

# Novel effects of Brefeldin A (BFA) in signaling through the insulin receptor (IR) pathway and regulating FoxO1-mediated transcription

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Brefeldin A (BFA) is a fungal metabolite best known for its ability to inhibit activation of ADP-ribosylation factor (Arf) and thereby inhibit secretory traffic. BFA also appears to regulate the trafficking of the GLUT4 glucose transporter by inducing its relocation from intracellular stores to the cell surface. Such redistribution of GLUT4 is normally regulated by insulin-mediated signaling. Hence, we tested whether BFA may intersect with the insulin pathway. We report that BFA causes the activation of the insulin receptor (IR), IRS-1, Akt-2, and AS160 components of the insulin pathway. The response is mediated through phosphoinositol-3-kinase (PI3K) and Akt kinase since the PI3K inhibitor wortmannin and the Akt inhibitors MK2206 and perifosine inhibit the BFA effect. BFA-mediated activation of the insulin pathway results in Akt-mediated phosphorylation of the insulin-responsive transcription factor FoxO1. This leads to nuclear exclusion of FoxO1 and a decrease in transcription of the insulin-responsive gene SIRT-1. Our findings suggest novel effects for BFA in signaling and transcription, and imply that BFA has multiple intracellular targets and can be used to regulate diverse cellular responses that include vesicular trafficking, signaling and transcription.

## Introduction

The fungal metabolite BFA has been characterized extensively as a modulator of intracellular trafficking pathways. BFA acts by directly binding to an interface formed by a complex of a large GDP/GTP exchange factor (GBF1, BIG1 or BIG2) bound to its substrate Arf-GDP and preventing GDP/GTP exchange on the Arf.<sup>1,2</sup> BFA is best known as an inhibitor of trafficking at the ER-Golgi interface where it prevents the association of the COPI coat with membranes to generate transport vesicles, leading to inhibition in ER-Golgi trafficking and subsequent disassembly of the Golgi.<sup>3-6</sup> BFA also inhibits binding of the AP1 and GGA coat adaptors within the endosomal pathway, causing disruption in endosomal trafficking leading to the collapse of recycling endosomes.<sup>7</sup> Thus, BFA appears to influence multiple trafficking pathways by targeting GBF1 within the secretory pathway and BIG1/2 within the endocytic pathway. In addition, BFA has been shown to induce the relocation of GLUT4 from intracellular compartments to cell surface in rat adipocytes<sup>8</sup> through as yet uncharacterized mechanism.

Physiological GLUT4 trafficking is known to be regulated by insulin in a process that directly controls blood glucose levels. GLUT4 is a 12 membrane-spanning protein preferentially expressed in adipocyte and muscle cells that under basal

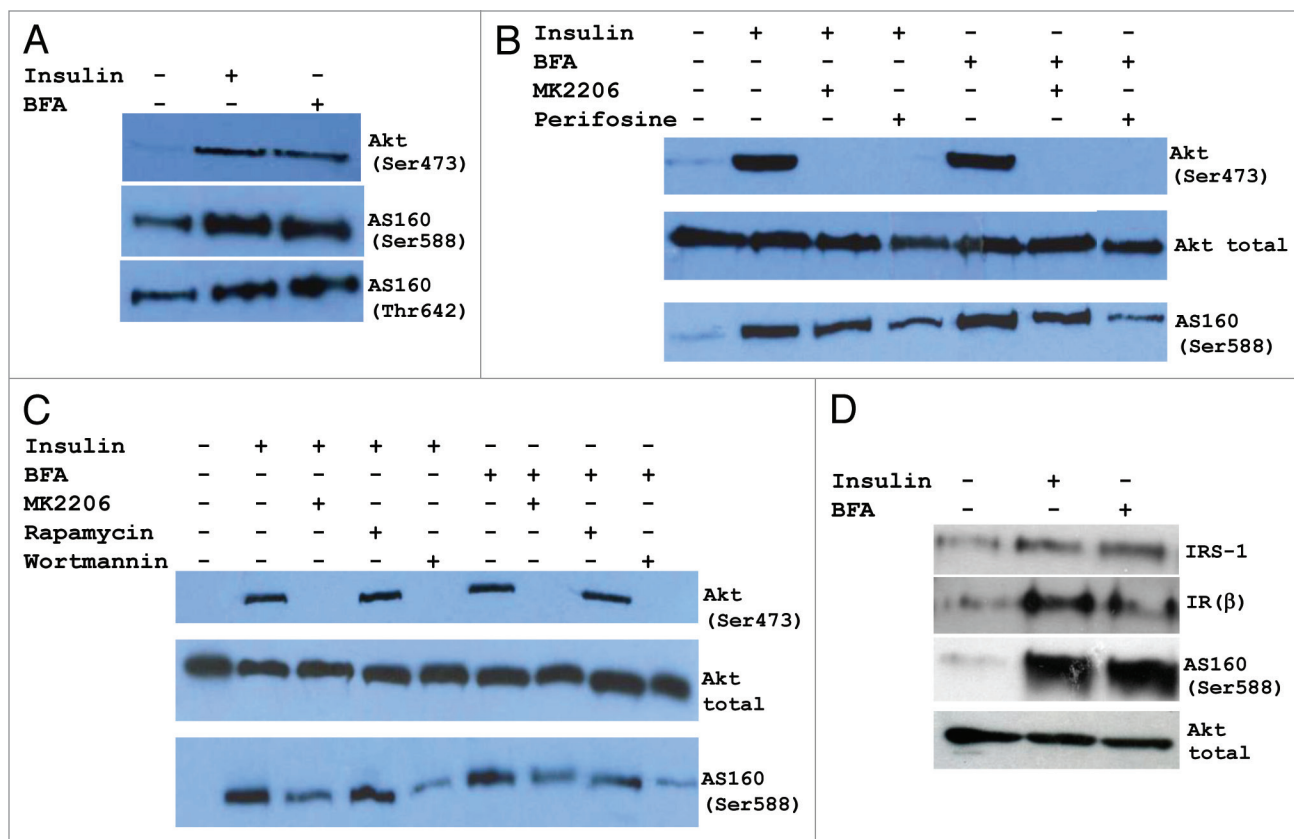
conditions resides in the trans-Golgi network (TGN)/endosomal compartments and small vesicles (called insulin-responsive vesicles or IRVs).<sup>9-13</sup> Insulin signaling targets only IRVs, causing their translocation and fusion with the plasma membrane, and the insertion of GLUT4 into the plasma membrane. At the cell surface, GLUT4 facilitates glucose uptake. Termination of insulin signaling causes GLUT4 to be internalized and re-sequestered into IRVs.

