RNAi

Double-stranded RNAs (dsRNAs) were made using *gld-2* cDNAs (pJK830, exons 2–8 or pJK831, exons 16–18) as templates. Young adults were either injected with 2 μg μl^{-1} *gld-2* dsRNA or soaked in 10 μl of 2 μg μl^{-1} *gld-2* dsRNA for 12 h at 20 °C or mock-treated by injection with M9 buffer. Embryos were collected at defined intervals after treatment and processed together.

Poly(A) polymerase assay

Proteins were *in vitro* translated using the TNT coupled transcription–translation system (Promega), and assayed using buffer conditions essentially as described[26]. For scintillation counting, poly(A) (Roche) was used as substrate. For gel assays, we used RNA oligo, $C_{35}A_{10}$ (Dharmacon), a 45-nucleotide and supplemental 1 mM $MgCl_2$. Products were analysed on 12% sequencing gels.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress

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Reactive oxygen species are required for cell proliferation but can also induce apoptosis[1]. In proliferating cells this paradox is solved by the activation of protein kinase B (PKB; also known as c-Akt), which protects cells from apoptosis[2]. By contrast, it is unknown how quiescent cells that lack PKB activity are protected against cell death induced by reactive oxygen species. Here we show that the PKB-regulated Forkhead transcription factor FOXO3a (also known as FKHR-L1) protects quiescent cells from oxidative stress by directly increasing their quantities of manganese superoxide dismutase (MnSOD) messenger RNA and protein. This increase in protection from reactive oxygen species antagonizes apoptosis caused by glucose deprivation. In quiescent cells that lack the protective mechanism of PKB-mediated signalling, an alternative mechanism is induced as a consequence of PKB inactivity. This mechanism entails the activation of Forkhead transcription factors, the transcriptional activation of MnSOD and the subsequent reduction of reactive oxygen species. Increased resistance to oxidative stress is associated with longevity. The model of Forkhead involvement in regulating longevity stems from genetic analysis in *Caenorhabditis elegans*[3–6], and we conclude that this model also extends to mammalian systems.

Reactive oxygen species (ROS) are a primary cause of cellular damage that leads to cell death[1]. In proliferating cells, protection from cell death is mediated by activity of the phosphatidylinositol-3-OH kinase (PI(3)K)–PKB signalling pathway, which is dependent on the presence of glucose[2]. In the absence of PI(3)K–PKB signalling, the FOXO subfamily of Forkhead transcription factors, con-

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sisting of FOXO4 (also known as AFX), FOXO1 (FKHR) and FOXO3a (FKHR-L1), is activated[7–9]. In most cell types, this leads to cell-cycle arrest and quiescence but not apoptosis, despite the absence of PKB activity[10,11]. Similarly, in *C. elegans* an absence of PKB signalling activates the FOXO homologue DAF-16 and causes dauer formation—a complex phenotype characterized by increased resistance to oxidative stress[3–6].

We have established an inducible FOXO3a cell line through stable transfection of a conditionally active HA–FOXO3a–A3–ER fusion into DLD-1 human colon carcinoma cells[11]. The HA–FOXO3a–A3–ER fusion protein is constitutively expressed in quantities comparable to the endogenous protein in clone DL23 (Supplementary Information Fig. 1), but remains inhibited unless presented with a modified ligand for the estrogen receptor (ER), 4-hydroxy-tamoxifen (4-OHT)[12]. Treatment of the DL23 cells with 4-OHT for 24 h resulted in the specific activation of FOXO3a, a strong increase in the amounts of $p27^{kip1}$ protein and a decrease in cell proliferation (Supplementary Information Fig. 1), and forced these cells into a state of quiescence[11]. Notably, 4-OHT had no effect on the control DLD-1 cells (Supplementary Information Fig. 1).

To measure the effect of Forkhead activity on cellular ROS, we loaded the DL23 cell line, either untreated or treated with 4-OHT for 16 h, with the lipophilic C11-$BODIPY^{581/591}$ probe, which can be used to quantify lipid oxidation in single living cells[13]. The initial rate of oxidation was 4 times lower, and the total oxidized fraction of the probe after a 50-min treatment with hydrogen peroxide (H_2O_2) was 2–3 times lower, in cells containing active Forkhead than in control cells (Fig, 1a, b). It should be noted that activation of PKB can also protect cells against various stresses, and that H_2O_2 treatment has been shown to induce PKB activation[14]. In DLD-1 and DL23 cells, however, we observed no PKB activation by H_2O_2 at concentrations used to induce radical formation (200 μM; Supplementary Information Fig. 1). Because treatment of the DLD-1 control cell-line with 4-OHT had no effect, these results show that the specific activation of Forkheads increases cellular protection against ROS.

