to arsinite induced expression of Sqst1–GFP, followed by accumulation of the fusion protein in large aggregates that were degraded by autophagy. Furthermore, suppression of autophagy in Sqst1–GFPK1 mice caused accumulation of Sqst1–GFP and formation of GFP-positive aggregate structures, leading to severe hepatic failure. These results indicate that Sqst1–GFPK1 mice are a useful tool for analyzing Sqst1 in living cells and intact animals.

INTRODUCTION

Sqst1 (also known as p62) is conserved among metazoans, but not in plants or fungi. Sqst1 contains multiple domains, including a Phox1 and Bem1p (PB1) domain, a zinc finger, two nuclear localization signals, a TRAF6-binding domain, a nuclear export signals, a TRAF6-binding domain, a Nuclear export factor nuclear factor erythroid 2 related factor 2 (Nrf2, also known as NFE2L2) (Ishii et al., 2000; Jain et al., 2010). Through its PB1 domain, Sqst1 protein forms helical filaments (Ciuffa et al., 2015) that are translocated to sites of autophagosome formation (Itakura and Mizushima, 2011), where they serve as molecular templates for nucleation of the growing autophagosomal membrane (Ciuffa et al., 2015). At these sites, Sqst1 eventually interacts with the autophagosome-localized protein LC3B (also known as MAP1LC3B; hereafter referred to as LC3) through its LIR, leading to its autophagic degradation (Ichimura et al., 2008; Pankiv et al., 2007; Shvets et al., 2008).

Sqst1 also contributes to selective autophagy for ubiquitylated cargos (Bjorkoy et al., 2005; Rogov et al., 2014). In response to stressors, Sqst1 is sequentially phosphorylated at Ser409 (corresponding to human Ser407) and Ser405 (corresponding to human Ser403) of the UBA domain, which increases the binding affinity of Sqst1 for ubiquitin chains. As a result, Sqst1 is translocated to autophagy substrates, such as ubiquitin-positive protein aggregates, damaged mitochondria and infecting bacterial cells (Lim et al., 2015; Matsumoto et al., 2015, 2011; Pilli et al., 2012). Recognition of the ubiquitin chain causes conversion from large helical filaments into shorter and less compact helical ones, which play a role in exclusive sequestration of ubiquitylated cargo in forming autophagosomes (Ciuffa et al., 2015). In addition, Sqst1 self-oligomerizes in a PB1-domain-dependent manner to promote packaging of ubiquitylated cargos (Ichimura et al., 2008; Lamark et al., 2003; Pankiv et al., 2007). Meanwhile, Sqst1 interacts with Nbr1, which has similar structural domains to those of Sqst1 and serves as a receptor for selective autophagy through hetero-oligomerization mediated by PB1 domains (Kirkin et al., 2009). Interaction with LC3 is required for sufficient removal of ubiquitylated cargos during the process of selective autophagy (Bjorkoy et al., 2005; Ichimura et al., 2008).

Under selective autophagy-inducing conditions, mammalian target of rapamycin complex 1 (mTORC1) phosphorylates a specific serine residue (Ser351, corresponding to human Ser349) in KIR of Sqst1 on the autophagic cargos (Ichimura et al., 2013). Phosphorylated Sqst1 increases its binding affinity for Kelch-like ECH-associated protein 1 (Keap1), an adaptor of the ubiquitin ligase complex for Nrf2 and competitively abrogates the interaction between Nrf2 and Keap1. Consequently, Nrf2 translocates into the nucleus to induce the transcription of numerous cytoprotective genes encoding antioxidant proteins, detoxifying enzymes and multidrug transporters (Ichimura et al., 2013; Ishimura et al., 2014). Phosphorylated Sqst1 and Keap1, together with autophagic cargos, are degraded by autophagy (Jain et al., 2015; Taguchi et al., 2012). This process enhances the positive-feedback loop resulting from Nrf2-mediated activation of Sqst1 gene expression (Jain et al., 2010), that is two major stress response pathways,
selective autophagy and the Keap1–Nrf2 system, are coupled to each other through Ser351-phosphorylation ofSqstm1.

