Nuclear retention of importin α coordinates cell fate through changes in gene expression

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Various cellular stresses including oxidative stress induce a collapse of the Ran gradient, which causes accumulation of importin α in the nucleus and a subsequent block of nuclear protein import. However, it is unknown whether accumulated importin α performs roles in the nucleus after its migration in response to stress. In this study, we found that nuclear-retained importin $\alpha 2$ binds with DNase I-sensitive nuclear component(s) and exhibits selective upregulation of mRNA encoding *Serine/threonine kinase 35* (STK35) by microarray analysis. Chromatin immunoprecipitation and promoter analysis demonstrated that importin $\alpha 2$ can access to the promoter region of STK35 and accelerate its transcription in response to hydrogen peroxide exposure. Furthermore, constitutive overexpression of STK35 proteins enhances caspase-independent cell death under oxidative stress conditions. These results collectively reveal that nuclear-localized importin $\alpha 2$ influences gene expression and contributes directly to cell fate outcomes including non-apoptotic cell death.

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mediated by soluble factors that specifically recognize their cargoes and facilitate the passage of receptor–substrate complexes. Most import/export pathways use members of a family of carrier proteins, with those that facilitate nuclear import called importins and those mediating nuclear export termed exportins. Importins function by recognizing a specific signal sequence, the nuclear localization signal (NLS), on cargo proteins which typically consists of basic amino-acid clusters (Lange *et al*, 2007). Within the cytoplasm, importin α functions as an adaptor molecule between importin $\beta 1$ and a protein bearing a classical NLS (cNLS). Interaction of this trimeric import complex with the NPC is mediated by importin $\beta 1$, which enables its translocation into the nucleoplasm. In the nucleus, GTP-bound Ran (RanGTP) binds to importin $\beta 1$ and dissociates the import complex to release the cargo. After dissociation, importin $\beta 1$ –RanGTP is recycled to the cytoplasm, while importin α is transported back to the cytoplasm in a trimeric export complex with RanGTP by the cellular apoptosis susceptibility gene product, CAS (Kuersten *et al*, 2001; Sorokin *et al*, 2007).

Seven importin α s have been identified in human and six in mouse, and these are classified into three subtypes (Goldfarb *et al*, 2004; Hu *et al*, 2010; Kelley *et al*, 2010). In the present study, we use the term ‘importin $\alpha 1$ ’ that has been termed karyopherin $\alpha 1$ (KPNA1), NPI-1 in human or $\alpha S1$ in mouse; ‘importin $\alpha 2$ ’ that has been termed KPNA2, Rch1 in human or $\alpha P1$ in mouse; ‘importin $\alpha 3$ ’ that has been termed KPNA3, hSRP1 γ in human or $\alpha Q2$ in mouse; ‘importin $\alpha 4$ ’ that has been termed KPNA4, Qip1 in human or $\alpha Q1$ in mouse; ‘importin $\alpha 6$ ’ that has been termed KPNA6, NPI-2 in human or $\alpha S2$ in mouse as a consensus for both human and mouse, based on previous reports (Mason *et al*, 2009; Major *et al*, 2011).

Importantly, the importin α proteins show differences in their ability to interact with specific cNLS-containing cargoes, and also show selective expression patterns in cells and tissues at both RNA and protein levels, indicating that the cargo proteins differentially access the nucleus depending on the cohort of importins present (Jans *et al*, 2000; Itman *et al*, 2009; Yasuhara *et al*, 2009).

In addition to mediating cargo transport into the nucleus, the potential for importin α s to serve non-transport functions within the nucleus has been suggested by reports that importin α s rapidly accumulate in the nucleus in response to cellular stresses, including oxidative stress and heat shock (Stochaj *et al*, 2000; Furuta *et al*, 2004; Kodiha *et al*, 2004; Miyamoto *et al*, 2004). In these conditions, a collapse of the Ran gradient has been observed which leads to suppression of the CAS-mediated nuclear export of importin α in stress-exposed cells, causing a block of the classical nuclear import pathway. In addition, we found that the rapid nuclear accumulation of importin α is accelerated by importin $\beta 1$ /Ran-independent import (Miyamoto *et al*, 2002, 2004). While the molecular details of the Ran gradient decay are not completely clear, we have demonstrated that a stress-induced

Introduction

Nucleocytoplasmic transport of macromolecules occurs through the nuclear pore complex (NPC; Alber *et al*, 2007),

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decrease in cellular ATP contributes to the Ran gradient collapse (Yasuda *et al*, 2006).

Intriguingly, we discovered that importin $\alpha 2$ remains in the nucleus of HeLa cells after stress exposure, even when RanGTP is co-injected into the nucleus to supply nuclear Ran (Miyamoto *et al*, 2004). This observation strongly suggested that retention of importin $\alpha 2$ in the nucleus after its translocation induced by Ran gradient collapse is of functional significance. However, little is known about how importin $\alpha 2$ is retained in the nucleus and whether nuclear-retained importin $\alpha 2$ can serve roles which are different from its well-known nuclear transport function.

In the present study, we show that importin $\alpha 2$ is retained in the nucleus through binding to DNase I-sensitive nuclear components in response to stress. Microarray analysis revealed that nuclear importin $\alpha 2$ has a significant and selective effect on gene expression. *STK35*, which drives caspase-independent cell death upon oxidative stress, was upregulated. These results identify a novel role for importin $\alpha 2$ in changing gene expression in response to stress, which acts to regulate cell death. In the light of these and other recent discoveries, it is clear that importin α has multiple functions in addition to that of cargo nuclear trafficking.

Results

Stress-induced nuclear importin $\alpha 2$ is eluted with DNase I treatment

To know whether importin α binds to particular nuclear component(s) after migration into the nucleus in response to stresses, HeLa cells were permeabilized with Triton X-100 for 5 min after exposure to ultraviolet (UV), hydrogen peroxide (H_2O_2) or 42°C, and the cells were then treated with DNase I, RNase or NaCl without fixation. Following each treatment, endogenous importin $\alpha 2$ was detected using a specific antibody to identify the solubilization or retention of importin $\alpha 2$. The efficacy of either DNase I or RNase treatment was tested by observing either the decrease in DNA staining with Hoechst 33342 or the disappearance of poly(A)⁺RNA signals detected by *in situ* hybridization, respectively (data not shown). As shown in Figure 1A, DNase I treatment dramatically abolished nuclear importin $\alpha 2$ localization, while treatment with RNase or NaCl had only a slight effect. The sensitivity against DNase I was similar to that of endogenous RCC1, which is known as a chromatin-associated protein and to be mainly transported into the nucleus by importin $\alpha 3$ or $\alpha 4$, not by importin $\alpha 2$ (Kohler *et al*, 1999), while the nuclear export factor CRM1 was released by the treatment with Triton X-100 alone (Figure 1B). These data indicate that importin $\alpha 2$ is retained within the nucleus through direct or indirect binding to some DNase I-sensitive components such as chromatin in response to the three stresses applied in this study. Intriguingly, while oxidative stress induced the greatest extent of importin $\alpha 2$ loss following DNase I treatment, the reduction in importin $\alpha 2$ signal was common to all the tested stressors, indicating that at least a fraction of the nuclear importin $\alpha 2$ is associated with DNase I-sensitive component(s) in each stress condition. These results suggest that nuclear importin α may function in nuclear events such as DNA replication and RNA transcription.