We examined whether Forkheads increase cellular antioxidant capacity through the regulation of antioxidant enzymes, notably SODs. Treating the DL23 cell line with 4-OHT for up to 24 h showed a gradual increase in the amount of the mitochondrial MnSOD protein but no change in protein quantities of the cytoplasmic copper/zinc SOD (CuZnSOD; Fig. 1c). The increase in MnSOD expression was not a secondary event caused by $p27^{kip1}$-induced cell-cycle arrest, because wild-type and $p27^{kip1-/-}$ mouse embryo fibroblasts (MEFs) showed a similar increase in MnSOD protein on infection with a retrovirus expressing FOXO3a–A3 (Fig. 1d). Similar effects were seen when we used a retrovirus expressing HA–FOXO4.

We investigated whether the regulation of MnSOD protein expression is under the control of endogenous PI(3)K–PKB–Forkhead signalling. Serum deprivation of 3T3-L6 cells increased the amounts of MnSOD protein (Fig. 1e), and subsequent activation of PI(3)K–PKB and inactivation of FOXO3a (indicated by phosphorylation of Thr 32) by the addition of insulin (Fig. 1e) reduced the amounts of MnSOD (Fig. 1e). This reduction was abolished when cells were pretreated with the PI(3)K inhibitor LY294002. These effects were also observed with the DLD-1 cell line (Supplementary Information Fig. 1). To establish the involvement of endogenous FOXO in the control of MnSOD, we expressed dominant-negative mutants of FOXO3a in 293T cells[15]. Notably, we could show that the LY294002-induced increase in MnSOD protein was prevented by expression of these dominant-negative mutants, which indicates that endogenous FOXO regulates the expression of MnSOD (Fig. 1f).

To investigate whether MnSOD is transcriptionally regulated by Forkheads, we examined the expression of MnSOD in the DL23 cell line. The levels of MnSOD mRNA were increased between 8 and 16 h of 4-OHT treatment and a roughly tenfold increase was seen after 24 h of FOXO3a activation (Fig. 2a). Similarly, when 3T3-L6 cells were serum starved for 48 h, the levels of MnSOD mRNA increased 3.5-fold, but were decreased after insulin treatment to

