Hydrogen peroxide stimulates nuclear import of the POU homeodomain protein Oct-1 and its repressive effect on the expression of Cdx-2

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Abstract

Background: The ubiquitously expressed POU homeodomain protein Oct-1 serves as a sensor for stress induced by irradiation. We found recently that in pancreatic and intestinal endocrine cells, Oct-1 also functions as a sensor for cyclic AMP (cAMP). The caudal homeobox gene Cdx-2 is a transactivator of proglucagon (gcg) and pro-insulin genes. Oct-1 binds to Cdx-2 promoter and represses its expression. cAMP elevation leads to increased nuclear exclusion of Oct-1, associated with reduced recruitment of nuclear co-repressors to the Cdx-2 promoter and increased Cdx-2 expression.

Results: We show in this study that inducing oxidative stress by hydrogen peroxide (H₂O₂) increased nuclear Oct-1 content in both pancreatic α and β cell lines, as well as in a battery of other cells. This increase was then attributed to accelerated nuclear import of Oct-1, assessed by Fluorescence Recovery After Photobleaching (FRAP) using green fluorescence protein (EGFP) tagged Oct-1 molecule. H₂O₂ treatment was then shown to stimulate the activities of DNA-dependent protein kinase (DNA-PK) and c-jun N-terminal kinase (JNK). Finally, increased Oct-1 nuclear content upon H₂O₂ treatment in a pancreatic α cell line was associated with reduced Cdx-2 and gcg mRNA expression.

Conclusion: These observations suggest that Oct-1 functions as a sensor for both metabolic and stress/survival signaling pathways via altering its nuclear-cytoplasmic shuttling.

Background

A transcription factor may serve as a sensor for different signaling pathways via altering gene expression profiles. For example, members of Foxo protein family were shown to mediate stress signaling via promoting its nuclear translocation and Foxo pathway downstream target gene expression, while insulin and insulin-like growth factor-1 (IGF-1) can block this pathway via stimulating Foxo protein phosphorylation at certain Ser/Thr residues, followed by its nuclear exclusion and degradation [1,2].

Oct-1 is a member of the POU domain transcription factor [3,4]. The protein in this family typically contains a bipartite DNA binding domain, in which two sub-domains are covalently connected by a flexible linker. These two sub-domains normally recognize DNA through major groove interaction on the opposite sides of the helix. The classical recognition sequence is known as the octamer motif “ATGCWWAT”, where W can be either “A” or “T”. This ubiquitously expressed transcription factor exerts multiple biological functions via up- or down-regulating the expression of a large profile of target genes in different cell lineages [5-8]. Recent studies indicated that Oct-1 functions as a sensor for radiation mediated stress via enhanced phosphorylation at multiple Ser/Thr sites in the N terminus of the molecule by DNA-dependent protein kinase (DNA-PK) [9-11]. Lately, we reported that Oct-1 binds to the promoter region of Cdx-2, a homeobox gene expressed in pancreatic islets and intestinal endocrine L cells, via the typical ATGCWTAAT motif. We observed that nuclear content of Oct-1 can be reduced in response to cyclic AMP (cAMP) elevation in pancreatic and intestinal endocrine cells, and this reduction is associated with increased expression of Cdx-2 and its downstream target gene, the proglucagon gene (gcg). Furthermore, cAMP
elevation reduced the binding of Oct-1 to Cdx-2 promoter and the recruitment of nuclear co-repressors, including silencing mediator of retinoid and thyroid hormone receptors (SMRT) and histone deacetylase 1 (HDAC1)[12]. These observations suggest that Oct-1 functions as a transcriptional repressor for a set of target genes, while cAMP elevation in response to the stimulation by peptide hormones leads to the release of the repressive effect.