Nuclear importin $\alpha 2$ leads to altered transcript levels

To test whether nuclear-localized importin $\alpha 2$ influences transcriptional outcomes, we sought to examine gene expression changes in cells with nuclear accumulation of importin $\alpha 2$ by performing microarray analysis. We designed an experiment in which EGFP fused full-length importin $\alpha 2$ was transfected into HeLa cells (Figure 2A). In contrast to endogenous importin $\alpha 2$ which have been observed as predominantly cytoplasmic in HeLa cells (Figure 1), the exogenous full-length importin $\alpha 2$ protein showed striking nuclear localization in cells with high expression levels following transient transfection (Figure 2B, upper two cells in short exposure). In contrast, the signal was evenly distributed between the nucleus and cytoplasm in cells with low expression levels (Figure 2B, bottom two cells in long exposure). To assess the feature of overexpressed importin $\alpha 2$ statistically, we captured its fluorescence automatically using ArrayScan instrument and then the nuclear to cytoplasmic ratio (N/C) was estimated by calculating average pixel intensity of the fluorescence within the nucleus or the cytoplasm. As expected, the EGFP protein alone evenly distributed throughout the cells and the N/C ratio did not depend on its expression levels (Supplementary Figure S-I). On the other hand, EGFP–importin $\alpha 2$ was concentrated predominantly in the nucleus, in particular, of highly expressing cells, while its total intensity was relatively lower than EGFP alone, coinciding with the previous report (Wu *et al*, 2009).

The exogenous full-length importin $\alpha 2$ protein can enhance nuclear transport of karyophilic proteins such as transcription factors and may thereby directly influence gene expression. To exclude this possibility, an isoform of EGFP–importin $\alpha 2$, which is mutated in the C-terminal CAS binding domain, was also transfected (Figure 2A). Although the fluorescence intensity of the protein was comparatively lower than EGFP alone or full-length EGFP–importin $\alpha 2$ throughout the cells, the mutant EGFP–importin $\alpha 2$ lacking the CAS binding domain, which is never recycled back to the cytoplasm, showed complete nuclear localization (Figure 2B; Supplementary Figure S-I). We considered that the feature of these proteins to localize in the nucleus mimicked the stress-induced nuclear accumulation of importin α , and this enabled us to analyse the effect of nuclear importin α , independent of effects of other cellular stress-response reactions. Therefore, we next undertook to investigate whether there were changes in gene expression common to cells expressing the full-length importin $\alpha 2$ and the C-terminal mutant (C-mutant) isoform.

By microarray analysis, we found that cells containing either construct exhibited >2-fold elevation of two transcripts, *Serine/threonine kinase 35* (*STK35*) and *Serine PI Kazal type 5 like 3* (*SPINK5L3*) (Supplementary Table S-I). On the other hand, transcripts encoded by 62 genes were scored as downregulated by >2-fold. Among them, nine genes were stress-response genes or apoptosis-related genes such as *Growth arrest and DNA damage-inducible alpha* (*GADD45A*) or *HARAKIRI* (*HRK*). All tested stress-responsive or apoptosis-related genes exhibited reduced mRNA levels in cells transfected with either full-length or C-mutant isoform of importin $\alpha 2$. On the other hand, *p21*, which previously exhibited little or no change in human importin $\alpha 1$ -expressing HeLa cells without Zac1 (Huang *et al*, 2007), showed no apparent alteration by quantitative RT–PCR (qPCR; Supplementary Figure S-II). In addition, 22 genes encoding

Figure 1 Importin $\alpha 2$ accumulation in the nucleus in response to the stress is efficiently released by DNase I treatment. **(A)** HeLa cells were either exposed to UV (254 nm, 0.3 J/cm²) or incubated with 200 μ M H₂O₂ for 1 h or at 42°C for 1 h, as indicated. The cells were permeabilized with 0.5% Triton X-100 for 5 min and then incubated for 1 h at 30°C with DNase I, RNase or NaCl. After incubation, the cells were fixed with 3.7% formalin and indirect immunofluorescence was performed to detect endogenous importin $\alpha 2$ using a specific antibody. **(B)** HeLa cells were permeabilized with 0.5% Triton X-100 for 5 min and then incubated for 1 h at 30°C with DNase I, RNase or NaCl. After incubation, the cells were fixed with 3.7% formalin and indirect immunofluorescence was performed to detect either endogenous RCC1 or CRM1 using specific antibodies. DNA was detected with DAPI.

replication-dependent histones were listed as downregulated genes. Since the histone genes are known to be clustered together in the genome (Marzluff *et al*, 2002), we assumed that nuclear importin $\alpha 2$ may access specific gene clusters. Hence, we surveyed the expression of all histone genes in the microarray and examined whether the downregulated histone genes were localized at specific loci (Figure 3; Supplementary Table S-II). However, we did not detect any cluster specificity, since the expression of almost all of the histone genes was decreased. To further understand the biological significance of the microarray data, gene set enrichment analysis (GSEA) was used as a complementary

approach (Subramanian *et al*, 2005). The GSEA provided four significantly enriched upregulated gene sets, under an NOM *P*-value of 0.05 and an FDR *q*-value of 0.25, three genes of which were associated with haematopoietic stem cell differentiation (Supplementary Table S-IV).

Nuclear importin $\alpha 2$ can drive *STK35* mRNA expression

Building on the microarray results, we focused on the upregulated *STK35* gene and assessed its expression by qPCR. The findings support the array results by revealing a slight but significant elevation in the level of *STK35* (Figure 3). Therefore, we investigated whether nuclear localization of

Figure 2 Transfection of importin $\alpha 2$ into HeLa cells. **(A)** Schematic illustration of enhanced green fluorescent protein (EGFP)-fused importin $\alpha 2$ proteins used in this transfection analysis. The C-mutant was mutated between 469 and 474 a.a., as indicated. IBB: importin $\beta 1$ -binding, ARM repeats: armadillo repeats. **(B)** Cellular localization of these encoded proteins in transiently transfected HeLa cells. HeLa cells expressing the EGFP-importin $\alpha 2$ full-length proteins are shown with different exposure times for the same image.