Recent studies ofSqstm1 have clarified its unique functions (Jiang et al., 2015; Moscat and Díaz-Meco, 2011; Rogov et al., 2014). However, given the diversity in characteristic properties ofSqstm1, such as stress-inducible expression, self-oligomerization, autophagic degradation and dynamic intracellular translocation, it is difficult to determine the roles ofSqstm1 in living cells and in tissues of intact animals. To overcome this issue, we developedSqstm1-GFP knock-in mice and used them to investigate the dynamic features ofSqstm1 in cells and tissues under stress conditions.

**RESULTS**

**Generation ofSqstm1-GFP knock-in mice**

To monitor dynamics ofSqstm1 during autophagy in vivo, we generated knock-in mice that expressSqstm1 C-terminally fused to GFP (Sqstm1–GFP) (Fig. 1A). We verified germ-line transmission of the knock-in (Sqstm1-GFPKI/+) by Southern blot analysis (Fig. 1B). Sqstm1-GFPKI/+ mice were fertile and showed no obvious pathological phenotypes for at least 2 years. To test the expression level ofSqstm1–GFP, we isolated mouse embryonic fibroblasts (MEFs) from wild-type, Sqstm1-GFPKI/+, and Sqstm1-GFPKI/KI embryos and immortalized them by introducing simian virus 40 (SV40) T (large T) antigen. Immunoblot analysis with anti-Sqstm1 antibody revealed that Sqstm1-GFPKI/+ MEFs expressed both Sqstm1–GFP and Sqstm1 (Fig. 1C), whereas wild-type MEFs expressed only Sqstm1, and Sqstm1-GFPKI/KI MEFs expressed only Sqstm1–GFP (Fig. 1C). Next, to determine whether GFP-tagging ofSqstm1 affected its ability to bind endogenous proteins, we performed immunoprecipitation assays with anti-GFP antibody. Sqstm1–GFP from Sqstm1-GFPKI/+ MEFs formed a complex with endogenous Sqstm1 (Fig. 1D), and Sqstm1–GFP in Sqstm1-GFPKI/+, and Sqstm1-GFPKI/KI MEFs had the ability to interact with endogenous ubiquitylated proteins and Nbr1 (Fig. 1D). We hardly detected any LC3 signal in immunoprecipitates prepared from Sqstm1-GFPKI/+ and Sqstm1-GFPKI/KI MEFs (Fig. 1D), probably due to their transient interaction at autophagosome formation site and rapid degradation through autophagy (Itakura and Mizushima, 2011). However, Sqstm1–GFP extensively colocalized with LC3-positive structures and was then degraded (see Figs 2 and 3). On the basis
of these results, we concluded that GFP tagging did not influence the interactions with endogenous proteins, at least in regard to autophagy-related proteins.