In this study, we assessed the effect of hydrogen peroxide (H$_2$O$_2$) on Oct-1 cytoplasmic-nucleus shuttling. H$_2$O$_2$ treatment in pancreatic glucagon and insulin producing cell lines, as well as a battery of other cell lines and primary smooth muscle cells, was shown to increase nuclear Oct-1 content and Oct-1 nuclear translocation. In the Cdx-2 and Gcg expressing pancreatic islet $\alpha$ cell line, this was associated with increased c-jun N-terminal kinase (JNK) activation and DNA-PK activity, and decreased Cdx-2 and gcg mRNA expression. We suggest that Oct-1 exerts an important role in metabolic homeostasis by functioning as a sensor not only for cAMP, but also for oxidative stress.

**Results**

**H$_2$O$_2$ treatment increases nuclear Oct-1 levels**

Given that oxidative stress in pancreatic islet cells is a major contributor of islet cell damage and subsequent diabetic hyperglycemia, and that Oct-1 is a known sensor for radiation mediated and other types of stress, we assessed whether oxidative stress affects Oct-1 subcellular distribution by Western blotting. The InR1-G9 cell line was treated with 100 or 500 µM H$_2$O$_2$ for 2 or 4 h, and Oct-1 contents in whole cell lysates, as well as in nuclear and cytoplasmic fractions were examined. As shown in Figure 1A, although Oct-1 levels in whole cell lysates were not notably altered by H$_2$O$_2$ treatment, the content of Oct-1 in nuclei was substantially increased. For cells exposed to 100 µM H$_2$O$_2$ for 2 h, cytosolic Oct-1 expression was almost undetectable. However, when the exposure time was extended to 4 h, cytosolic Oct-1 expression was restored (Figure 1A). We have also investigated whether oxidative stress would affect Oct-1 nuclear content in other cell lineages. Indeed, H$_2$O$_2$ treatment enhanced nuclear Oct-1 contents in the intestinal Gcg-expressing GLUTag cell line [13], the pancreatic insulin-expressing Ins-1 cell line, two non-endocrine cell lines, COS-7 and Caco-2 (Figure 1B), as well as primary rat smooth muscle cells (Figure 1C).

**H$_2$O$_2$ treatment induces Oct-1 shuttling from cytoplasm into nuclei**

To assess the effect of H$_2$O$_2$/oxidative stress and further examine the effect of cAMP elevation on Oct-1 cytoplasmic-nuclear shuttling, we generated a fusion protein of Oct-1 and enhanced green fluorescence protein (EGFP) (Oct-1-EGFP). The empty EGFP-C3 vector or Oct-1-EGFP construct were transiently transfected into

![Figure 1](http://www.biomedcentral.com/1471-2121/11/56)
the InR1-G9 cell line. Twenty-four hrs after the transfection, cells were treated with the cAMP promoting agents Forsklin/IBMX (F/I) for 2 h. The expression and sub-cellular compartmentalization of the EGFP tag was visualized using confocal microscopy. A representation result with EGFP empty vector transfection by vehicle and F/I treatment was shown as additional file 1. It appears that EGFP mainly remains in the cytoplasm, regardless of the presence of F/I (Additional File 1A). However, Oct-1-EGFP showed two distinct patterns with regard to fluorescence distribution (Additional File 1B). For Pattern I, fluorescence was observed in a limited cell compartment, which represents Oct-1-EGFP nuclear localization. For Pattern II (after F/I treatment), fluorescence was mainly observed in the cytoplasm, indicating that most of Oct-1 molecules are outside of the nuclei. Additional file 2 top panel shows representative images with Oct-1-EGFP transfected InR1-G9 cells that were treated with F/I, 8-bromo-cAMP and Epac pathway specific cAMP analogue 8-pMeOPT-2′-O-MecAMP (Epac). Without a treatment, we observed approximately 58% of Pattern I and 42% of Pattern II of Oct-1 distribution. Following an above treatment for 2 h, significant pattern change was observed. The Pattern I decreased to 17-22% while Pattern II increased to 78-83% (Additional File 2 bottom panel), indicating a stimulated Oct-1 EGFP nuclear exclusion in response to cAMP elevation or Epac activation, which is consistent with our previous observations [12].