Figure 3 Validation of mRNA expression levels of *STK35* and histone genes by quantitative PCR. HeLa cells were transfected with EGFP as a control, EGFP–importin $\alpha 2$ full-length or C-mutant constructs, and mRNA expression levels of *STK35*, each histone gene and β -actin (*ACTB*) as a control were analysed by qPCR. The results were from three independent experiments and were presented in comparison with values in the EGFP-expressing cells as the mean $\pm$ s.e.m. ($n = 3$ each). ** $P < 0.01$; Student's *t*-test. Primers are shown in Supplementary Table S-III.

importin $\alpha 2$ can directly drive *STK35* mRNA expression through an interaction with chromatin in response to stress. To achieve this, we tested whether nuclear importin $\alpha 2$ can access the promoter region of *STK35* and effectively enhance its transcription. We first sought to identify the functional promoter of *STK35* and examined whether importin α s can impact on its activity in terms of gene regulation. Upstream sequences from the first exon (ENSMUSG00000037885,

Figure 4A) corresponding to -0.25 , -0.5 , -1 or -2 kbp were amplified by PCR from adult mouse testis genomic DNA, because the *STK35* transcript has been identified to have exclusively high expression in the testis (Vallenius and Makela, 2002). The products were cloned into pGL3 Basic luciferase reporter plasmid and the constructs were transfected into the GC-2 mouse male germ-cell line. As an internal control, a plasmid pRL-TK that contains Renilla luciferase was co-transfected, and the relative promoter activity was determined by the ratio of the activities of firefly luciferase and Renilla luciferase. As shown in Figure 4A, constructs that consist of either -0.5 or -0.25 kbp sequences upstream from the first exon showed the highest signal intensities, at > 8 -fold elevation over control values. In addition, constructs lacking regions of either -0.5 or -0.285 kbp were not active, indicating that the -0.25 kbp sequence could function as a core promoter of *Stk35*.

To address whether the promoter activity is influenced by the presence of importin α s, the -2 kbp construct was co-transfected with constructs encoding each of five mouse importin α proteins into GC-2 cells. The luciferase intensity was enhanced ~ 2 -fold in the presence of either importin $\alpha 2$ or $\alpha 4$ (Figure 4B). In addition, promoter activity was also enhanced by either of the C-mutant, importin $\beta 1$ binding (IBB) domain-deleted (Δ IBB) mutants of importin $\alpha 2$ and $\alpha 4$, and also by the cNLSs binding-deficient mutant of importin $\alpha 2$ (ED mutant) (Figure 4C). These results indicate that the promoter activity is facilitated by a gene regulation function of nuclear-localized importin α and not by the nuclear transport of karyophilic proteins such as transcription factors. In contrast to the -2 kbp construct, a more modest upregulation was observed when the -0.25 kbp construct was co-transfected with importin $\alpha 2$ or $\alpha 4$ into GC-2 cells (Figure 4D).

Figure 4 Importin $\alpha 2$ can access to a promoter region of *STK35*. (A) Genomic DNA sequences of *Stk35* described were constructed into the pGL3-Basic luciferase vector and were transfected into GC2 cells with an internal control plasmid pRL-TK. After 24 h incubation, the relative luciferase activity was determined by the ratio of the activities of firefly luciferase and Renilla luciferase. Values are means $\pm$ s.e.m. ($n = 3$ each). $*P < 0.05$ and $***P < 0.001$; Student's t -test. (B) pGL3-Basic/-2 kbp plasmid was co-transfected with EGFP as a control (Cont.) or each EGFP-importin α construct, as indicated and with pRL-TK into GC2 cells and incubated for 48 h. Values are means $\pm$ s.e.m. ($n = 3$ each). $*P < 0.05$ and $***P < 0.001$. (C) pGL3-Basic/-2 kbp plasmid was co-transfected with EGFP as a control (Cont.), EGFP-importin $\alpha 2$ C-mutant, EGFP-importin $\alpha 2$ Δ IBB-mutant, EGFP-importin $\alpha 4$ Δ IBB-mutant or EGFP-importin $\alpha 2$ ED-mutant constructs and with pRL-TK into GC2 cells and incubated for 24 h. Values are means $\pm$ s.e.m. ($n = 3$ each). $**P < 0.01$ and $***P < 0.001$. Because the transfection condition for the ED mutant was different from that for the other constructs as described in Materials and methods, the data are separated by a vertical line. (D) pGL3-Basic/-0.25 kbp plasmid was co-transfected with EGFP as a control (Cont.), EGFP-importin $\alpha 2$ or EGFP-importin $\alpha 4$ and with pRL-TK into GC2 cells and incubated for 48 h. Values are means $\pm$ s.e.m. ($n = 3$ each). $*P < 0.05$ and $**P < 0.01$. (E) HeLa cells were exposed to 200 μ M H_2O_2 for 30 min and subjected to immunoprecipitation using an anti-importin $\alpha 2$ antibody or anti-importin $\alpha 3$ antibody. After DNA purification from the precipitated the chromatin sample, PCR was performed to amplify sequences in the *STK35* promoter region. *GAPDH* was used as a negative control.

Next, we conducted a chromatin immunoprecipitation (ChIP) analysis. Treatment with H_2O_2 was selected as the cellular stress, because this led to the most notable release of nuclear importin $\alpha 2$ following DNase I treatment (Figure 1). As shown in Figure 4E, importin $\alpha 2$ binding to the *STK35* promoter region was recorded as the amplification of promoter sequences by PCR following ChIP with the antibody to importin $\alpha 2$. On the other hand, the PCR product was not detected in samples precipitated with either of the antibodies specific for p53, which successfully pulled down the *p21* promoter region as expected (Supplementary Figure S-IIIB), or for importin $\alpha 3$ (Figure 4E). This is consistent with the result of the luciferase assay shown in Figure 4B. Taken together, these results argue that nuclear-localized importin $\alpha 2$ can access the promoter of *STK35* and enhance its transcription through upstream sequences from the core

promoter, while it remains to be established whether this interaction is mediated by other proteins or through direct binding of importin α to DNA.