**Dynamics of Sqstm1–GFP during starvation-induced autophagy**

In the next series of experiments, we investigated Sqstm1-GFP in *Sqstm1-GFP*<sup>+/−</sup> MEFs under nutrient-deprived conditions. Upon nutrient deprivation of *Sqstm1-GFP*<sup>+/−</sup> MEFs, the levels of Sqstm1-GFP and Sqstm1 decreased (Fig. 2A). Free GFP, which is derived from degradation of Sqstm1-GFP in lysosomes (Ishimura et al., 2014), disappeared in response to nutrient deprivation (Fig. 2A). Treatment of the MEFs with lysosomal enzyme inhibitors, E64d and pepstatin A increased levels of Sqstm1–GFP and Sqstm1, as well as of LC3-II (Fig. 2A), but decreased the level of free GFP (Fig. 2A). These results imply that Sqstm1–GFP is subject to autophagic degradation. Fluorescence microscopic analysis revealed that upon nutrient deprivation, structures positive for Sqstm1–GFP with a diameter ranging from 0.5 to 1 μm appeared in the cytoplasm (Fig. 2A). Immunofluorescence analysis with anti-LC3 antibody revealed that the number of LC3-positive puncta dramatically increased upon nutrient deprivation.
Fig. 3. Dissection of Sqstm1-GFP<sup>Wt</sup> MEFs under stress conditions. (A) Relative mRNA levels of Sqstm1, GFP, and Nqo1 and Ho-1. Total RNAs were prepared from Sqstm1-GFP<sup>Wt</sup> MEFs cultured for 12 h in the presence or absence of 10 µM sodium arsenite (As[III]) and then reverse-transcribed into cDNAs, which were used as templates for real-time quantitative PCR analysis. Values were normalized to the amount of each mRNA in the non-treated MEFs. The experiments were performed three times; data are mean±s.e.m. **P<0.01; ***P<0.001 (Welch test). (B) Immunoblot analysis. Sqstm1-GFP<sup>Wt</sup> MEFs were cultured as described in A. Total cell lysates and nuclear fractions were prepared and subjected to immunoblot analysis with the specified antibodies. Data are representative of three independent experiments. (C) Immunoprecipitation (IP) analysis. Sqstm1-GFP<sup>Wt</sup> MEFs were cultured as described in A. Immunoprecipitates obtained with anti-GFP antibody were analyzed by immunoblotting with the specified antibodies. Data are representative of three independent experiments. (D) Immunoblot analysis. Sqstm1-GFP<sup>Wt</sup> and Atg7<sup>−/−</sup>Sqstm1-GFP<sup>Wt</sup> MEFs were challenged by As[III]. After removal of As[III], cells were cultured in regular medium for the indicated time. Cell lysates were prepared and subjected to immunoblot analysis with the specified antibodies. Data are representative of three independent experiments. (E) Immunofluorescence staining. Sqstm1-GFP<sup>Wt</sup> MEFs were cultured as described in D, and then immunostained with anti-LC3 antibody. Each inset is a magnified image. Scale bar: 10 µm. (F) Time-lapse video microscopic analysis with Sqstm1-GFP<sup>Wt</sup> MEFs cultured as shown in D. Scale bar: 10 µm. (G) Immunoelectron microscopy. Sqstm1-GFP<sup>Wt</sup> MEFs were cultured as described in D, and then immunostained with anti-LC3 antibody, followed by secondary antibody conjugated to colloidal gold particles (indicated by arrowheads for c and d). The boxed region in a was magnified and is shown in the inset. For better recognition of colloidal gold particles on dark background area, gamma correction was performed for c and d. Scale bars: 100 nm.
and 20.7±2.5% (mean±s.e.m., n=30 cells) of these puncta were colocalized with Sqstm1–GFP (Fig 2B). These results corresponded approximately to those obtained upon immunofluorescence staining of endogenous Sqstm1 with a specific antibody (Bjorkoy et al., 2005; Komatsu et al., 2007). To directly determine the lifespan of these Sqstm1–GFP puncta in Sqstm1-GFPKO/+ MEFs, we carried out time-lapse video microscopic analysis. In response to nutrient deprivation, the Sqstm1–GFP dots appeared at random in the cytosol, persisted for a time, and then disappeared. The mean (±s.e.m.) duration of this process was 9.49±6.46 min (Fig 2C; Movie 1), similar to the life-time of the autophagosome (Mizushima et al., 2001). To explore the localization of Sqstm1–GFP on the membrane structures more precisely in cells, we first carried out analysis using structured illumination microscopy (SIM). SIM revealed cup-shaped LC3 structures, in which the LC3 intensity differed among sites, and Sqstm1–GFP translocated onto restricted parts of LC3-positive structures (Fig 2D). Next, we performed immunoelectron microscopy analyses with anti-GFP antibody. Sqstm1–GFP was localized both inside and on the restricted part of the outer membranes of autophagosomes. Counting of colloidal gold particles indicated that the majority of the signal (74.5±38.8%; mean±s.d., n=20) was in the engulfed region (Fig 2E). Aggregate-like structures positive for Sqstm1–GFP were occasionally found on the autophagosomal membranes (Fig 2E).