We then examined whether H2O2 treatment affects sub-cellular distributions of Oct-1-EGFP. Oct-1-EGFP was transiently transfected into the InR1-G9 cell line for 24 h, followed by H2O2 treatment. We found that the treatment of InR1-G9 cells with 100 μM H2O2 for 0.5, 1, and 2 h led to increased percentages of pattern I cells. Figure 2A (left panel) shows our representative results after a 1 h H2O2 treatment, cells with nuclear Oct-1-EGFP expression (Pattern I) being sharply increased from 58% to 94% (right panel). We therefore suggest that H2O2 treatment significantly stimulates nuclear import of Oct-1, which is opposite to the effect induced by cAMP elevation. We then transfected InR1-G9 cells with myc-tagged Oct-1. 24 h after the transfection, cells were treated with vehicle or 100 μM H2O2 for 1 h before immunofluorescence staining, assessing the distribution of myc-tagged Oct-1. Representative images in Figure 2B indicate that H2O2 treatment increased nuclear myc-Oct-1 immunofluorescence signal.

H2O2 treatment enhances Oct-1 recovery into nuclei
Recent studies have indicated, with irradiation, DNA-PK activation could lead to enhanced phosphorylation of Oct-1 at its selected Ser/Thr residues [9]. This phosphorylation event may facilitate the binding of Oct-1 to its target gene promoters [9,14,15]. To investigate whether increased percentages of pattern I cells after H2O2 treatment is a result of increased Oct-1-EGFP nuclear import, we utilized Fluorescence Recovery After Photobleaching (FRAP). For FRAP analysis, the fluorescence molecule in the nuclei is bleached by a laser beam. By recording the time (normally in the ones to tens of seconds range) taken to reach 50% intensity when the fluorescence reaches a new plateau (or termed “50% recovery time”) for the same bleached area, one can measure the capability of a molecule to move around over time. The “recovery rate” refers to as the percentage of the recovery observed from total cells bleached. For this study we transfected Oct-1-EGFP into the InR1-G9 cell line. Twenty-four h after the transfection, cells were treated with vehicle or H2O2. The treated cells were then subjected to the laser photobleaching. The 50% recovery time was recorded as an indicator of Oct-1-EGFP to move from cytoplasm to nuclear. The effect of H2O2 treatment on Oct-1-EGFP recovery time and the recovery rate by FRAP are shown in Figure. 3A and 3B. As summarized, for cells treated with vehicle, the 50% recovery time was about 10.12 seconds while the recovery rate is only about 33% (4 out of 12). However, for cells received H2O2 treatment, 50% recovery time was only 3.5 seconds and the recovery rate reached 79% (11 out of 14). These results clearly indicate that H2O2 treatment stimulates the import of Oct-1 from the cytoplasm into the nuclei, and further suggest that Oct-1 serve as a sensor for oxidative stress.

H2O2 treatment activates DNA-PK and JNK
Stress induced by irradiation was shown to cause DNA double strand break (DSB) and the activation of DNA-PK, followed by the recruitment of Ku protein complex to the DNA break ends. Zeocine, an antibiotic, was also shown to cause DSB. We then assessed whether oxidative stress induced by H2O2 treatment leads to increased DNA-PK activity. For this purpose, we have firstly verified that treating InR1-G9 cells with Zeocine, similar to H2O2 treatment, led to Oct-1 nuclear accumulation (Figure. 4A). This result allowed us to utilize Zeocine as a positive control to assess DNA-PK activity. As shown in Figure. 4B, DNA-PK activity in H2O2 treated InR1-G9 cells reached to a similar level as that in cells treated with Zeocine. We therefore conclude that in the pancreatic islet cell line InR1-G9, H2O2 treatment leads to a moderate but significant DNA-PK activation, associated with enhanced Oct-1 nuclear import.