Nuclear accumulation of importin α is a feature in caspase-independent cell death

We previously demonstrated that several stresses, including H_2O_2 , decreased intracellular ATP levels in HeLa cells, resulting in collapse of the Ran gradient and subsequent nuclear accumulation of importin α (Yasuda *et al*, 2006). Alteration of intracellular ATP levels is often observed to trigger cell death; in particular, a decrease in ATP leads to non-apoptotic cell death (Lee and Shacter, 1999; Troyano *et al*, 2003). This suggests that the nuclear accumulation of importin α attributed to a decrease in ATP could provoke non-apoptotic cell death. To test this hypothesis, we firstly measured

intracellular ATP levels in HeLa cells exposed either to H_2O_2 or to staurosporine (STS), an apoptosis-inducing reagent that has been reported to increase intracellular ATP levels prior to apoptosis (Ferrando-May *et al*, 2001; Zamaraeva *et al*, 2005). As expected, exposure to 5 mM H_2O_2 caused a dramatic decrease in ATP levels at 30 min after treatment to <5% of pretreatment values (Figure 5A). This observation agrees with our previous data in which treatment with 200 μ M H_2O_2 decreased the ATP levels to 30% of normal in HeLa cells (Yasuda *et al*, 2006). On the other hand, treatment with 0.5 μ M of STS sustained ATP levels for 2 h at ~90% and only slightly decreased to 70% after 4 h (Figure 5A).

To investigate the correlation between altered ATP levels and nuclear accumulation of importin α , we observed the subcellular localization of importin $\alpha 2$ in HeLa cells exposed to 5 mM H_2O_2 or 0.5 μ M STS for 4 h. As measured by an immunofluorescence analysis, STS treatment did not cause significant nuclear localization of importin $\alpha 2$ in HeLa cells in comparison with H_2O_2 treatment (Figure 5B). Thus, nuclear accumulation of importin $\alpha 2$ occurred in stress conditions, which were clearly associated with a decrease in intracellular ATP levels.

Next, we sought to determine whether these stress conditions could induce the apoptotic cell death pathway. HeLa cells were exposed to 5 mM H_2O_2 or to 0.5 μ M STS for 4 h and then examined for the presence of the cleaved product of Poly

(ADP-ribose) polymerase (PARP), which is a substrate for caspase-3 during the execution phase of apoptosis. As expected, cleaved PARP was readily detected only in STS-treated HeLa cells, and its appearance was prevented by pretreatment with a caspase inhibitor, zVAD-fmk (Figure 5C), coinciding with a previous report (Ferrando-May *et al*, 2001). While oxidative stress treatment did not induce PARP cleavage, the proportion of propidium iodide (PI)-positive cells was dramatically increased to 40% of total cell number (Figure 5D), suggesting that H_2O_2 treatment induces caspase-independent cell death. These results indicate that the importin α nuclear accumulation attributed to the decrease in intracellular ATP following H_2O_2 exposure is a feature of non-apoptotic cell death.

STK35 affects non-apoptotic cell death in response to oxidative stress

We next examined whether *STK35* transcript levels are altered in response to oxidative and non-oxidative stresses. HeLa cells were exposed to 1 mM H_2O_2 and then placed in fresh medium; the *STK35* mRNA was quantified by qPCR from 0 to 8 h later. In the treated cells, this transcript was significantly increased by 2 h and reached a level about 2.5 times higher at 8 h (Figure 6A). On the other hand, exposure to STS led to downregulation of *STK35* transcripts (Figure 6B), while expression of β -ACTIN was unchanged

Figure 5 Nuclear accumulation of importin α is a feature in caspase-independent cell death mode. **(A)** HeLa cells were exposed either to 5 mM H_2O_2 or to 0.5 μ M staurosporine (STS) at the indicated times. After incubation, the intracellular ATP levels were measured using CellTiter-Glo assay kit. The results were risen from three independent experiments and presented in comparison with the values in 0 h cells as the mean $\pm$ s.e.m. ($n = 3$ each). **(B)** HeLa cells were exposed either to 5 mM H_2O_2 or to 0.5 μ M STS for 4 h. After incubation, the cells were fixed with 3.7% formalin and indirect immunofluorescence was performed to detect endogenous importin $\alpha 2$ using a specific antibody. DNA was visualized by Hoechst 33342. **(C)** HeLa cells were pretreated in the presence or absence of 50 μ M zVAD-fmk for 30 min and treated with 5 mM H_2O_2 or 0.5 μ M STS for 4 h. Equal amounts of cellular proteins contained in total cell extracts were subjected to SDS-PAGE and analysed by western blotting for PARP and GAPDH. **(D)** HeLa cells were exposed to 5 mM H_2O_2 for 4 h and were stained with PI and then sorted by FACS. Untreated cells were showed as a control (Cont.). Values are means $\pm$ s.e.m. ($n = 3$ each) of PI-positive cells. ** $P < 0.01$; Student's *t*-test.

Figure 6 Ectopic expression of *STK35* enhances non-apoptotic cell death during oxidative stress. **(A)** HeLa cells were treated with 1 mM H₂O₂ for 1 h and then placed in fresh medium. The *STK35* mRNA was measured by qPCR at the indicated times. Quantification of *GAPDH* was used as a control for data normalization. Values are means $\pm$ s.e.m. ($n = 3$ each). ** $P < 0.01$ and *** $P < 0.001$; Student's *t*-test. **(B)** HeLa cells were exposed to 0.5 μ M STS at the indicated times and the mRNA expression levels were analysed by qPCR. Quantification of *GAPDH* was used as a control for data normalization. Values are means $\pm$ s.e.m. ($n = 3$ each); *** $P < 0.001$. **(C)** 293F stably expressing wttag as a control (Cont.), wttag-STK35S or wttag-STK35L1 was pretreated for 30 min in the absence or presence of 50 μ M zVAD-fmk and exposed to 2 mM H₂O₂ for 2 h. Cells were stained with PI and then sorted by FACS. Values are means $\pm$ s.e.m. ($n = 4$ each) of PI-positive cells; * $P < 0.05$. **(D)** HeLa cells transfected with indicated siRNA oligonucleotides were exposed to 5 mM H₂O₂ for 4 h. Cells were stained with PI and then sorted by FACS. Values are means $\pm$ s.e.m. ($n = 3$ each) of PI-positive cells. ** $P < 0.01$; Student's *t*-test.

(data not shown). These data collectively indicate that importin $\alpha 2$ can access the promoter region of *STK35* to act as a stress-responsive transcriptional enhancer following its nuclear migration during oxidative stress.