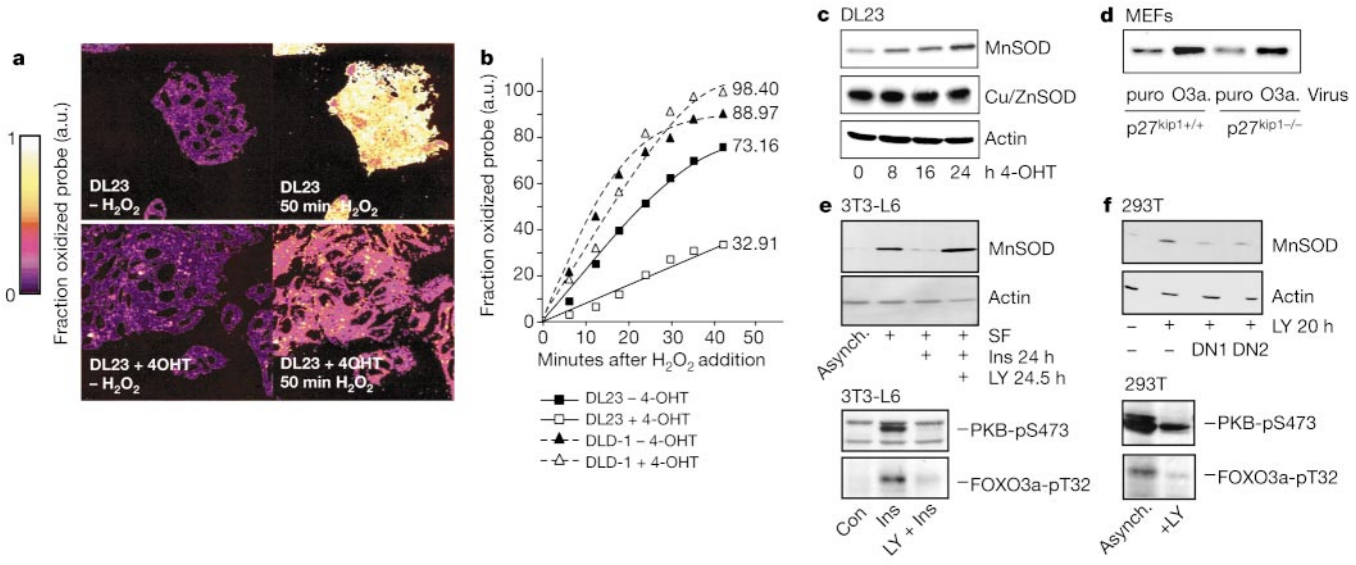

Figure 1 Forkhead transcription factors increase cellular protection against ROS. **a**, DL23 cells left untreated or treated with 500 nM 4-OHT for 16 h were loaded with the C11-$BODIPY^{581/591}$ probe, and treated with 200 μM H_2O_2 for 50 min. Images were taken by confocal microscopy[13]. Yellow areas represent high oxidation, purple areas represent low oxidation. **b**, DL23 (unbroken lines) and DLD-1 (broken lines) cells were treated as in **a**, and the fraction of oxidized probe per time point in arbitrary units (a.u.) was calculated. **c**, Total lysates of DL23 cells left untreated or treated with 500 nM 4-OHT were analysed for expression of MnSOD, Cu/ZnSOD and actin as a loading control. **d**, MnSOD expression in wild-type or $p27^{kip1-/-}$ MEFs infected with control retrovirus (puro) or FOXO3a–A3 (O3a) 24 h after infection. **e**, 3T3-L6 cells were grown asynchronously or serum starved (SF, serum free) for 48 h, and stimulated with insulin (Ins) for 24 h (top) or 10 min (bottom), with or without a 30-min pretreatment with LY294002 (LY). Lysates were analysed for expression of MnSOD and actin (top) or for phosphorylated PKB (PKB-pS473) and FOXO3a (FOXO3a-pT32; bottom). **f**, 293T cells were transfected with dominant-negative mutants of FOXO3a (DN1 or DN2). Cells were left untreated or treated with LY294002 for 16 h. Expression of MnSOD and actin (loading control), and phosphorylation of PKB on Ser 473 and FOXO3a on Thr 32 were analysed by immunoblotting.

almost basal amounts (Fig. 2b). Again, LY294002 fully reversed the inhibition induced by insulin.

We analysed the effect of specific Forkhead activation on a promoter fragment of 3,340 base pairs (bp) of the human *SOD2* gene for MnSOD (pSODLUC-3340)[16]. Luciferase expression controlled by this fragment was increased 3–4-fold on cotransfection with the various Forkhead transcription factors in several cell lines (data not shown), and also after 16 h of 4-OHT addition to DL23 but not DLD-1 cells (Fig. 2c). No effect was seen on a similar reporter construct lacking the sequences upstream of the transcription start site (pSODLUC-1; Fig. 2c). We could identify one inverse FOXO-binding element (DBE)[17] at position −1,249 (GTAAACAA; DBE1) and one suboptimal DBE at position −997 (TTGTTTAA; DBE2) in the human *SOD2* promoter (Fig. 2d). A single G to C substitution in the TTGTTT sequence of DBE2 had no effect on FOXO3a-induced luciferase activity of the *SOD2* reporter construct, but a similar substitution in the AAACAA sequence of DBE1 completely abolished FOXO3a-mediated expression of the *SOD2* gene (Fig. 2d). Chromatin immunoprecipitation assays also showed that FOXO3a could bind *in vivo* to the MnSOD promoter region encompassing DBE1 (Fig. 2e). Binding had already occurred as early as 4 h after 4-OHT treatment, which indicates that binding precedes the increase in mRNA MnSOD as determined by northern blotting (Supplementary Information Fig. 2). Together, these results show that expression of MnSOD is controlled transcriptionally by FOXO3a binding directly to DBE1.

