**GNG11 (G-protein γ subunit 11) suppresses cell growth with induction of reactive oxygen species and abnormal nuclear morphology in human SUSM-1 cells**

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GNG11 (G-protein γ subunit 11) suppresses cell growth with induction of reactive oxygen species and abnormal nuclear morphology in human SUSM-1 cells

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Abstract

Enforced expression of \textit{GNG11}, G-protein \(\gamma\) subunit 11, induces cellular senescence in normal human diploid fibroblasts. We here examined the effect of the expression of \textit{GNG11} on the growth of immortalized human cell lines, and found that it suppressed the growth of SUSM-1 cells, but not of HeLa cells. We then compared these two cell lines to understand the molecular basis for the action of \textit{GNG11}. We found that expression of \textit{GNG11} induced the generation of reactive oxygen species (ROS) and abnormal nuclear morphology in SUSM-1 cells but not in HeLa cells. Increased ROS generation by \textit{GNG11} would likely be caused by the down-regulation of the antioxidant enzymes in SUSM-1 cells. We also found that SUSM-1 cells, even under normal culture conditions, showed higher levels of ROS and higher incidence of abnormal nuclear morphology than HeLa cells, and that abnormal nuclear morphology was relevant to the increased ROS generation in SUSM-1 cells.

Thus, SUSM-1 and HeLa cells showed differences in the regulation of ROS and nuclear morphology, which might account for their different responses to the expression of \textit{GNG11}. Then, SUSM-1 cells may provide a unique system to study the regulatory relationship between ROS generation, nuclear morphology, and G-protein signaling.

\textbf{Key words:} \textit{GNG11}; cellular senescence; cellular immortalization; nuclear shape; reactive oxygen species;
Introduction

Normal types of human somatic cell undergo terminal growth arrest after a limited number of cell divisions in vitro, a phenomenon termed cellular senescence or replicative senescence (Hayflick and Moorhead 1961). Cellular senescence can also be induced by various means such as reactive oxygen species (ROS), DNA damages, cell cycle perturbations, chromatin destabilization, etc (Michishita et al. 1999; Young and Smith 2000). Cells can escape from cellular senescence by mutational events and acquire an ability to grow indefinitely. For instance, mutations in tumor suppressor genes such as p53, RB, and INK4a/ARF are well-known to help cells escape from cellular senescence. Then, immortalized cell lines have different genetic backgrounds, and the difference in their genetic backgrounds would be causative for their distinct responses to various stimuli or treatments.

We have shown that 5-bromodeoxyuridine (BrdU), an analogue of thymidine, effectively induces cellular senescence in various types of cell (Michishita et al. 1999). BrdU is a thymidine analogue and thus exerts its effects upon incorporation into genomic DNA in the place of thymidine. Since BrdU decondenses heterochromatin through destabilization or disruption of nucleosome positioning (Miki et al. 2008; Miki et al. 2010; Zakharov et al. 1974), dysregulation of heterochromatin seems to be involved in the induction of cellular senescence. Heterochromatin is tethered to the nuclear envelope and has roles in the organization of the nuclear envelope. To analyze
the molecular basis for the action of BrdU in cellular senescence, we screened for the genes
up-regulated in BrdU-induced senescent cells, and identified \textit{GNG11}, $\gamma$-subunit 11 of the
derotrimeric G-protein (Hossain et al. 2006; Suzuki et al. 2001). The heterotrimeric G-proteins are
composed of the $G_\alpha$, $G_\beta$, and $G_\gamma$ subunits, and the $G_\alpha$ and $G_{\beta\gamma}$ subunits work synergistically or
independently to transduce signals upon receptor activation (Clapham and Neer 1997). $G_{\beta\gamma}$ subunits
reside not only in the plasma membrane but also in endosomes, endoplasmic reticulum, the Golgi
apparatus, mitochondria, and nuclei (Khan et al. 2013). \textit{GNG11} ($G_{\gamma11}$) forms a complex with the $G_{\beta1}$
subunit and shuttles between the plasma membrane and the Golgi apparatus (Cho et al. 2011; Saini
et al. 2010); however, the function of \textit{GNG11} has been largely unidentified.