What is the physiological significance of the high level of *STK35* in response to stress? A previous report suggested that STK35, a protein of 401 amino acids (named as STK35S here), could be recruited by the PDZ-LIM protein CLP-36 to regulate actin stress fibres (Vallénius and Makela, 2002). Moreover, Goyal *et al* (2009) identified an additional transcript encoding a longer STK35 isoform with an N-terminal extension of 133 amino acids referred to as STK35L1. However, there is no knowledge of the biological functions served by these STK35 isoforms under stress conditions. To address this, we examined the contribution of these proteins to cell viability following exposure to H₂O₂. We first established two independent stable 293F cell lines expressing either *STK35S* or *STK35L1* (Supplementary Figure S-IVA). By counting the number of dead cells stained with PI using flow cytometry, exposure to 2 mM H₂O₂ for 2 h resulted in an increase from <10% to over 40% of PI-positive cells (Figure 6C). These cells appeared to have died by a non-apoptotic mode, because the H₂O₂ treatment did not induce PARP cleavage (Supplementary

Figure S-IVB). In addition, the *STK35*-expressing cells displayed significantly elevated susceptibility to oxidative stress compared with control cells, while no differences were observed between the cell lines containing *STK35S* and *STK35L1* (Figure 6C). Moreover, the enhancement revealed in both *STK35*-expressing cell populations was not affected by pretreatment with zVAD-fmk (Figure 6C), which also inhibited the cleavage of PARP in cells treated with STS (Supplementary Figure S-IVB). These results strongly imply that the *STK35* proteins can accelerate non-apoptotic cell death in response to oxidative stress by H₂O₂.

To further ascertain the functional importance of the *STK35* protein in cell viability, we designed a knockdown experiment for *STK35* gene using RNAi oligonucleotides. HeLa cells were transfected with either control siRNA against Luciferase (siLUC.) or two kinds of *STK35* siRNA oligonucleotides (siSTK35-1 or siSTK35-2), and the mRNA expression levels of *STK35* were analysed by qPCR. The level of the *STK35* mRNA was decreased to 40% by siSTK35-1 or to 20% by siSTK35-2, respectively (Supplementary Figure S-IVD). Using these cells, we examined cell viability after treatment with H₂O₂. Cells transfected with siSTK35-1 showed no difference compared with control cells after exposure to

5 mM H₂O₂ for 4 h, while the number of PI-positive cells was increased in the siSTK35-2 cells (Figure 6D). These results together with the results from overexpression experiments indicate that the nuclear accumulation of importin α 2 is a feature in non-apoptotic cell death and that the specific impact on gene expression, in particular upregulation of *STK35*, could be a determinant of the cell death mode.

Discussion

A novel function of importin α 2 in gene expression

In this study, we demonstrated that nuclear-localized importin α 2 has the capacity to regulate expression of specific genes. *STK35* was identified through microarray analysis as increased >20-fold in cells transfected with full-length importin α 2 and >5-fold in cells expressing a C-mutant (Supplementary Table S-I). In addition, promoter analysis and ChIP assay demonstrated that importin α 2 binds the promoter region of *STK35*. Intriguingly, all tested importin α 2 mutants exhibited a significant upregulation of *Stk35* promoter activity (Figure 4C). Reflecting the observation that the importin α mutants accumulated predominantly in the nucleus, we propose that importin α has the ability to migrate into the nucleus in an importin β 1-independent manner when it is not bound to cNLS-containing proteins (Miyamoto *et al*, 2002), the nuclear distribution of overexpressed importin α proteins, including the ED mutant, might be attributed to excess amount of the cargo-free importin α . Our data using several mutants provide strong evidence that importin α influences transcriptional outcomes independent of cNLS-containing cargo transport. However, we still cannot rule out the possibility that C-mutant might mediate a single round of cargo nuclear import. In addition, there remains the possibility that importin α transports a non-classical NLS-containing protein such as CaMKIV (Kotera *et al*, 2005).

Interestingly, the luciferase activity elicited by the -0.25 kbp construct was about twice that of the -2 kbp construct, in spite of the presence of core promoter sequences in the longer construct (Figure 4A). This suggests that the upstream sequences from the core promoter serve a repressor function. Remarkably, while the -2 kbp construct exhibited a two-fold enhanced activity in the presence of importin α 2 or α 4 (Figure 4B), the -0.25 kbp core promoter exhibited a lower, but significant, elevation in presence of the importin α proteins (Figure 4D). These results strongly imply that importin α enhances *STK35* promoter activity through regions in the -2 kbp, particularly upstream from the core promoter. To address the structural features of the promoter sequences, we predicted transcription factor binding sites in the -2 kbp of the upstream region from the first exon in human and mouse using AliBaba2.1 program (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>). Intriguingly, certain transcription factor binding sites are relatively concentrated in the sequences upstream from -1 kbp (data not shown). This suggests that the upstream sequences may function as a transcriptional modulation area for the *STK35* core promoter and that importin α might access the region via transcription factors. Further work is required to address whether this interaction is mediated by classical/non-classical NLSs-containing proteins or through direct binding of importin α to DNA.

Importin α as a multifunctional molecule

There are now several lines of evidence indicating that importin α s serve functions additional to their classical role as transport machinery (Goldfarb *et al*, 2004). These well-studied roles include negative regulation of spindle assembly factor activity, including TPX2, NuMA or XCTK2 proteins, which normally occurs in association with importin β 1 under Ran GTPase control (Askjaer *et al*, 2002; Geles *et al*, 2002; Schatz *et al*, 2003; Ems-McClung *et al*, 2004). In addition, GAL4-DNA binding (Chan and Jans, 1999), mRNA biogenesis by binding with cap methyltransferase (Wen and Shatkin, 2000), ubiquitin-mediated protein degradation (Tabb *et al*, 2000) and downregulation of DNA synthesis (Kim and Lee, 2008) mediated by importin α s have all been reported to occur in an importin β 1/Ran-independent manner. In *Drosophila*, importin α 2 plays a role in ring canal assembly by regulating the deposition of Kelch (Gorjanacz *et al*, 2002, 2006), while additional studies have implicated importin α s in nuclear envelope assembly or nuclear lamina formation (Hachet *et al*, 2004; Adam *et al*, 2008). Thus, our new findings contribute to the growing understanding that importin α serves a variety of functions, some of which like classical nuclear import occur in the presence of importin β 1 under Ran gradient influence, while others occur in the absence of regulation by Ran.

Physiological importance of downregulation of replication-dependent histone genes

Surprisingly, 22 among the 62 transcripts were measured as >2-fold downregulated in the microarray analysis encoded replication-dependent histones. Although the decrease in level was highest for *H2A*, two replacement variant genes, *H2AZ* and *H2AX*, which have been well studied in relation to DNA damage responses (Redon *et al*, 2002), showed little change in transcript abundance (data not shown), suggesting that nuclear importin α may be specifically related to expression of the replication-dependent histones. It has been demonstrated that CHO-K1 cells transfected with GFP-fused human importin α 2 exhibit an increase in the sub-G1 population (Kim *et al*, 2000). It will be important to investigate how nuclear importin α can influence the activity of some cell cycle-related protein(s), thereby resulting in the decrease of replication-dependent histone transcripts. Alternatively, the downregulation of *euchromatic histone-lysine N-methyltransferase 2* (*EHMT2/G9a*), which was observed to be downregulated in this microarray analysis (Supplementary Table S-I), might induce cell growth defects and change the cell cycle (Tachibana *et al*, 2001), leading to the downregulation of replication-dependent histone transcripts.