### Dynamics of Sqstm1-GFP under stress conditions

Consistent with a previous report (Ichimura et al., 2013), gene expression of Sqstm1 in Sqstm1-GFPKO/+ MEFs was dramatically induced upon exposure to sodium arsenite (As[III]) (Fig 3A). Likewise, treatment of the MEFs with As[III] induced expression of Sqstm1-GFP (Fig 3A). We confirmed prominent upregulation of Sqstm1 and Sqstm1–GFP proteins, both of which were phosphorylated at Ser351, in response to As[III] (Fig 3B). Immunoprecipitation assay with anti-GFP antibody revealed that As[III] treatment significantly enhanced the interaction between Keap1 and Sqstm1–GFP (Fig 3C). Thus, the exposure of As[III] was accompanied by nuclear accumulation of Nrf2, as well as induction of Nrf2 targets such as Nqo1 and Ho-1 (also known as HMOX1) (Fig 3A,B). The properties of Sqstm1–GFP and the Keap1–Nrf2 pathway in Sqstm1-GFPKO/+ MEFs were consistent with those in wild-type MEFs (Ichimura et al., 2013; Lau et al., 2010). As with non-tagged endogenous Sqstm1, the levels of Sqstm1–GFP and its phosphorylated form in Sqstm1-GFPKO/+ MEFs decreased 6 h after removal of As[III], whereas levels of LC3-II increased (Fig 3D), suggesting autophagic turnover of Sqstm1–GFP. No such downregulation was observed in the case of Atg7+/−;Sqstm1-GFPKO/+ MEFs (Fig 3D), in which autophagy is impaired (Komatsu et al., 2005). Remarkably, removal of As[III] decreased levels of Ser351-phosphorylated Sqstm1 even in Atg7+/−;Sqstm1-GFPKO/+ MEFs (Fig 3D), suggesting that there are phosphatase(s) that recognize Ser351-phosphorylated Sqstm1.

Loss of autophagy in mouse tissues is usually accompanied by formation of large aggregate structures positive for Sqstm1, in particular in quiescent cells such as neurons and hepatocytes (Komatsu et al., 2007). Likewise, immunofluorescence analysis revealed aggregates positive for Sqstm1–GFP in Atg7−/−;Sqstm1-GFPKO/+ MEFs (Fig 3E), but the number and size were obviously less and smaller than those in Atg7−/−-deficient hepatocytes and neurons. This result might be accounted for by dilution of aggregate structures by rapid cell division of MEFs. Upon exposure of As[III], aggregate structures positive for Sqstm1–GFP with a diameter ranging from 1 to 5 μm formed in the cytoplasm of both Sqstm1-GFPKO/+ and Atg7−/−;Sqstm1-GFPKO/+ MEFs (Fig 3E). Most of these structures in Sqstm1-GFPKO/+ MEFs contained LC3, whereas the aggregates in Atg7−/−;Sqstm1-GFPKO/+ MEFs hardly contained any (Fig 3E). Both the number and size of such structures in Sqstm1-GFPKO/+ MEFs were not significantly different from those in Atg7−/−;Sqstm1-GFPKO/+ MEFs after 6 h of As[III] treatment, and both number and size increased with time (Movie 2). In the case of Atg7−/−;Sqstm1-GFPKO/+ MEFs, such structures were formed sooner (Movie 3). Most of these structures in Sqstm1-GFPKO/+ MEFs persisted for several hours after removal of As[III], and they frequently became small aggregates (<1 μm) by 4 h after the removal. Subsequently, several small aggregates became crescent-like structures and eventually disappeared (Fig 3F; Movie 4). Even after 12 h after the removal of As[III], a small number of large aggregate structures was still observed (Fig 3F; Movie 4). By contrast, most large aggregate structures in Atg7−/−;Sqstm1-GFPKO/+ MEFs remained even 12 h after the removal of As[III] (Movie 5). Immunoelectron microscopy indicated that Sqstm1–GFP in Sqstm1-GFPKO/+ MEFs localized in aggregated structures (Fig 3Ga) and autolysosomal structures (Fig 3Gb), and was occasionally associated with phagophore profiles (Fig 3Gc,d). Taken together, we conclude that the Sqstm1-GFPKO/+ MEFs immortalized by SV40 T-antigen are available for dynamic analysis of Sqstm1 during starvation- and stress-induced autophagy. We note that because the SV40 T-antigen targets multiple cellular pathways, primary cultured cells derived from Sqstm1-GFPKO/+ mice should be used to explore a role of Sqstm1 in signal transduction pathways (e.g. atypical PKC, ERK1, NF-κB and caspase-8) in which Sqstm1 serves as a signaling hub (Moscat and Diaz-Meco, 2011).