We have previously shown that enforced expression of \textit{GNG11} suppresses the growth of
normal human diploid fibroblast TIG-7 cells (Hossain et al. 2006). We then examined whether
expression of \textit{GNG11} suppresses the growth of immortalized cell lines. For this, we employed
human immortal SUSM-1 and HeLa cells, and found that expression of \textit{GNG11} suppressed the
growth of SUSM-1 cells, but not of HeLa cells. We then compared SUSM-1 cells with HeLa cells to
understand the mechanisms for the growth-suppressive effect of \textit{GNG11} in SUSM-1 cells, and found
that ROS and nuclear morphology were regulated differently in SUSM-1 cells than in HeLa cells,
and these differences may account for the different responses to the expression of \textit{GNG11} in
SUSM-1 cells and HeLa cells.
Materials and Methods

Cell culture

Cervical tumor-derived HeLa cells were obtained from the Japanese Collection of Research Bioresources (JCRB). SUSM-1 cells were established from fetal human diploid fibroblast (AD387) by repeated mutagen treatments and were kindly provided by Dr. Namba, M (Namba et al. 1993). Cells were cultured in plastic dishes (Thermo Scientific, Nunc) containing Dulbecco's modified Eagle's Medium (DMEM) (Nissui Seiyaku) supplemented with 5% fetal calf serum (Cell culture bioscience) at 37°C in 5% CO\textsubscript{2} and 95% humidity as described previously (Michishita et al. 1999). Paraquat (Sigma-Aldrich) and BrdU (Wako) were added to the cells at the concentrations of 5-40 μM and 50 μM, respectively.

Colony formation assay

To determine the survival of cells, appropriate numbers of cells (1 x 10\textsuperscript{3} - 1 x 10\textsuperscript{4}) were plated on 30- or 60-mm dishes, and allowed to grow for 1-2 weeks. To observe colony formation, cells were fixed with methanol and stained with Coomassie Brilliant Blue (CBB, Bio-Rad), and subjected to photography.

Plasmids
The plasmid encoding YFP-tagged GNG11 was kindly provided by Dr. Gautam (Washington University school of Medicine) (Cho et al. 2011; Saini et al. 2010). Construction of the plasmid that expresses GNG11 or GFP from the human cytomegalovirus promoter was previously described (Hossain et al. 2006).

DNA transfection

Plasmids (10 µg) were introduced into cells (10⁶ cells) by electroporation with a high-efficiency electroporator (type NEPA21, Nepa Gene), and appropriate numbers of the cells (1×10³-10⁶ cells) were seeded on dishes or cover slips for further analyses.

Senescence-associated β-Galactosidase assay

Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde at room temperature for 5 min, and incubated at 37°C with a fresh staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂] as previously described (Michishita et al. 1999).

Assay of ROS
The cells were washed twice with PBS (phosphate buffered saline), and incubated in serum free medium supplemented with 5 µM of a ROS-sensitive fluorescence probe, 2′7′-dichloro-fluorescein diacetate (H$_2$DCFDA, Molecular probes) for 30 min at 37°C in dark. Then, cells were washed twice with PBS, and the fluorescence signals were photographed with a fluorescence microscope (IX-70, Olympus) equipped with a standard filter set for fluorescein.

**Immunofluorescence analysis**

Cells grown on a cover slip were fixed with 100% methanol for 15 min at -20°C, washed three times with PBS, and incubated with 1% bovine serum albumin in PBS at room temperature for 1 h. After washing with PBS, the primary antibody against GFP (Bio Vision) was mounted on a cover slip for 18 h. The cells were washed three times with PBS, and incubated with an alexa 568-conjugated secondary antibody (Molecular Probes) for 3 h. Finally, the cells were washed three times with PBS, incubated with DAPI (4′,6-diamidino-2-phenylindole) for 30 min, and mounted with an anti-fading reagent (Molecular probes). Immunofluorescence images were obtained by fluorescence microscopy (BZ-9000, Keyence).