Cell fate determination by nuclear importin α 2

The microarray data revealed two different features of nuclear importin α 2 relating to cell death mechanisms. One involves upregulation of *STK35* mRNA. Our data showed that the high level of *STK35* would facilitate caspase-independent cell death in response to oxidative stress (Figure 6C), but not apoptosis following STS treatment (Supplementary Figure S-IVC). The levels of *STK35* transcripts were strictly regulated in response to the stresses (Figure 6A and B). In addition, only the higher reduction of the *STK35* mRNA levels by siSTK35-2, and not by siSTK35-1, influenced cell fate outcomes in response to oxidative stress

(Figure 6D; Supplementary Figure S-IVD). Since silencing of STK35L1 affected cell cycle and cell migration in endothelial cells (Goyal *et al*, 2011), quantity control of STK35 transcripts might strongly contribute to cell viability. Another possible outcome of nuclear importin $\alpha 2$ localization is avoidance of apoptosis, through downregulation of specific apoptosis-related genes (Supplementary Table S-I; Supplementary Figure S-II), while a molecular mechanism for this pattern of gene expression remains to be elucidated.

From these findings, and based on previously reported results, we propose that modulation of gene expression by nuclear importin $\alpha 2$ directs cell fate towards a cell death pathway that bypasses apoptosis, such as necrosis upon stress exposure. Reflecting the requirement for ATP in cellular homeostasis, intracellular ATP depletion itself has been known to induce necrosis or apoptosis-independent cell death (Eguchi *et al*, 1997; Nicotera *et al*, 1998; Kumagai *et al*, 2008; Miyoshi *et al*, 2008). Such findings correlate well with the transcriptional regulation documented here. Furthermore, our data strengthen the understanding that apoptosis requires active nuclear import (Yasuhara *et al*, 1997), because nuclear migration of importin α in response to stresses blocks classical nuclear import, resulting in the inhibition of apoptosis. Our finding in this article provides a new molecular understanding of how non-apoptotic cell death is elicited by a decrease of intracellular ATP under stress. This is the first report to demonstrate that nuclear localization of importin α in response to stress bypasses apoptosis and provokes apoptosis-independent cell death through changes in both gene expression and nuclear transport.

Materials and methods

Constructs

Five full-length mouse importin α s and IBB domain-deleted (Δ IBB) mutants of importin $\alpha 2$ (51–529 a.a.) or importin $\alpha 4$ (51–521 a.a.) were constructed within the pEGFP-C1 plasmid (Clontech). The CAS-binding defective mutants (termed C-mutant or C-mut) (G469E/L470A/D471A/K472A/I473A/E474G) of importin $\alpha 2$ was generated using the QuikChange site-directed mutagenesis kit (Stratagene) with mouse importin $\alpha 2$ cDNA as a template (Herold *et al*, 1998). Synthetic oligonucleotides were as follows: 5'-ATAATG ATTGAAGAGTGTGGAGAACGGCTGCAGCTGGTGCCTACAGAGGC ATGAAAAC-3' and 5'-GTTTTCATGCTCTGTAGTCACACAGCTGCAG CCGCTTCTCCACACTCTTCAATCATTAT-3'. The reaction was performed with 1 cycle of 30 s at 95°C followed by 16 cycles of 30 s at 95°C, 1 min at 50°C, 14 min at 68°C, and the products were subcloned into pEGFP-C1. The ED mutant (D192K/E396R) of importin $\alpha 2$ was generated using the QuikChange site-directed mutagenesis kit (Toyobo) with mouse importin $\alpha 2$ cDNA as a template. Synthetic oligonucleotides were as follows: D192K, 5'-GC TCTTGGAAACATTTGCAGGTAAAGGTTTCAGCTTCCGAGAC-3' and 5'-GTCTCGGAAAGCTGAACCTTTACCTGCAATGTTCCTCAAGAGC-3'; E396R, 5'-GCGGACTTTAAGACACAGAAGCGGGCCGCTGGGCTATA ACC-3' and 5'-GGTTATAGCCACGCGGCCGCTTCTGTGTCTTAAAG TCCGC-3'. The reaction was performed with 1 cycle of 30 s at 95°C followed by 16 cycles of 30 s at 95°C, 1 min at 50°C, 16 min at 68°C. The PCR products were confirmed by sequencing and subcloned into pEGFP-C1.

Full-length human STK35S was amplified from HeLa cell cDNA using Pyrobest DNA Polymerase (Takara). Primers were 5'-CTCAGA TCTATGGAAACGGGAAGGACGCGCC-3' and 5'-ACCGTCGACTTA AGCAGCATGTGACCTGGTCC-3'. The PCR consisted of 1 cycle of 1 min at 95°C, then 30 cycles of 10 s at 98°C, 30 s at 61°C and 120 s at 72°C. The amplified product was loaded onto 1% agarose gel and a band of around 1.2 kbp was extracted. PCR was performed again using the extracted DNA as a template: 1 cycle of 1 min at 95°C,

then 35 cycles of 10 s at 98°C and 120 s at 68°C. The PCR product was cut with *Bgl*II and *Sall*, and then subcloned into pEGFP-C1 or pCAG-wtag vectors which encoded N-terminal GST and 3 $\times$ Flag fusion proteins (Takeda *et al*, 2005) at *Bam*HI and *Xho*I. The products were confirmed by sequencing.

Full-length STK35L1 was obtained using a multistep cloning strategy as previously described (Goyal *et al*, 2009). Primers were 5'-GACAGATCTATGGGCCACCAGGAGTCTCC-3' (F-1), 5'-GGGC CTCGGCCGCCACCGGATC-3' (R-520), 5'-ATGGAACGGGGAAGGAC GGCGCCGC-3' (F-400) and 5'-CAAGTCGACTTAAGCAGCACATGTG ACCTG-3' (R-1605). In the first PCR step, the 3'-end of STK35L1 (400–1605 bp) was amplified with F-400 and R-1605. In the second step, we amplified the 5'-end (520 bp) of STK35L1 gene with F-1 and R-520. In third step, the PCR product from the first step and the second step were mixed and used as a PCR template for amplification of the full-length STK35L1 gene with F-1 and R-1605. The PCR product was digested with *Bgl*II and *Sall*, and then cloned into the *Bam*HI and *Xho*I sites of the pCAG-wtag vector.