### Sqstm1-GFP in autophagy-deficient tissues

Finally, we tested the utility of Sqstm1-GFP knock-in mice for in vivo studies in intact animals. To this end, we crossedbreed the Sqstm1-GFPKO/+ with hepatocyte-specific Atg7-knockout mice (Atg7−/−;Albumin-Cre) (Komatsu et al., 2007), because the pathogenic roles of Sqstm1 have been extensively investigated in mice with liver-specific defects in autophagy (Komatsu et al., 2010, 2007; Ni et al., 2014; Takamura et al., 2011). Like endogenous Sqstm1, Sqstm1–GFP and its phosphorylated form prominently accumulated in livers of Atg7−/−;Albumin-Cre;Sqstm1-GFPKO/+ mice (Fig 4A). Immunohistofluorescence revealed aggregate structures positive for Sqstm1–GFP with a diameter ranging from 1 to 10 μm in the livers of the mutant mice (Fig 4B). These aggregates were also colabeled by antibodies against Sqstm1, Ser351-phosphorylated Sqstm1, and/or Keap1 (Fig 4B). Their size and number, as well as composition, were comparable to those of aggregates formed in livers of Atg7−/−;Albumin-Cre (Fig 4B).

Persistent activation of Nrf2 due to sequestration of Keap1 into Sqstm1-positive structure causes hepatomegaly and liver failure in Atg7−/−;Albumin-Cre and Atg7−/−;Albumin-Cre mice (Komatsu et al., 2010; Ni et al., 2014). Therefore, we investigated whether GFP-tagging of Sqstm1 affected the liver phenotypes of Atg7−/−;Albumin-Cre. Gene expression of Nrf2 targets, including Nqo1, Gstm and Ho-1, was significantly induced in livers of Atg7−/−;Albumin-Cre;Sqstm1-GFPKO/+ and the induced levels were quite similar to those in Atg7−/−;Albumin-Cre livers (Fig 4C). As in Atg7−/−;Albumin-Cre livers, the level of Nqo1 protein was elevated in Atg7−/−;Albumin-Cre;Sqstm1-GFPKO/+ livers (Fig 4A). Hematoxylin & eosin (H&E)
staining revealed hepatocytic swelling, as well as infiltration of inflammatory cells, in both Atg7f/f;Albumin-Cre;Sqstm1-GFPKI/+ and Atg7f/f;Albumin-Cre livers (Fig. 4D). As predicted, Atg7f/f;Albumin-Cre;Sqstm1-GFPKI/+ mice exhibited liver enlargement and leakage of hepatic enzymes (Fig. 4E,F), and there was no statistical difference in the extent of damage between Atg7f/f;Albumin-Cre;Sqstm1-GFPKI/+ and Atg7f/f;Albumin-Cre mice (Fig. 4E,F). These results indicate that Sqstm1-GFPKI/+ mice are
useful for in vivo analysis in intact animals, at least under conditions in which autophagy is impaired.