**Quantitative real time RT-PCR**

Total RNA samples were prepared from cells with an RNA extraction kit (Isogen, Nippon Gene). RNA was converted to cDNA by a reverse transcriptase (PrimeScript 1st strand cDNA synthesis kit, Takara), and transcripts were quantified by quantitative real time PCR with a kit (QuantiFast SYBR green PCR kit, Qiagen) on a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer’s protocol. The primers used were as follows:

1. 5'-GAAGGTTGTGGGAAGCATT-3' and 5'-ACATTGCCCAAGTCTCAAC-3' for SOD1;
2. 5'-CGTCACCAGAGGAGATACC-3' and 5'-CTGATTGGAGCAAGCAGCAA-3' for SOD2;
3. 5'-CTGCTGAATGAGAAGTACC-3' and 5'-AGTCAGGGTGACCTCAGTG-3' for catalase;
4. 5'-CCAAGCTCATCACCTGGTCT-3' and 5'-TCGATGTCATGGTCTGGAA-3' for GPX1;
5. 5'-TGCAACCAATTGGACATCAG-3' and 5'-AGACAGGATGCTGTCTGC-3' for GPX2;
6. 5'-AGAGATCAAGAGTCCGCCGC-3' and 5'-TCTTCATCCACTCCACAGCG-3' for GPX4;
7. 5'-CCACTGTAGGCGCCCTAAGTT-3' and 5'-CTCAGCGGATCCAGATCC-3' for NOX1;
8. 5'-GCCAAAGGTGTCCAAGCT-3' and 5'-TCCCCAACGATCGGATAT-3' for NOX2;
9. 5'-CTCTCTGTGAAGACCGGTATGCA-3' and 5'-GACCACAGGCCTAAAATCCA-3' for
   NOX3; 5'-CTCAGCCGAATCAATCAGCTGTG-3' and
10. 5'-AGAGGAAACCGACAATCAGCCTAG-3' for NOX4;
11. 5'-CAGGCACCAGAAAGAAACAT-3' and 5'-TGTTGATCCAGATAAGTCCTCCT-3' for
   NOX5; 5'-AGCTGCTTGGACCCAGTCTC-3' and 5'-GCCTTTCTCAAGTCCCTGC-3' for
1 \textit{GNG11}; 5'-GAAGGTGAAGGTCGGAGTCAA-3' and 5'-GACAAGCTCCGTTCTCAG-3' for

2 \textit{GAPDH} (Ahmad et al. 2013; Juhasz et al. 2009; Lei et al. 2016). Results were normalizes with the

3 expression of \textit{GAPDH}.
Results

Growth suppression by expression of GNG11 in SUSM-1 cells

We examined whether enforced expression of GNG11 suppresses the growth of immortalized human cell lines. For this, we employed SUSM-1 cells and HeLa cells. We transfected these cells with the GNG11-expressing vector or an empty vector (Hossain et al. 2006), and subsequently cultured them with G418 to select the cells that stably expressed GNG11. SUSM-1 cells formed much less colonies when transfected with the GNG11-expressing vector than when transfected with an empty vector (Fig. 1A). By contrast, HeLa cells showed similar colony formation when transfected with the GNG11-expressing vector or an empty vector (Fig. 1A). Thus, expression of GNG11 effectively suppressed the growth of SUSM-1 cells, but not of HeLa cells. Importantly, SUSM-1 cells transfected with the GNG11-expression vector were stained with senescence-associated β-galactosidase (Fig. 1B), which observation suggested the possibility that enforced expression of GNG11 induced cellular senescence in SUSM-1 cells. We also found that GNG11 was endogenously expressed at detectable levels in both SUSM-1 and HeLa cells, but its expression level was lower in SUSM-1 cells than in HeLa cells (Fig. 1C).