Mitogens, G-protein coupled receptors, and stress were all shown to use complex mitogen activated protein kinase (MAPK) signaling cascades to exert their regulatory functions. Indeed, both cAMP and H2O2 have been demonstrated to activate MAPK signaling
**Figure 2** H$_2$O$_2$ treatment leads to increased nuclear Oct-1 content. (A) Left panel, InR1-G9 cells were transfected with Oct-1-EGFP and treated with vehicle, or H$_2$O$_2$ (100 μM) for 1 h before confocal microscopy examination. The right panel shows the counting results. (B) InR1-G9 cells were transfected with myc-Oct-1 for 24 h, followed by vehicle or H$_2$O$_2$ (100 μM) treatment for 1 h. Immunofluorescence staining were conducted, Blue, nuclei, red, myc-Oct-1. Arrow indicates enhanced nuclear myc-Oct-1 or Oct-1-EGFP expression, while triangle indicates mixed expression in both nuclei and cytosol.

**Figure 3** H$_2$O$_2$ stimulates OCT-1-EGFP nuclear import. (A) InR1-G9 cells were transfected with Oct-1-EGFP, and treated with vehicle (control), or H$_2$O$_2$ (100 μM). After photo bleaching of the nuclear fluorescence, the time was recorded for the area to recover to the 50% intensity. (B) Comparison of recovery time and recovery rate between vehicle and H$_2$O$_2$ treated cells.
pathways [16-18]. Among the MAPKs, JNK is a known effector of stress induced by genotoxic agents [19]. We have learned that cAMP elevation in InR1-G9 cells led to increased ERK activation, associated with reduced nuclear Oct-1 content [12]. To initiate the examination why cAMP elevation and H2O2 treatment trigger Oct-1 sub-cellular localization in opposite directions, we examined the effects of these two treatments on ERK and JNK activation in the InR1-G9 cell line. We found that although cAMP elevation induced by F/I treatment, stimulated ERK phosphorylation [12,20], the treatment did not affect JNK phosphorylation (Figure, 4C). H2O2 treatment, however, generated a stimulatory effect on JNK phosphorylation (Figure, 4D), consistent with our knowledge that JNK signaling is among the effectors of oxidative stress [19,21,22]. The treatment, however, did not affect ERK phosphorylation (Figure, 4D). These observations would further suggest that cAMP signaling and oxidative stress utilize different MAPK in exerting their effects on Oct-1 nuclear-cytoplasmic shuttling, and therefore generate different effects on hormone gene expression.

Oxidative stress represses Cdx-2 and gcg expression in the InR1-G9 cells

Since Oct-1 serves as a transcriptional repressor of Cdx-2, expressed in pancreatic and intestinal endocrine cells, we wonder whether increased nuclear content of Oct-1 in response to oxidative stress reduces Cdx-2 expression in such an endocrine cell line. While H2O2 treatment increased nuclear Oct-1 content in the InR1-G9 cell line (Figure 5A, B), the inhibitory effect on Cdx-2 protein expression, however, was not notable until 6 h (Figure, 5A). This delay could be due to increased stability of
Cdx-2 protein in response to a stress [23,24]. We therefore directly assessed the effect of H2O2 treatment on Cdx-2 mRNA expression. As shown in Figure 5C, H2O2 treatment resulted in about 40% reduction in Cdx-2 mRNA level over the entire 6 h experimental period, confirming that oxidative stress represses Cdx-2 mRNA expression. Additionally, the expression of Gcg mRNA, which is a known downstream target of Cdx-2 in pancreatic α cells [25], is also reduced by H2O2 treatment, to approximately 50% level (Figure 5D).