**DISCUSSION**

In this study, we developed a line of mice that express GFP-fused Sqstm1 under the control of the Sqstm1 promoter, and demonstrated that Sqstm1–GFP in Sqstm1–GFP/KI/− mice and derived cells closely resembles the properties of endogenous Sqstm1. Dysfunction of Sqstm1 is involved in the pathogenesis of human diseases. For example, various mutations of Sqstm1 have been identified in patients with Paget disease of bone (PDB), amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (Rea et al., 2014). Moreover, aberrant accumulation of Sqstm1-positive aggregated structures has been detected in patients with liver disorders including non-alcoholic steatohepatitis and α1-antitrypsin deficiency, tumors such as hepatocellular carcinoma and various neurodegenerative diseases (Zatitouakal et al., 2002). Furthermore, Sqstm1 has been identified as a pathogenic target of 5q copy number gains in kidney cancer (Li et al., 2013). Thus, the Sqstm1–GFP/KI/− mice could become useful tools for elucidating the pathophysiological roles of Sqstm1 in the aforementioned human diseases by crossbreeding them with disease-related model mice. At the molecular level, it remains unknown how Sqstm1 is selectively degraded by autophagy and how it regulates multiple signaling pathways. For example, it is unclear howSqstm1 is translocated to sites of autophasogosome formation prior to the formation of membranes, and how Sqstm1 asymmetrically localizes on the inner membrane of the autophagosome. Our Sqstm1–GFP/KI/− cells should be useful tools for addressing such fundamental questions.

**MATERIALS AND METHODS**

**Cell culture and knockdown**

Mouse embryonic fibroblasts (MEFs) were prepared as described previously (Komatsu et al., 2007). Immortalized MEFs were established by infecting MEFs with a recombinant retrovirus carrying a temperature-sensitive simian virus 40 large T antigen. MEFs were grown in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 5 U/ml penicillin, and 50 µg/ml streptomycin. We used Earle’s balanced salt solution (EBSS) as a starvation medium. Sodium arsenite was purchased from Wako Pure Chemical Industries (Osaka, Japan). E64d and Pepstatin A solution (EBSS) as a starvation medium. Sodium arsenite was purchased from Wako Pure Chemical Industries (Osaka, Japan). E64d and Pepstatin A were from Peptide Institute, Inc (Osaka, Japan).

**Mice**

The targeting vector for Sqstm1–GFP knock-in mice was constructed by inserting the cDNA fragment encoded by exons 3–8 (302–1326) of Sqstm1, the GFP cDNA, and a poly(A) signal sequence after exon 2 of the endogenous Sqstm1 gene (Fig. 1A). A neo resistance gene cassette (mcl-neo-pa) was ligated behind the polyA signal sequence. We electroporated the targeting vector into mouse TT2 embryonic stem cells, selected with G418 (250 µg/ml; Thermo Fisher Scientific), and then screened for homologous recombinants by Southern blot analyses. Southern blots were performed by digestion of genomic DNA with BamHI and hybridization with the probe shown in Fig. 1A. Genotyping of mice by PCR was performed using the following primers: 5′-TGAGGCCCTATACCCACATCT-3′; Sqstm1 right, 5′-CGCCCTTCATCCG-GAAAC-3′; GFP left, 5′-CAGCAGACACCCCCCCCATC-3′; GFP right, 5′-TGTTGTTCAGATGTTGTT-3′; Nqo1 left, 5′-AGCGTGCGTAT-TAGACC-3′; Nqo1 right, 5′-AGTACACGAGGGCCTCTTCG-3′; Gstm left, 5′-CTACCTTGCGGAAAGAC-3′; Gstm right, 5′-AGTGC- TGCAGCGATTCCTC-3′; Ho-1 left, 5′-GGTACGAGTCCAGAAGAGAAGG-3′; Ho-1 right, 5′-CTCCAGGGCCGTGATGATA-3′; Gusb left, 5′-GATGTTGGTCGTGCGCAAT-3′ and Gusb right, 5′-GTGGTTGACATGACGTCCT-3′.

**Histological examination**

Fixation and embedding procedures for immunohistofluorescence were as described previously (Waguri and Komatsu, 2009). Briefly, mouse livers were quickly excised, cut into small pieces, and then fixed by immersing in 4% parafomaldehyde with 4% sucrose in 0.1 M phosphate buffer, pH 7.4. After rinsing, the livers were embedded in paraffin for staining with H&E and in OCT compound for immunohistofluorescence. For immunolabeling, sections were processed for antigen retrieval in Immunosaver (Nissin EM Laboratories) antibodies. Immunofluorescence images were acquired with a Zeiss ELYRA S.1 microscope equipped with a Plan Apochromat oil-immersion objective (63×, 1.4 NA, Carl Zeiss). Image contrast and brightness were adjusted using Photoshop CS4 (Adobe).