STK35 promoter sequences were generated by PCR from adult mouse testis genomic DNA (ENSMUSG00000037885). Forward primers are as follows: –2 kbp, 5'-GAGGTACCGGTTCCACTGAGCC ATTACC-3'; –1 kbp, 5'-GAGGTACCTTAACTGTGAGACATCTACG-3'; –0.5 kbp, 5'-GAGGTACCAAAATCCAAGCCAAAGCAGC-3'; –0.25 kbp, 5'-GAGGTACCCAGCATCAAGTGCCGAGTGCG-3' and Reverse primer: 5'-ATCTCGAGGGAGATTCTGTGGTGGCCATC-3'. The PCR consisted of 1 cycle of 2 min at 94°C, then 40 cycles of 15 s at 94°C and 3 min at 68°C using Platinum Pfx DNA polymerase (Invitrogen). The PCR products were digested with *Kpn*I and *Xho*I, and then cloned into the pGL3-Basic vector (Promega). All constructs were confirmed by sequencing. The pGL3-Basic/–2 kbp was digested with *Bst*XI and *Bgl*II or *Pvu*II and *Xho*I, respectively, and blunt-end sites generated by T4 DNA polymerase (New England Biolabs) were ligated to generate the pGL3-Basic/–2 kbp delta 500 bp or delta 285 bp.

Cell culture

HeLa cells and the GC-2 germ-cell line (Hofmann *et al*, 1994) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FCS) at 37°C under an atmosphere of 5% CO₂.

Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously (Yasuda *et al*, 2006). The antibodies used in this study were as follows: importin $\alpha 2$ /Rch1 (BD Biosciences, at a concentration of 1:250), RCC1 (N-19, Santa Cruz Biotechnology, 1:50) and CRM1 (BD Biosciences, 1:100). The antibody was detected either with Alexa Fluor 488-labelled goat anti-mouse IgG (Molecular Probes) for importin $\alpha 2$ and CRM1 or with Alexa Fluor 488-labelled donkey anti-goat IgG for RCC1. Images were obtained using an Axiophot fluorescent microscope (Carl Zeiss), IX71 fluorescence microscope (Olympus) or a LSM510 microscope (Carl Zeiss).

Treatment of HeLa cells with DNase I, RNase and NaCl

HeLa cells plated on coverslips were treated with UV (254 nm, 0.3 J/cm²), 200 μ M H₂O₂ for 1 h or 42°C for 1 h, washed with PBS and then permeabilized with 0.5% Triton X-100 in Buffer A (20 mM HEPES-KOH (pH 7.3), 110 mM CH₃COOK, 5 mM CH₃COONa, 2 mM (CH₃COO)₂Mg, 2 mM DTT, 1 μ g/ml each of leupeptin, pepstatin and aprotinin) for 5 min at 4°C. The cells were incubated with 0.2 mg/ml DNase I (Roche, Grade II, RNase free), 0.2 mg/ml RNase A (Roche, DNase free) or 0.1 M NaCl in Buffer A for 1 h at 37°C, and washed twice with Buffer A. After fixation with 3.7% formaldehyde in Buffer A, endogenous importin $\alpha 2$ was detected by indirect immunofluorescence.

Microarray analysis

HeLa cells (6×10^5) were grown in 100 mm dish for 24 h and then transfected with pEGFP, pEGFP-mouse importin $\alpha 2$ full-length and pEGFP-mouse importin $\alpha 2$ C-mutant constructs using Effectene Transfection Reagent (Qiagen). After 24 h, the medium was changed to DMEM containing 10% FCS and 1 mg/ml G418 and the cells were cultured for 24 h. The number of the collected cells was 4×10^5 from each transfection. Total RNA was extracted using Qiagen RNeasy micro kits (Qiagen). The RNA quality was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies). High-quality RNA samples (300 ng each) acquired from HeLa cells transfected with pEGFP

were amplified and labelled with Cyanine3 CTP, and RNAs from HeLa cells transfected with either pEGFP–importin $\alpha 2$ full-length or C-mutant constructs were amplified and labelled with Cyanine5 CTP, respectively, to produce labelled cRNA using Agilent Low RNA Fluorescent Linear Amplification Kit following the manufacturers protocol. After purification of labelled cRNAs by RNeasy mini spin columns (Qiagen), 750 ng of the Cy3-labelled control EGFP sample and 750 ng of either the Cy5-labelled EGFP–importin α full-length or C-mutant sample were mixed and incubated with an Agilent 60-mer oligo microarray slide (Whole Human Genome, Agilent Technologies) for 17 h. Slides were scanned using the Agilent DNA microarray scanner and expression data were obtained by the Agilent Feature Extraction software. Microarray results from this study have been submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) under series accession GSE25303.

Quantitative real-time PCR

HeLa cells were plated on 60 mm dishes 2 days before stress treatment. After the cells were exposed to 1 mM H₂O₂ for 1 h at 37°C and washed twice with PBS, 1 ml of Trizol (Invitrogen) was added. Total RNA was extracted following the manufacturer's protocol. One microgram of total RNA was used to perform first-strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed for *STK35* using FastStart universal SYBR Green Master (Roche) on an ABI PRISM 7900HT. Primers are shown in Supplementary Table S-III. Reactions consisted of 1 cycle of 10 min at 95°C, 40 cycles of 15 min at 98°C, 30 s at 60°C and 30 s at 72°C, followed by 15 s at 95°C, 15 s at 60°C and 15 s at 95°C.

Chromatin immunoprecipitation

A 150-mm dish of HeLa cells exposed to H₂O₂ (200 μ M, 30 min) was fixed with 1% formaldehyde for 4 min at 37°C. Cells were washed twice in ice-cold PBS and collected by scraping in 5 ml of lysis buffer 1 (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) containing protease inhibitors (1 μ g/ml each of leupeptin, pepstatin and aprotinin). Cells were collected by centrifugation and washed with 10 ml lysis buffer 1 and 10 ml lysis buffer 2 (10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) containing protease inhibitors. Cells were collected by centrifugation and the pellets suspended in 1.5 ml lysis buffer 3 (10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS) containing protease inhibitors. Each sample was sonicated for eight cycles (30 s on/90 s off, high-energy settings) using a Bioruptor UCD-250 (Cosmo Bio). The remaining insoluble material was removed by centrifugation at 20 500 g for 15 min at 4°C. The supernatant was precleared with protein A-Sepharose (Sigma-Aldrich) for either anti-importin $\alpha 2$ antibody (BD Biosciences) or anti-p53 antibody (Santa Cruz; FL-393x), or with protein G-Agarose (Sigma Aldrich) for anti-importin $\alpha 3$ (Abcam) antibody. Prior to use, the matrices were blocked with 1.6 mg/ml salmon sperm DNA (Invitrogen) and 5 mg/ml BSA (Sigma-Aldrich) in PBS for 3 h at 4°C and washed extensively with lysis buffer 3. The matrices were removed by centrifugation and the precleared chromatin was incubated overnight at 4°C with antibody-coated matrices. The matrices were incubated with 4 μ g of each anti-importin $\alpha 2$, anti-importin $\alpha 3$, anti-p53 antibody, mouse normal IgG, rabbit normal IgG or goat normal IgG in PBS containing 1.6 mg/ml salmon sperm DNA and 5 mg/ml BSA for 3 h at 4°C, and washed extensively with lysis buffer 3 before use. Immune complexes were washed five times in wash buffer (50 mM HEPES-KOH (pH 7.0), 0.5 M LiCl, 1 mM EDTA, 0.7% Na-Deoxycholate, 1% NP-40) containing protease inhibitors and were eluted from Sepharose by adding 100 μ l elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS) and heated at 65°C for 30 min twice. Reverse crosslinking was performed by overnight incubation at 65°C. Samples were diluted with an equal volume of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and then digested with 0.2 mg/ml RNase A (Sigma-Aldrich) for 1 h at 37°C and 0.2 mg/ml proteinase K for 2 h at 42°C. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and then precipitated with 0.2 M NaCl and two volumes of ethanol. The pellets were collected by centrifugation and suspended in 20 μ l H₂O. Approximately 4% of the input chromatin sample and 20% of the ChIP sample were used as a template for PCR with the recombinant Taq polymerase