Abnormal nuclear morphology in SUSM-1 cells

To explore the mechanisms for the growth-suppressive effect of GNG11 in SUSM-1 cells, we
examined the subcellular localization of GNG11 in SUSM-1 and HeLa cells. We expressed the YFP-fused GNG11 protein (YFP-GNG11) in cells (Cho et al. 2011; Saini et al. 2010); however, YFP fluorescence signals were found to be too weak to precisely determine the localization of GNG11 in both SUSM-1 and HeLa cells, though GFP alone was efficiently expressed in both cell lines (Supplementary Fig. 1). We then enhanced the signals by indirect immunofluorescence with an antibody against YFP/GFP. The YFP-GNG11 protein was observed in the nuclei and at the perinuclear regions in both SUSM-1 and HeLa cells, and thus SUSM-1 and HeLa cells did not appear to show differences in the subcellular localization of GNG11 (Fig. 2A). However, we unexpectedly found that expression of GNG11 significantly increased the cells with abnormal nuclear morphology, which was revealed by staining the nuclei with DAPI, in SUSM-1 cells (Fig. 2A, B). Interestingly, the YFP-GNG11 signals frequently accumulated in the bending regions of the nuclei (Fig. 2A). Abnormal nuclear morphology in SUSM-1 cells was also confirmed by staining the nuclear envelope with an antibody against lamin B receptor (LBR) (Fig. 2C). Additionally, we found that considerable numbers of SUSM-1 cells showed abnormal nuclear morphology even under normal culture conditions (Fig. 2B, 5D). However, by contrast, HeLa cells showed almost normal nuclear morphology when GNG11 was expressed or not (Fig. 2A). Thereby, expression of GNG11 increased the cells with abnormal nuclear morphology in SUSM-1 cells, but not in HeLa cells.
Increased ROS generation by GNG11 in SUSM-1 cells

We next examined the generation of ROS in SUSM-1 and HeLa cells by staining the cells with a ROS-sensitive fluorescent probe, H$_2$DCFDA, because ROS are frequently involved in the regulation of cell growth. We found that expression of GNG11 clearly induced ROS generation in SUSM-1 cells, but not in HeLa cells, and further that SUSM-1 cells showed higher basal levels of ROS than HeLa cells under normal culture conditions (Fig. 3A, B). Thus, SUSM-1 cells showed a clear induction of ROS by GNG11 as well as high basal levels of ROS, whereas HeLa cells showed no detectable induction of ROS by GNG11 as well as low basal levels of ROS. This observation suggested that ROS were regulated differently in SUSM-1 cells than in HeLa cells. We then examined the sensitivity to oxidative stress in SUSM-1 and HeLa cells by culturing them in medium supplemented with paraquat, which generates superoxide anions in cells. SUSM-1 cells were more sensitive to paraquat than HeLa cells (Fig. 3C), and thus SUSM-1 cells would be more susceptible to the growth defect due to oxidative stress than HeLa cells. Since the growth-suppressive effect of paraquat was diminished by simultaneous addition of an antioxidant, N-acetyl-L-cysteine, in SUSM-1 and HeLa cells, ROS were causally involved in the growth suppression by paraquat in these cells (Fig. 3D). Then, ROS were regulated differently in SUSM-1 cells than in HeLa cells, and GNG11-induced growth suppression in SUSM-1 cells may be at least partly explained by the increased generation of ROS by GNG11.
Expression of antioxidant and pro-oxidant enzymes in SUSM-1 and HeLa cells