**Discussion**

An early study by our group indicated that Oct-1 co-transfection stimulated Cdx-2 promoter expression in the pancreatic and intestinal gcg producing cell lines [26]. More recent investigations suggested that Oct-1 could function as a transcriptional repressor [8]. We noticed that cAMP promoting agents, forskolin and IBMX, reduced nuclear Oct-1 content but stimulated Cdx-2 expression. Our further investigations revealed that Oct-1 is able to recruit nuclear co-repressors to Cdx-2 promoter and represses its transcription, while the activation of cAMP-Epac signaling increases Oct-1 nuclear-cytoplasmic shuttling [12]. Thus, Oct-1 serves as a repressor of Cdx-2 and its downstream target Gcg in pancreatic and intestinal endocrine cells [12]. As an abundantly expressed homeobox gene in gut, Cdx-2 plays a critical role in intestinal cell differentiation [23,24,27]. We have shown that it is expressed in intestinal endocrine L cells and stimulates Gcg transcription [25].

It has been shown that Oct1−/− cells are more hypersensitive to stress-inducing agents or treatment, such as ionizing radiation [15]. In the present study, we have assessed the effect of H2O2 on cytoplasmic-nuclear shuttling of Oct-1. We show in the current study that H2O2 treatment led to increased Oct-1 nuclear localization by Western blotting, following sub-cellular fractionation; and confocal and fluorescence microscopy, following exogenous expression of EGFP or myc- tagged Oct-1. Increased nuclear shuttling was then directly assessed by FRAP. We found that H2O2 treatment stimulated the activity of DNA-PK; and that opposite to cAMP elevation, H2O2 stimulated JNK activity but not ERK activity. Finally, we show that increased nuclear Oct-1 content upon H2O2 treatment led to approximately 40-50% reduction of Cdx-2 and gcg mRNA expression, which was consistent with our notion that Oct-1 represses Cdx-2 expression. Since DNA-PK activity is responsible for Oct-1 phosphorylation upon irradiation [9], we speculate that increased Oct-1 nuclear shuttling in response to H2O2...
treatment is a result of its phosphorylation by DNA-PK [9]. This, nevertheless, needs to be further investigated.

Intensive investigations have shown that Oct-1 up- or down-regulates the expression of a large profile of target genes in different cell lineages and that this ubiquitously expressed transcription factor is involved in different categories of cellular and molecular activities, from transcriptional regulation to embryonic development [8,28-30]. The role of Oct-1 in mediating metabolic as well as stress/survival signaling pathways, however, was recognized only recently [8,14,15,31]. In addition to the repression of Cdx-2 expression, we have also shown that Oct-1 can repress the expression of the transcription factor carbohydrate responsive element binding protein (ChREBP) [32], which is important in facilitating liver lipogenesis [33]. Insulin, however, stimulated ChREBP transcription. More importantly, the stimulatory effect of insulin was at least partially mediated by attenuating the repressive effect of Oct-1 [32]. These observations collectively suggest that Oct-1 serves as a sensor for metabolic signaling pathways. It represses the expression of important master control genes, such as Cdx-2 in pancreatic islets and ChREBP in hepatocytes. Following the stimulation by a peptide hormone, such as insulin or those that utilize cAMP as the second messenger, Oct-1 is phosphorylated at certain Ser/Thr residues and excluded from the nucleus. This may represent a novel mechanism for peptide hormones in regulating gene expression.

Oct-1 is also known to act as a sensor for stress. Oct-1 deficiency in mice (Oct-1−/−) is embryonically lethal [29]. Utilizing microarray expression profiling, Tantin et al. found that in Oct-1−/− fibroblasts a large profile of genes associated with cellular stress exhibited altered expression pattern [15]. Furthermore, Tantin et al. [15] and Schild-Poulter et al. [34] have shown that in radiation induced stress, Oct-1 could be phosphorylated by DNA-PK at 13 potential Ser/Thr residues within the N terminus of the Oct-1 molecule. Consistent with this finding, Oct-1−/− fibroblasts are hypersensitive to γ irradiation, doxorubicin and H2O2 treatment, and contained elevated level of reactive oxygen species (ROS). Very recently, Kang et al. have demonstrated that Oct-1 is dynamically modulated by phosphorylation in vivo following the response to genotoxic and oxidative stress [14]. The stress induced phosphorylated Oct-1 has a higher affinity for DNA binding. Additionally, the interaction between Oct-1 and a distinct group of target promoters is inducible by oxidative stress and these target promoters frequently contain conserved octamer binding sites [14]. We present here that in a battery of cell lines and primary cells, nuclear Oct-1 content is elevated after H2O2 treatment. We suggest this is due to the result of Oct-1 shuttling from cytoplasm to nuclei, following the phosphorylation by DNA-PK.