(Takara) and the following primers: *STK35* forward primer 5'-GGTG TTCTGCCAGTGATTG-3' and reverse primer 5'-GCCAGCTGAGCCC TTTACAT-3'. As a negative control, *GAPDH* flanking region was used by the following primers: forward 5'-ATGGTTGCCACTGGGGA TCT-3' and reverse 5'-TGCCAAAGCCTAGGGGAAGA-3'. *p21* promoter forward and reverse primers were as follows: 5'-CTGGACTGGGC ACTCTTGTC-3' and 5'-CTCTACCATCCCCCTTCCTC-3'. Reaction mixtures were initially melted at 95°C for 5 min followed by 33 cycles for anti-importin $\alpha 2$ antibody or 30 cycles for anti-importin $\alpha 3$ antibody of 95°C for 20 s, 55°C for 20 s, 72°C for 25 s and a final extension of 72°C for 30 s. Amplicons were visualized with ethidium bromide in 2% agarose gels.

Luciferase assay

GC-2 cells (1.0×10^6) were transiently transfected with each pGL3-Basic plasmid described in *Constructs* together with the pRL-TK plasmid (Promega) by Amaxa Nucleofector II device (Cell Line kit V (VCA-1003), Program T-020, Lonza), except for the ED mutant in Figure 4C. Either pEGFP or pEGFP–importin $\alpha 2$ ED construct was transfected with the pGL3-Basic/–2 kbp and the pRL-TK plasmids into GC-2 cells (6×10^5) by Neon transfection system (Resuspension Buffer R, Electrolytic Buffer E2, Pulse voltage 1400, Pulse width 30, Pulse no. 1, Invitrogen).

After the transfections, cells were then placed in DMEM with 10% FCS and were transferred into 24-well plates at 1×10^5 /well each and incubated for times described in the figure legends.

The luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega). Transfected cells were washed with PBS once and incubated for 20 min at RT with 100 μ l of Passive Lysis Buffer. Twenty-microlitre aliquots of cell extracts were transferred into 96-well white plate (BD Falcon) and luminescence was measured using a FLUOstar OPTIMA (BMG Labtech). The following steps were used for luminescence measurements: 60 μ l of the firefly luciferase reagent (LARII) was added to the test sample, and luminescence measured with a 10-s interval time, followed by addition of 60 μ l of the Renilla luciferase reagent and firefly quenching (Stop & Glo), and measurement of luminescence with a 10-s interval time. The data are represented as the ratio of firefly to Renilla luciferase activity (Fluc/RLuc).

Intracellular ATP measurement

HeLa cells were exposed either to 5 mM H₂O₂ or to 0.5 μ M STS (Biomol) at the indicated times as described in Figure 5A. After incubation, the intracellular ATP levels were measured using the CellTiter-Glo assay kit (Promega).

Detection of cleaved PARP

HeLa cells were plated at 2×10^5 in a 60-mm dish. After 2 days, the cells were incubated for 30 min in the presence or absence of 50 μ M zVAD-fmk (Peptide Institute, Inc.) and exposed to 5 mM H₂O₂ or 0.5 μ M STS for 4 h. Cells were harvested by centrifugation and the pellets were resuspended in lysis buffer (20 mM HEPES-KOH (pH 7.3), 150 mM NaCl, 0.5% NP-40, 1 μ g/ml each of leupeptin, pepstatin and aprotinin). The resuspended cell pellets were sonicated for 20 s and centrifuged at 20 400 g for 15 min. After determination of the supernatant protein concentration using the BCA assay (Pierce), equal cell extracts were subjected to western blotting using antibodies specific for either PARP (BD Transduction) or GAPDH (Ambion). Proteins were visualized by chemiluminescence (ECL kit, Amersham Pharmacia Biotech).

293F stable cell lines

pCAG-wtag, pCAG-wtag-STK35S or pCAG-wtag-STK35L1 was cut by *AhdI* and transfected into 293F cells by Effectene Transfection Reagent (Qiagen). The cells were selected in medium containing 0.8 mg/ml G418 (Nacalai Tesque, Inc.), after which single cell clones were isolated. The expressed proteins were ascertained by western blotting using anti-GST antibody (Santa Cruz).

Cell viability assay

The 293F stable cells were seeded at 2×10^5 in 60 mm dishes. After 2 days, cells were incubated for 30 min in the presence or absence of 50 μ M zVAD-fmk and exposed to 2 mM H₂O₂ for 2 h. The cells were stained with PI (Beckman coulter). Briefly, cells were harvested according to the manufacturer's protocol, washed with PBS, and suspended in binding buffer (Annexin V-FITC kit; Beckman coulter). PI was added to individual samples and incubated for

10 min on ice. Cells were measured using flow cytometric analysis (FACSsort; Becton Dickinson). Percentages of PI-positive cells were analysed with WinMDI software.

RNA interference

HeLa cells were transfected with synthesized siRNAs. The siRNA sequences were as follows: Luciferase, 5'-UCAGAGAGAUCCUCAUA AATT-3' (siLUC.); STK35, 5'-UCAAGCAGCUCUUGAAAGATT-3' (siSTK35-1) and 5'-CGAGCGCCAUUGCCUCCUTT-3' (siSTK35-2). Transfection with each siRNA was performed using RNAi MAX (Invitrogen) in accordance with the manufacturer's instructions. The transfected cells were harvested 48 h after transfection and used for experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: YY and YM designed and performed the experiments and wrote the manuscript. TY, MA, AM and CW carried out parts of the experiments and analysed the data. KLL wrote the manuscript with discussion from the co-authors. YY supervised the project, designed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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