**Real-time quantitative PCR analysis**

Using a Transcriptor First-Strand cDNA synthesis kit (Roche Applied Science), cDNA was synthesized from 1 µg of total RNA. Quantitative PCR was performed using LightCycler® 480 Probes Master mix (Roche Applied Science) on a LightCycler® 480 (Roche Applied Science). Signals were normalized to the corresponding levels of 18S rRNA (encoding β-glucuronidase). The sequences of the primers used were: Sqstm1 left, 5′-GCTGCCCTATACCCACATCT-3′; Sqstm1 right, 5′-CGCCCTTCATCCGGAAAC-3′; GFP left, 5′-CAGCAGACACCCCCCCCATC-3′; GFP right, 5′-TGTTGTTCAGATGTTGTT-3′; Nqo1 left, 5′-AGCGTGCGTATTTAGACC-3′; Nqo1 right, 5′-AGTACACGAGGGCCTCTTCG-3′; Gstm left, 5′-CTACCTTGCGGAAAGAC-3′; Gstm right, 5′-AGTGCCTGACGATTCCTC-3′; Ho-1 left, 5′-GGTACGAGTCCAGAAGAGAAGG-3′; Ho-1 right, 5′-CTCCAGGGCCGTGATGATA-3′; Gusb left, 5′-GATGTTGGTCGTGCGCAAT-3′ and Gusb right, 5′-GTGGTTGACATGACGTCCT-3′.
a laser scanning confocal microscope (FV1000, Olympus) with a 40× objective lens (UPlanSapo, oil, NA 1.3, Olympus). After image acquisition, contrast and brightness were adjusted using Photoshop CS4 (Adobe).

Electron microscopic analysis

Sgst1n-GFPpf62 MEFs were cultured in amino-acid-free medium for 1 h, then fixed with 0.1 M phosphate buffer pH 7.4 containing 4% paraformaldehyde and 0.03–0.06% glutaraldehyde. In the experiment using As[III], the cells were fixed with 0.1 M phosphate buffer pH 7.4 containing 4% paraformaldehyde alone. Ultrathin cryosections were prepared and immunolabeled with anti-GFP antibody (Abcam), followed by incubation with secondary antibody conjugated with colloidal gold particles (12-nm diameter; Jackson ImmunoResearch laboratories). Detailed procedures used for cell freezing, sectioning and immunoreactions are as given previously (Waguri and Komatsu, 2009). Sections were viewed using an electron microscope (JEM1200EX, JEOL, Tokyo, Japan). In some electron microscopy images, gamma correction was performed using the Adobe Photoshop software for better recognition of colloidal gold particles on dark background area, as indicated in the figure legend.

Statistical analysis

Values, including those displayed in the graphs, are mean±s.e.m. or mean±s.d., as indicated. Statistical analysis was performed using the unpaired t-test (Welch test). *P<0.05 denoted statistical significance.

Acknowledgements

We thank T. Kouno (Niigata University Graduate School of Medical and Dental Sciences) for her assistance in biochemistry, M. Nozumi (Niigata University Graduate School of Medical and Dental Sciences) for his help with SIM studies, and K. Kanno and A. Yabashi (Fukushima Medical University School of Medicine) for his help with SIM studies. We thank T. Kouno (Niigata University Graduate School of Medical and Dental Sciences) for his help with SIM studies.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.K. designed and directed the study. M.K. and S.W. wrote the manuscript. A.E. and S.W. performed histological and microscopic analyses. I.N. provided intellectual support. All authors discussed the results and commented on the manuscript.

Funding

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (grant number 251101008; a Grant-in-Aid for Scientific Research (A) [grant number 26253019; a Human Frontier Science Program [grant number RGP0055/2014]; and the Takeda Science Foundation. Deposited in PMC for immediate release.

Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.180174/-/DC1

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