The molecular cascade leading to insulin-mediated GLUT4 translocation has been partially defined: activation of the insulin pathway is initiated by insulin binding to the extracellular domain of the insulin receptor (IR) causing autophosphorylation of the intracellular domains. Activated IR then phosphorylates IRS-1, which creates docking sites for PIP3K. Activated PIP3K catalyzes the formation of PtdIns(3,4,5) $P_3$  from PtdIns(4,5) $P_2$  leading to the recruitment of PDK1 and Akt kinases to the membrane. PDK1 phosphorylates and activates Akt, and Akt subsequently phosphorylates numerous substrates, among them AS160 (TBC1D4). AS160 has been characterized as a Rab Gap and has activity on Rab10 important in GLUT4 trafficking in adipocytes, and on Rab8 and Rab14, which regulate GLUT4 trafficking in muscle cells.<sup>14</sup> Phosphorylation of AS160 leads to its inactivation and causes persistent activation of the Rab, an event required for the release of GLUT4-containing IRVs from

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**Figure 1.** BFA causes phosphorylation of insulin-signaling pathway components. (A-C) Differentiated 3T3-L1 adipocytes were serum starved for 4 h and treated with insulin or BFA for 30 min (A), or pretreated for 1 h with Akt inhibitors (MK2206 and perifosine), and then treated with insulin or BFA (B), or pretreated with PI3 kinase inhibitor (wortmannin), Akt inhibitor (MK2206), or mTOR pathway inhibitor (rapamycin) for 1 h, and then treated with insulin or BFA (C). Cells were lysed and lysates were immunoblotted to detect Akt (Ser 473) and AS160 (Ser 588 and Thr 642). Both, insulin and BFA induce phosphorylation of Akt (Ser 473) and AS160 (Ser 588 and Thr 642), and both events are inhibited by inactivating Akt or PI3K. (D) Differentiated 3T3-L1 adipocytes were serum starved for 4 h and then treated with insulin or BFA for 30 min. Cells were lysed and lysates were immunoprecipitated with anti-phosphotyrosine antibodies. The immunoprecipitates were immunoblotted with anti-IRS1 and anti-IR ( $\beta$ -subunit) antibodies. Both, IRS-1 and IR are phosphorylated in response to insulin or BFA. Representative blots from at least 3 experiments are shown.

the peri-nuclear internal stores and their translocation to and fusion with the plasma membrane.

Because BFA, like insulin, caused redistribution of GLUT4, we tested whether BFA may intersect with the insulin pathway. Herein, we report that treating 3T3-L1 adipocytes with BFA stimulates phosphorylation of the IR, IRS-1, Akt kinase and AS160. We implicate PI3K in the BFA-mediated activation of the insulin pathway response because the PI3K inhibitor wortmannin inhibits the BFA-induced phosphorylation of Akt and diminishes the phosphorylation of AS160. We also show that the BFA effect is propagated through Akt since the BFA-induced phosphorylation was inhibited by Akt inhibitors (MK2206 and Perifosine).

The unexpected finding that BFA activates components of the insulin-signaling pathway led us to investigate whether BFA may regulate cellular responses that are under insulin control. As mentioned above, insulin stimulates the relocation of GLUT4 from perinuclear stores to the plasma membrane and significantly stimulates glucose uptake into cells. Thus, we examined whether BFA similarly causes GLUT4 relocation and increases glucose uptake. We show that BFA causes the redistribution of GLUT4

to the plasma membrane, but has minimal effects on glucose uptake, in agreement with previous reports<sup>8,15</sup> (). Thus, despite activating the insulin-signaling pathway, BFA doesn't recapitulate the insulin-mediated increase in glucose uptake.