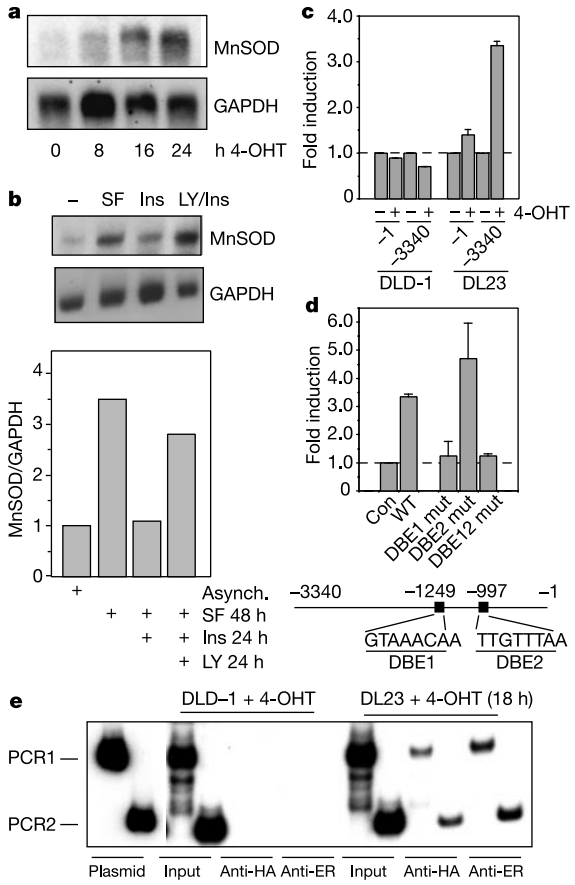

Figure 2 FOXO3a directly regulates the MnSOD promoter through an inverse DBE. **a**, mRNA (2 μg) isolated from DL23 cells left untreated or treated with 500 nM 4-OHT was analysed by northern blotting for MnSOD and GAPDH (loading control). **b**, 3T3-L6 cells were treated as in Fig. 1e. Total RNA (30 μg) was analysed for MnSOD and GAPDH mRNA by northern blotting. A representative experiment is shown; the histogram shows the mean of two independent experiments (SF, serum free; Ins, insulin; LY, LY294002). **c**, pSODLUC-3340 and pSODLUC-1 were transfected into DL23 or DLD-1 cells, which were left untreated or treated with 500 nM 4-OHT for 16 h before the luciferase activity was measured. **d**, pSODLUC-3340 carrying a point mutation at indicated positions was transfected into DL23 cells and luciferase activity was measured as in **c**. Data in **c** and **d** are the mean of three independent experiments. The linearized *SOD2* promoter fragment containing the two DBEs is also shown. DBE12mut, double mutant. **e**, DLD-1 and DL23 cells were treated with 4-OHT for 16 h, and then chromatin-bound DNA was isolated and immunoprecipitated with antibodies against the ER moiety or the HA epitope of HA–FOXO3a–ER. Immunoprecipitated DNA was analysed by PCR using two primer combinations, both encompassing DBE1. PCR was carried out on pSODluc-3340 as a control for migration of the PCR fragments.

The availability of glucose may be essential for growth-factor- and PKB-mediated protection from apoptosis[2]. Because Forkheads become active in the absence of PKB activity, we reasoned that the regulation of antioxidant capacity by Forkhead-induced MnSOD regulation may protect cells from apoptosis, particularly under conditions of low glucose availability. We therefore analysed the effect of glucose deprivation on mitochondrial membrane integrity, an early marker for apoptosis, by rhodamine-1,2,3 staining[18]. Various cell types including DLD-1 and MEFs, which were cultured in the presence of 8% fetal calf serum (FCS) to activate PKB but were deprived of glucose for 16 or 72 h, respectively, showed a clear loss of mitochondrial membrane integrity (Fig. 3a). Pretreating the cells with 10 mM *N*-acetyl-L-cysteine (NAC), which enhances the scavenging of oxygen radicals, largely prevented apoptosis induced by glucose deprivation. This indicates that, in agreement with other reports[19–21], mitochondrial damage induced by glucose deprivation proceeds through increased production of ROS. Increased ROS production by glucose deprivation was also confirmed by means of an *in vitro* fluorescent assay similar to the *in vivo* assay described in Fig. 1 (Supplementary Information Fig. 3). We also showed that this effect of glucose deprivation was not due to depletion of cellular ATP resulting from diminished activity of the trichloroacetic acid cycle, as addition of pyruvate could not prevent the induction of mitochondrial instability (Fig. 3a). Mitochondrial leakage correlated with the induction of apoptosis in these cells, as determined by the fraction of cells containing a sub-G1 DNA content (Fig. 3a). Notably, whereas 4-OHT treatment was ineffective in DLD-1 cells, activation of FOXO3a in DL23 cells, either by the addition of 4-OHT or by LY294002 treatment, efficiently protected the cells from apoptosis induced by glucose deprivation (Fig. 3b).