To understand the mechanisms for the increased generation of ROS by GNG11 in SUSM-1 cells, we analyzed the expression of the enzymes that scavenge or produce ROS: i.e., superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and NADPH oxidase (NOX). NOX produces ROS but the others scavenge ROS. Of these, we examined the expression of the intracellular enzymes such as SOD1, SOD2, catalase, GPX1, GPX2, GPX4, and NOX1-5, and found that GNG11 significantly down-regulated the expression of SOD1, SOD2, catalase, and NOX5 but not of GPX1, GPX4, and NOX4 in SUSM-1 cells (Fig. 4). The expression of GPX2 and NOX1-3 was not detected in SUSM-1 cells (data not shown). Further, we also compared the expression of these enzymes between SUSM-1 cells and HeLa cells. We found that expression of SOD1, catalase, GPX2 and NOX5 was significantly lower in SUSM-1 cells, but that of NOX4 was higher in SUSM-1 cells, than in HeLa cells (Fig. 4). The expression of NOX1-4 was not detected in HeLa cells (data not shown). Thereby, decreased expression of the antioxidant genes appeared to be involved in the increased ROS generation in SUSM-1 cells upon expression of GNG11 and the increased basal levels of ROS in SUSM-1 cells as compared with those of HeLa cells under normal culture conditions, though the roles of the NOX genes in ROS generation remain elusive.
Regulatory interplay between ROS generation and nuclear morphology in SUSM-1 cells

To examine the relationship between increased ROS generation and abnormal nuclear morphology in SUSM-1 cells, we treated SUSM-1 cells with paraquat and examined their nuclear shapes. We then found that paraquat significantly increased the cells with abnormal nuclear morphology in SUSM-1 cells (Fig. 5A, D). This observation indicated that ROS, at least partly, regulated the nuclear shapes in SUSM-1 cells, and thus suggested that expression of GNG11 increased the cells with abnormal nuclear morphology by up-regulating ROS generation in SUSM-1 cells. Interestingly, we also found that SUSM-1 cells, when treated with BrdU, showed ameliorated nuclear morphology and decreased basal levels of ROS (Fig. 5B, C, D). BrdU might affect the nuclear envelope organization because BrdU is an agent that decondenses heterochromatin which interacts with nuclear envelope proteins (Zakharov et al. 1974). Collectively, these findings suggested that increased ROS generation might couple with abnormal nuclear morphology in SUSM-1 cells. However, in HeLa cells, ROS alone were not sufficient to induce abnormal nuclear morphology (Fig. 5A). Thus, nuclear envelope organization as well as ROS generation was regulated differently in SUSM-1 cells than in HeLa cells.
In this study, we showed that enforced expression of GNG11 suppressed the growth of SUSM-1 cells, but not of HeLa cells. Since different responses to the expression of GNG11 in SUSM-1 and HeLa cells would be caused by their different genetic backgrounds, we compared these two cell lines to understand the molecular mechanisms for the growth-suppressive effect of GNG11 in SUSM-1 cells. We then found that SUSM-1 cells showed a clear induction of ROS by GNG11 as well as high basal levels of ROS, whereas HeLa cells showed no detectable induction of ROS by GNG11 as well as low basal levels of ROS. Since ROS impair cellular functions, increased ROS seemed to be involved in the growth suppression by GNG11 in SUSM-1 cells. Given that GNG11 encodes a member of the γ-subunit family of the heterotrimeric G-proteins, GNG11 would likely participate in the regulation of the reactions that erase or produce ROS, rather than GNG11 directly catalyzes the reactions that produce ROS. Indeed, we showed that GNG11 down-regulated the expression of the antioxidant enzymes in SUSM-1 cells, though its regulatory mechanisms are currently unidentified. The activity of transcription factors that regulate the expression of these antioxidant genes might be regulated differently in SUSM-1 cells than in HeLa cells.