Although the activation on DNA-PK by H2O2 is moderate, the stimulation was comparable with that of Zeocine treatment. Interestingly, Lebrun et al. have reported that DNA-PK could phosphorylate another pancreatic islet homeodomain protein PDX-1, and the phosphorylation accelerated PDX-1 proteasome degradation [35]. Therefore, DNA-PK activation in response to oxidative stress may affect pancreatic islet hormone-gene expression through affecting both homeodomain protein expression and degradation. Furthermore, we observed increased JNK phosphorylation in the InR1-G9 cells in response to H2O2 treatment. Whether this activation is related to Oct-1 nuclear-cytoplasmic shuttling deserves a further examination.

Our finding that cAMP elevation stimulates Oct-1 nuclear exclusion and that H2O2 treatment leads to increased nuclear Oct-1 content place Oct-1 in the centre of signaling cascades that are involved in response to both oxidative stress and hormones/neurotransmitters that utilize cAMP as the second messenger. For this matter, Oct-1 should not be simply considered as a repressor for a cluster of genes. Instead, it is a sensor for both metabolic and stress/survival signaling pathways. Indeed, a recent study shows that Oct-1 mediates the effect of oxidized LDL (oxLDL) in repressing the expression of vascular cytochrome P450 (CYP) monooxygenases [8]. In the coronary arterial endothelial cells, knockdown of Oct-1 expression prevented oxLDL-mediated silencing of CYP expression [8]. Therefore, Oct-1 activation in response to oxidative stress is among the pathological entity in metabolic dysfunction [8], and attenuating the function of Oct-1 improves the dysfunction.

Conclusion
Based on data presented in this study and elsewhere [8-12], we conclude that the ubiquitously expressed Oct-1 functions to control gene expression in response to cAMP elevation and oxidative stress via a similar nuclear-cytoplasmic shuttling system, which confines Oct-1 to either the nucleus or the cytoplasm.

Methods
Reagents, plasmids, cell cultures, and DNA transfection
Forskolin, 3-Isobutyl-1-methylxanthine (IBMX) and Hydrogen peroxide (H2O2) were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Epac pathway specific cAMP analogue 8-pMeOPT-2’-O-Me-cAMP (ESAC) was provided by BIOLOG Life Sciences Institute (Bremen, Germany). The antibiotics Zeocin was purchased from Invitrogen (Invitrogen Life Technology, Burlington, Ontario, Canada). The plasmid construct Oct-1-EGFP was generated by inserting a copy of human Oct-1 coding sequence [36] into the EGFP-C3
expression vector (Invitrogen Life Technology, Burlington, Ontario, Canada). Hamster pancreatic Inr1-G9, mouse large intestinal GLUTag, small intestinal STC-1, Monkey Kidney Fibroblast cell line Cos-7 and the human colon cancer Caco-2 cell lines were maintained as previously described [25,26,37]. The rat pancreatic β cell line Ins-1 and the rat primary islet cell cultures were grown in RPMI medium with 10% fetal bovine serum. The primary smooth muscle cells were isolated from rat aortas and cultured as previously described [38].