Increased cellular ROS can result in the activation of stress-activated kinases, including Jun amino-terminal kinase (JNK). Indeed, glucose deprivation induced activation of JNK and this activation was inhibited in part by NAC pretreatment (Fig. 3c). More notably, the activation of JNK induced by glucose deprivation was inhibited after activation of FOXO3a, which indicates that the increased tolerance against oxidative stress induced by FOXO3a activation observed in the experiments using rhodamine-1,2,3 staining is reflected by a reduction in the induction of JNK (Fig. 3c).

To show that MnSOD is involved in this Forkhead-induced survival signal, we infected MnSOD-deficient MEFs[22] with a retrovirus expressing FOXO3a–A3. Similar to DLD-1 cells, wild-type MEF cells deprived of glucose also lost mitochondrial membrane integrity unless infected with FOXO3a–A3 (Fig. 3d). In $Sod2^{-/-}$ cells, however, mitochondrial damage occurred irrespective of the presence of FOXO3a–A3. Similarly, whereas LY294002 pretreatment efficiently protected wild-type cells, it had much less effect on $Sod2^{-/-}$ cells. Ectopic expression of MnSOD in $Sod2^{-/-}$ cells in similar amounts to those observed after inducing Forkhead activity made these cells refractory to glucose deprivation. We confirmed these data by analysing cell survival after glucose deprivation. Wild-type cells infected with FOXO3a–A3 could survive prolonged glucose deprivation (up to 5 d), whereas control-infected and $Sod2^{-/-}$ cells expressing FOXOa3–A3 died in this period (Supplementary Information Fig. 3). FOXO3a-mediated survival is not a consequence of a reduced glucose requirement of arrested or

quiescent cells, because expression of the cell-cycle inhibitor p16 resulted in cell-cycle arrest but did not protect against glucose deprivation (Fig. 3d).

As reported previously[10,23], ectopic expression of FOXO3a reduces colony outgrowth of wild-type MEFs by inhibiting proliferation. When we carried out the same experiment in *Sod2*$^{-/-}$ cells, this resulted in the absence of any colony outgrowth (Fig. 3e), which indicates that cells entering quiescence through activation of Forkheads may require MnSOD for their survival. We therefore tested whether cells maintained in a quiescent state would show a similar