We also found that GNG11 or paraquat induced abnormal nuclear morphology in SUSM-1 cells. Then, ROS induced by GNG11 or paraquat would have a role in the induction of abnormal nuclear morphology in SUSM-1 cells. Abnormal nuclear morphology was also observed in a portion
of SUSM-1 cells cultured under normal culture conditions, and this phenomenon would be probably linked with high basal levels of ROS in SUSM-1 cells. Additionally, we showed that BrdU ameliorated nuclear shapes with decreased basal levels of ROS. Thereby, these observations suggested a regulatory interplay between ROS generation and nuclear envelope organization in SUSM-1 cells. Consistent with this, it is well known that the Hutchinson-Gilford progeria syndrome (HGPS) patient cells, which have a defective nuclear lamina due to a mutation in lamin A/C, show not only aberrant nuclear morphology but also increased levels of ROS and an increased sensitivity to ROS (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003; Richards et al. 2011; Viteri et al. 2010). Recent findings indicate that the defective nuclear lamina in HGPS cells impairs the NRF2 function through sequestration of NRF2 at the nuclear periphery and intracellular aggregates, and consequently increases chronic oxidative stress (Kubben et al. 2016). This finding indicates the crucial roles of the nuclear envelope organization in the regulation of oxidative stress because \textit{NRF2}

encodes a transcription factor that plays a central role in the response to oxidative stress by inducing many detoxification/antioxidant enzymes (Itoh et al. 1997). Further, increased generation of ROS due to the defects in the nuclear lamina, in turn, impairs lamin A/C through oxidation of its tail domain (Pekovic et al. 2011). Thus, ROS induced by the defective nuclear lamina amplify the defects in nuclear lamina in a positive feedback loop manner. Then, it would be interesting to speculate such a regulatory interplay between ROS and nuclear envelope proteins might be observed.
in SUSM-1, though it is unclear at present whether increased generation of ROS is a cause or
consequence of abnormal nuclear organization in SUSM-1 cells. However, ROS alone did not seem
to be sufficient to alter nuclear shapes because paraquat hardly affected nuclear shapes in HeLa cells;
thus, we prefer the possibility that altered nuclear envelope organization might cause increased
generation of ROS that consequently leads to an increased sensitivity to ROS in SUSM-1 cells.

Then, it is possible to speculate that SUSM-1 cells would have altered nuclear envelope
organization. In addition, we showed that heterochromatin would also be involved in the regulation
of nuclear shapes because abnormal nuclear shapes in SUSM-1 cells were ameliorated by BrdU,
which decondenses heterochromatin (Zakharov et al. 1974). Given that heterochromatin interacts
with the nuclear envelope proteins, abnormal nuclear shapes might be caused by abnormal
interaction between the nuclear envelope and heterochromatin in SUSM-1 cells. If so, BrdU might
ameliorate the nuclear shapes by unraveling the abnormal interaction between the nuclear envelope
and heterochromatin, and consequently down-regulate the increased basal levels of ROS in SUSM-1
cells. However, we found that, even though BrdU down-regulated the increased basal levels of ROS
in SUSM-1 cells, the ROS levels in BrdU-treated SUSM-1 cells seemed to be higher than those in
HeLa cells under normal culture conditions. This may be due to that BrdU-treated SUSM-1 cells
entered cellular senescence, in which increased ROS generation is frequently observed (Chen et al.
1995; Lee et al. 1999; Lee et al. 2002; Takauji et al. 2016).
To date, human immortalized cell lines are classified into, at least, four genetic complementation groups (A-D) by examining the immortal phenotype of the cell-cell hybrids constructed with a cell-cell fusion technique (Ning et al. 1991). This finding indicates that a limited number of genes are involved in cellular immortalization. We have previously demonstrated that introduction of human chromosome 7 by microcell-mediate chromosome transfer suppresses cell growth in SUSM-1 cells which belong to the complementation group D, but not in HT1080, HeLa, and TE85 cells, which belong to the other complementation groups (Ogata et al. 1993; Ogata et al. 1995). This indicates that a gene(s) that locates on chromosome 7 suppresses the growth of SUSM-1 cells. Since GNG11 locates on chromosome 7, it would be intriguing to speculate that GNG11 plays a role in the growth suppression by introduction of chromosome 7 in SUSM-1 cells.

In summary, we have shown that GNG11 suppressed cell growth with induction of ROS and abnormal nuclear morphology in SUSM-1 cells. Our findings suggested a regulatory interplay between ROS generation and nuclear envelope organization in SUSM-1 cells, and thus, SUSM-1 cells may provide a unique system to study the relationship between ROS generation, nuclear envelope organization, and G-protein signaling.