Real time RT-PCR
Complementary DNAs (cDNAs) were generated using a Superscript First-strand RT-PCR kit (Invitrogen Life Technology). Real time RT-PCR was conducted using the QuantiTect SYBR green PCR kit from Qiagen (Mississauga, Ontario, Canada). DNA sequences of the primers used for quantitatively assessing mRNA expression by real time RT-PCR are as follows: For hamster Cdx-2: Forward, 5’-CCTAGACAAGGACGTGAGCA-3’; Reverse, 5’-CCTAGACAGGACGTGAGCA-3’. For hamster gcg: Forward, 5’-AGAAGAAGTCGCCATTGCTG-3’; Reverse, 5’-CGCAGAGATGTTGTCAAGA-3’

Nuclear and cytosolic protein extraction
The nuclear and cytosolic proteins were extracted based on the method by Schreiber et al [39]. Briefly, approximately 1 × 10⁶ cells collected were washed with phosphate buffered saline (PBS) and pelleted by centrifugation (1500 g for 5 min). The pellet was then re-suspended in 500 μl cold buffer A [10 mM HEPES (pH 8.0), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF] and incubated on ice for 15 min. After the addition of 25 μl of 10% Nonident NP-40, the cells were vigorously vortexed for 10 sec. Following a centrifugation for 30 sec, the supernatant was collected and treated as the cytoplasmic fraction. The nuclear pellet was then resuspended in 60 μl ice-cold buffer C [20 mM HEPES (pH 8.0), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF] and the tube was vigorously rocked at 4°C for 15 min. Nuclear proteins were then collected by a 5 min centrifugation at 4°C.

Western blotting
The Cdx-2 antibody was generated as previously described [40]. Antibodies against Oct-1 (sc-8024), actin, ERK (sc-94), phosphorylated ERK (sc-7383), JNK (sc-571) phosphorylated JNK (sc-6254), PCNA, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Preparation of whole cell lysates, and Western blotting were carried out as described previously.

Confocal microscopy and FRAP analysis
For Confocal microscopy, Inr1-G9 cells were transfected with EGFP-C3 or Oct-1-EGFP construct. The cells were treated with Forskolin/IBMX (10 μM each), or 8-bromo-cAMP (100 μM), or the Epac pathway specific cAMP analogue 8pMeOPT-2’-O-ME-cAMP (ESCA, 20 μM), or H2O2 (100 μM) for the indicated time before visualizing the fluorescence on Zeiss LSM 510 for image capturing. For FRAP analysis, the imaging was carried out on Olympus FV1000 confocal (Olympus, USA). The Inr1-G9 cells were grown on the Lab-Tek II chamber plate (Nunc, NY, USA) and transfected with Oct-1-EGFP construct. After 24 hours, the transfected cells were treated with vehicle or 100 μM H2O2 immediately before imaging. The nuclear region was located and 405 nm diode laser was used for photobleaching. The time for 50% recovery of fluorescence intensity refers to as “50% recovery time”, which is the half time between T0 (the moment at bleach) and T1 (when the fluorescence reaches a new plateau, which is normally lower than the original intensity). The “recovery rate” refers to as the percentage of the recovery observed from total number of cells bleached.

DNA dependent protein kinase assay
The DNA PK assay was performed by using an assay kit from Promega (Promega Corporation, WI), according to manufacturer’s instruction. Briefly, Approximately 5 ×10⁶ Inr1-G9 cells treated with either vehicle, Zeocin (100 μg/ml), or H2O2 (100 μM) were harvested and nuclear extract was prepared. The endogenous DNA from the nuclear extract was removed by DEAE Sepharose column. The DNA PK activity from the nuclear extract was subsequently measured with γ-ATP as a probe, as per manufacturer’s instructions.

Additional material

Abbreviations
DNA-PK: DNA-dependent protein kinase; EGFP: enhanced green fluorescent protein; Epac: exchange protein directly activated by cAMP; IBMX: 3-isobutyl-1-methylxanthine; JNK: c-jun N-terminal kinase; MEK: mitogen-activated protein kinase; OCT: octamer-binding site; Oct-1: octamer transcription factor-1; RT: reverse transcription; H2O2: hydrogen peroxide.

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Authors’ contributions
PW did all the bench work and data analysis. TJ took overall responsibility for the project, and writing up the article. All authors read and approved the final version of the manuscript.

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