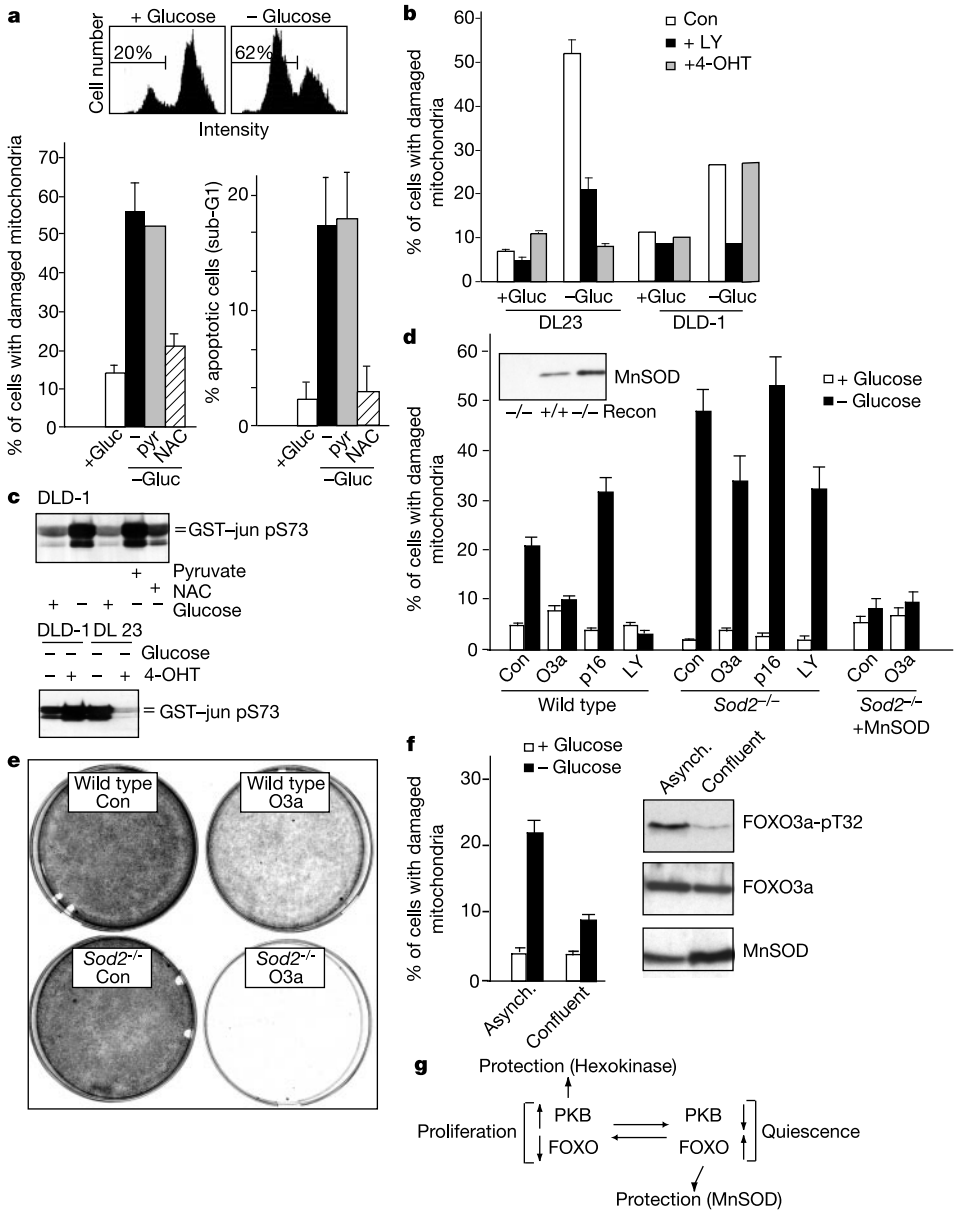

Figure 3 A Forkhead-mediated increase in MnSOD is required for the survival of arrested cells. **a**, DL23 cells were grown in DMEM medium supplemented with 8% FCS with or without glucose for 16 h, and mitochondrial membrane integrity was measured by rhodamine-1,2,3 staining. Typical cytometry profiles are shown on top. Left, DL23 cells were grown for 16 h with or without glucose, or without glucose and with pyruvate. Cells were left untreated or were treated with 10 mM NAC for 40 h (24 h before glucose withdrawal). Data are the means of at least three independent experiments. Right, cells were treated as above, but were fixed and stained with propidium iodide. The percentage of sub-G1 cells (representing apoptotic cells) was determined by flow cytometry. **b**, DLD-1 and DL23 cells were processed as in **a**. Cells were left untreated or were treated with LY294002 or 4-OHT for 40 h (24 h before glucose withdrawal). **c**, Cells were treated as indicated, and lysates were assayed for JNK activity. Treatment with 4-OHT, NAC and pyruvate was done as in **a** and **b**. **d**, Wild-type cells (+/+), *Sod2*$^{-/-}$ cells (−/−) or *Sod2*$^{-/-}$ cells with reconstituted MnSOD expression (−/− recon) were infected with a control virus (con) or with a virus expressing FOXO3a–A3 (O3a), and mitochondrial membrane integrity was measured after 72 h of glucose withdrawal. Inset, quantity of MnSOD protein in the three cell lines. **e**, Wild-type or *Sod2*$^{-/-}$ cells were infected as in **d**. After 2 weeks of selection, the cells were stained with crystal violet to visualize surviving colonies. **f**, Wild-type MEFs were grown to confluency, maintained for 4 d, and lysates were analysed for MnSOD and FOXO3a. Identical separate cultures were analysed by rhodamine staining for mitochondrial damage. **g**, Representation of the PKB–FOXO module, which functions as a molecular switch controlling cellular proliferation and protection from oxidative stress. Activation of PKB leads to inactivation of FOXO factors and cell-cycle entry. Simultaneously, cells become reliant on PKB-dependent survival signals that require the availability of glucose. Switching from active PKB to active FOXO factors will not only drive cells into a state of quiescence, but will replace simultaneously the PKB-dependent protection from oxidative insult by an alternative mechanism that no longer depends on glucose. This mechanism involves transcriptional upregulation of the MnSOD antioxidant enzyme.