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Figure legends

Fig. 1. Growth suppression by expression of GNG11 in SUSM-1 cells

A, SUSM-1 and HeLa cells were transfected with the GNG11-expressing vector or an empty vector and allowed to form colonies in the presence of G418 (200-400 µg/ml) for 2 weeks. Cells were subjected to photography and stained with CBB.

B, SUSM-1 cells transfected with the GNG11-expressing vector or an empty vector were stained with senescence-associated β-galactosidase.

C, Expression level of endogenous GNG11 was determined by quantitative RT-PCR in SUSM-1 and HeLa cells, and is expressed as a value relative to that of SUSM-1 cells. An error bar indicates S.D.

Fig. 2. Subcellular localization of GNG11 and nuclear morphology in SUSM-1 and HeLa cells upon expression of GNG11

A, SUSM-1 and HeLa cells were transfected with the vectors expressing YFP-GNG11 or GFP (control), and probed with an anti-GFP antibody. Nuclei were stained with DAPI. Arrowheads indicate abnormal nuclei, and arrows indicate the accumulated YFP-GNG11 signals at the bending regions of the nuclei.

B, The percentage of the cells with abnormal nuclear morphology (A) was determined. Approximately 50 nuclei of the YFP/GFP-positive cells were examined (n=3). An error bar indicates...
S.D., and an asterisk indicates statistical significance ($P<0.05$).

C, SUSM-1 cells were stained for the nuclear envelope with an antibody against a nuclear envelope protein, lamin B receptor (LBR).

**Fig. 3.** Regulation of ROS in SUSM-1 and HeLa cells

A, SUSM-1 and HeLa cells were transfected with the GNG11-expressing vector or an empty vector, and generation of ROS was determined by culturing these cells with a ROS-sensitive fluorescent probe, H$_2$DCFDA.

B, Basal levels of ROS were determined by culturing SUSM-1 and HeLa cells with H$_2$DCFDA.

C, SUSM-1 and HeLa cells were cultured with paraquat (5 µM) for 1 week, and stained with CBB.

D, SUSM-1 and HeLa cells were cultured with paraquat (5 µM for SUSM-1 cells and 10 µM for HeLa cells) in the presence or absence of 0.5 mM of N-acetyl-L-cysteine (NAC) for 1 week, and stained with CBB.

**Fig. 4.** Expression of ROS-scavenging and ROS-producing enzymes

Expression levels of ROS-scavenging and ROS-producing enzymes were determined by quantitative RT-PCR in SUSM-1 cells transfected with the GNG11-expressing vector (grey bars) or an empty vector (white bars), and in HeLa transfected with an empty vector (black bars). The expression level
is expressed as a value relative to that of SUSM-1 cells transfected with an empty vector. An error bar indicates S.D.

Fig. 5. Regulatory interplay between ROS generation and nuclear envelope organization

A, SUSM-1 and HeLa cells were treated with paraquat (40 µM) for 3 days and stained with DAPI. Arrowheads and squares indicate abnormal nuclei and slightly abnormal nuclei, respectively.

B, C, SUSM-1 and HeLa cells were treated with BrdU (50 µM) for 3 days and stained with H$_2$DCFDA (B) or DAPI (C). Arrowheads and squares indicate abnormal nuclei and slightly abnormal nuclei, respectively.

D, The percentage of the cells with abnormal and slightly abnormal nuclear morphology in SUSM-1 cells (A and C) was determined (> 100 cells, n=3). Error bars indicate S.D., and asterisks indicate statistical significance ($P<0.05$).

Supplementary figure 1. Transfection of SUSM-1 and HeLa cells with a GFP-expressing vector.

SUSM-1 and HeLa cells were transfected with a GFP-expression vector and GFP fluorescence was photographed.
Fig. 3 Takauchi et al.