resistance to glucose starvation by comparing contact-inhibited cultures with asynchronously growing MEFs. We found that contact-inhibited cells show increased protection against glucose deprivation, with a concomitant increase in their expression of MnSOD (Fig. 3f).

We have provided evidence that in the absence of PKB-mediated survival cells can be protected against oxidative damage by activating the Forkhead transcription factor FOXO3a, which directly increases the expression of MnSOD. These results show that the direct inverse coupling of Forkhead and PKB activity functions as a key switch in controlling the effects of cellular ROS. In a similar inverse manner, cell-cycle progression is controlled by PKB–Forkhead signalling. Activation of Forkheads results in entrance into a quiescent (G0) state[11], whereas inactivation of Forkheads by activation of PKB results in re-entry into the cell cycle and proliferation. Thus, regulation of proliferation and the appropriate protective mechanisms are coupled by the same PKB–Forkhead switch.

The dynamics of quiescence and proliferation set different requirements for the mechanism of ROS control, but also indicate why proliferation is coupled to glucose availability and why survival of quiescent cells must be relatively independent of their environment. This intricate link between proliferation or survival and glucose availability is also evident from results that we have obtained with tumour cells. In general, tumour cells are far more sensitive to glucose deprivation than are untransformed cells. In this respect, it is notable that tumour cells show high rates of aerobic glycolysis (the Warburg effect). In tumour cells, PKB is often constitutively activated either through deletions or mutations in PTEN (phosphatase and tensin homologue on chromosome 10) or through the activity of autocrine growth factors. Activation of PKB in tumour cells implies the inactivation of Forkhead and a loss of protection in the absence of glucose. Indeed, PTEN-negative U87MG glioblastoma cells show signs of substantial mitochondrial instability within a few hours of glucose deprivation (R.H.M., unpublished data), whereas MEFs show the same amount of mitochondrial damage only after prolonged deprivation (48–72 h). Others have reported an involvement of FOXO factors in oxidative stress responses[14]: expression of FOXO has been shown to increase cellular antioxidant activity, in keeping with the mechanism that we describe here. In contrast to that study, however, we did not observe any regulation of PKB activity (Supplementary Information Fig. 1) or FOXO transcriptional activity (data not shown) under conditions of low oxidative stress, such as glucose deprivation. In addition, whereas we have described a primary function of Forkheads in the defence against ROS (detoxification), others have shown that when ROS-induced DNA damage may occur Forkheads can contribute secondarily to defence by regulating the expression of proteins involved in the repair process[24].

In summary, we propose that the opposing action of PKB and the Forkhead transcription factors FOXO1, FOXO4 and FOXO3a on cell proliferation and their ability to provide protection against ROS-induced damage through differing mechanisms outlines a system that provides checks and balances for coupling cell-cycle regulation, glucose metabolism and protection from oxidative damage (Fig. 3g). In addition to the evolutionary conservation of the structure of this regulatory system in *Drosophila*, *C. elegans* and yeast (see refs 25, 26), we now show functional conservation as well. This indicates that the PKB–Forkhead pathway may be fundamental in coupling external cues to cellular response. □

Methods

General

Retroviral infections and maintenance of immortalized wild-type and $p27^{kip1-/-}$ MEFs and primary wild-type and $Sod2^{-/-}$ MEFs have been described[10,22]. The DL23 cell line has been described[11]. We created the plasmid pBabe–FOXO3a–A3 by ligating a Klenow-blunted *Hin*dIII/*Bam*HI fragment of pcDNA3–HA–FOXO3a–A3 into Klenow-blunted pBabe–puro plasmid cut by *Bam*HI.

Chromatin immunoprecipitation assay

We carried out CHIP as described[27]. About two 9-cm diameter dishes at 80% confluency were used per immunoprecipitation. Two sets of primers were designed to amplify by polymerase chain reaction (PCR) the region encompassing the FOXO3a-binding site in the *SOD2* promoter with the primers: PCR1, 5′-cagagcgaggctgtgtctc-3′ and 5′-gtcccagcctgaatttcc-3′; PCR2, 5′-ctaggcttccggtaagtg-3′ and 5′-gttgccccttttattgaaaag-3′.

Microscopy and fluorescence ratio imaging

We carried out microscopy and fluorescence ratio imaging essentially as described[13]. On oxidation, the fluorescence excitation/emission maxima of the C11-$BODIPY^{581/591}$ probe shift from 581/591 to 490/510 nm, which facilitates measurement of the fraction of oxidized probe versus time. After adding 200 μM H_2O_2 to induce radical formation, we followed cells in time with dual excitation and emission confocal laser scanning microscopy. It should be noted that the C11-$BODIPY^{581/591}$ probe is not oxidized by H_2O_2 itself, but by hydroxyl radicals formed in the Fenton reaction, $H_2O_2 + O_2^{\bullet -} \rightarrow HO^{\bullet} + OH^- + O_2$, which is catalysed by divalent metal cations.

Measurement of mitochondrial membrane integrity

DL23 cells or MEFs were glucose deprived for 16 h or 72 h, respectively, by adding DMEM medium lacking glucose and pyruvate, but supplemented with 8% FCS. Note that owing to the small amount of glucose in FCS, glucose amounts are estimated to be about 10–20-fold less in this medium than in normal culture conditions. We added NAC (10 mM), LY294002 (20 μM) or 4-OHT (500 nM) 24 h before withdrawing glucose. The cells were then digested with trypsin and incubated with 10 μg ml^{-1} rhodamine-1,2,3 (ref. 18) at 37 °C for 30 min. The cells were washed twice with PBS and mitochondrial membrane staining was measured by standard flow cytometry.

JNK activity

After being washed in PBS, the cells were lysed in RIPA buffer and lysates were incubated with 5 μg of glutathione *S*-transferase (GST)-labelled Jun (residues 1–135) coupled to glutathione beads for 1 h at 4 °C. Beads were washed three times and incubated in kinase buffer (100 μM ATP, 20 mM $MgCl_2$; 50 mM Tris, pH 7.5; 1 mM DTT) for 30 min at 30 °C. We detected phosphorylation of GST–Jun by immunoblotting and probing with an antibody specific for Jun phosphorylated on Ser 73 (anti-pS73-Jun).

Western blotting and antibodies

Western blotting of total lysates was done as described[8]. Antibodies to MnSOD and Cu/ZnSOD were from StressGen. The antibodies to the ER (C-20) and actin (I-19) were from SantaCruz. The antibody against $p27^{kip1}$ was from Transduction Labs. Antibody specific for PKB phosphorylated on Ser 473 (anti-pS473-PKB) and the anti-pS73-Jun antibody were from Cell Signaling. Antibody against FOXO3A phosphorylated on Thr 32 (anti-pT32-FOXO3A) was from Upstate Biotech. Before the immunblotting shown in Fig. 1f, transfected cells were isolated by magnetic activated cell sorting procedure[10].

Northern blotting

We ran 2 μg of mRNA (polyA-Tract, Promega) purified from 1 mg of total RNA (RNAZol, TEL-TEST; Fig. 2a) or 30 μg of total RNA (Fig. 2b) on a formaldehyde denaturing gel and transferred it to a GeneScreen-Plus nylon membrane (NEN). The blot was hybridized using radiolabelled probes for MnSOD (*Eco*RI fragment of pGEM3Z–MnSOD) and GAPDH (*Not*I-linearized pUC19–GAPDH). Plasmid pGEMZ–MnSOD was a gift from J. Wispe.

Luciferase assays

We carried out luciferase assays as described[8]. Plasmids pSODLUC-3340 and pSODLUC-1 (ref. 16) were a gift from M. Yim. We created pSODLUC-3340–DBE1mut and pSODLUC-3340–DBE2mut by site-directed mutagenesis using the primers 5′-ctgacgtctgtaaagaagcccagcccttc-3′ and 5′-cattcaggattgttctttaactgttgag-3′, respectively.

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Competing interests statement

The authors declare that they have no competing financial interests.

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