In addition to controlling blood glucose level through regulating GLUT4 trafficking, insulin also controls transcription of tissue-specific and ubiquitously expressed genes. Specifically, insulin regulates the Akt-mediated phosphorylation of the forkhead transcription factors that in mammals include FoxO1, FoxO3a and FoxO4.<sup>16-18</sup> Among the FoxO isoforms, FoxO1 is a major downstream target of insulin that mediates adaptive responses in many target tissues including liver, muscles, adipose tissue and pancreatic  $\beta$ -cells.<sup>19</sup> FoxO1 also mediates the response to insulin and leptin in the hypothalamus and controls the synthesis of a number of neuropeptides that regulate feeding behavior in mice.<sup>20</sup> FoxO1 regulates metabolic homeostasis by controlling the expression of multiple genes including the NAD<sup>+</sup>-dependent deacetylase sirtuin-1 (SIRT-1).<sup>21,22</sup> SIRT-1 exerts a variety of homeostatic functions by controlling transcription of target genes essential for regulating cell cycle, apoptosis, response to stress and autophagy.<sup>23-25</sup> Insulin/Akt-mediated phosphorylation of FoxO1

on serine 256 causes FoxO1 relocation from the nucleus to the cytosol and results in decreased expression of FoxO1-regulated genes including decreased transcription of SIRT-1 (reviewed in<sup>26</sup>).

Because BFA, like insulin, caused Akt activation, we assessed whether BFA can control phosphorylation of FoxO1 and regulate FoxO1-mediated transcriptional repertoire. We report that BFA causes FoxO1 phosphorylation on serine 256, the exclusion of phospho-FoxO1 from the nucleus and its relocation into the cytosol, and a consequent decrease in transcription of a SIRT-1 reporter construct. BFA stimulation of FoxO1 phosphorylation is Akt and PI3 kinase dependent, as inhibitors of these kinases abolish the BFA effect.

Our findings suggest that in addition to the well-known effects of BFA on GEF inactivation and inhibition of trafficking, BFA also targets cellular components that regulate signaling and transcriptional control. Thus, the numerous studies in which cellular phenotypes were analyzed after BFA treatment are likely to represent multi-component responses and must be interpreted with care. Future development of selective inhibitors that target only a single mechanism will allow the dissection of phenotypes caused by silencing of a single cellular pathway.

## Results

### BFA activates key components of the insulin-signaling pathway

Treatment of isolated rat adipocytes with BFA has been shown to cause the translocation of GLUT4 from intracellular compartments to cell surface.<sup>8,27</sup> This suggested that BFA may activate the insulin-signaling pathway, and we monitored phosphorylation of Akt and AS160 after treating differentiated 3T3-L1 adipocytes with insulin or 5 µg/ml BFA. This concentration of BFA has been shown previously to cause Golgi disassembly in a number of cell types,<sup>3,8,28-34</sup> and was used throughout this study. Insulin causes phosphorylation of Akt on threonine 308 and serine 473, and phosphorylation of AS160 on threonine 642 and serine 588.<sup>35,36</sup> As shown in **Figure 1A**, BFA mimics the effects of insulin and causes robust phosphorylation of Akt (Ser 473) and phosphorylation of AS160 (Thr 642 and Ser 588), as detected with antibodies specific for these phosphorylated forms of Akt and AS160.

To assess the role of Akt and PI3K in the BFA effect, the insulin and BFA treatments were done in the absence or presence of specific inhibitors of Akt (MK2206 and perifosine) or PI3K (wortmannin). MK-2206 is an allosteric inhibitor that targets the pleckstrin homology domain to inhibit auto-phosphorylation of both Akt threonine 308 and serine 473.<sup>37,38</sup> Perifosine inhibits both Akt and PI3K, and unlike most kinase inhibitors, which target the adenosine triphosphate-binding region, perifosine targets the pleckstrin homology domains of Akt and PI3K, thereby preventing their translocation to the plasma membrane.<sup>39</sup> Wortmannin is a fungal metabolite that covalently inhibits PI3K: this drug targets several enzymes in dose dependent manner, and at the concentration used in our studies (1 mM) selectively inhibits just PI3K.<sup>40</sup>

3T3-L1 adipocytes were serum starved for 4 h (to decrease signaling through serum-supplied growth factors, etc), left untreated or pretreated with inhibitors for 1 h, and then treated with either insulin or BFA for 30 min. As shown in **Figure 1B**, phosphorylation of Akt(Ser473) in response to either insulin or BFA was completely inhibited by MK2206 and perifosine. Phosphorylation of AS160 was significantly reduced by MK2206 and perifosine. Thus, BFA recapitulates insulin action with respect to regulating Akt activity and AS160 phosphorylation.

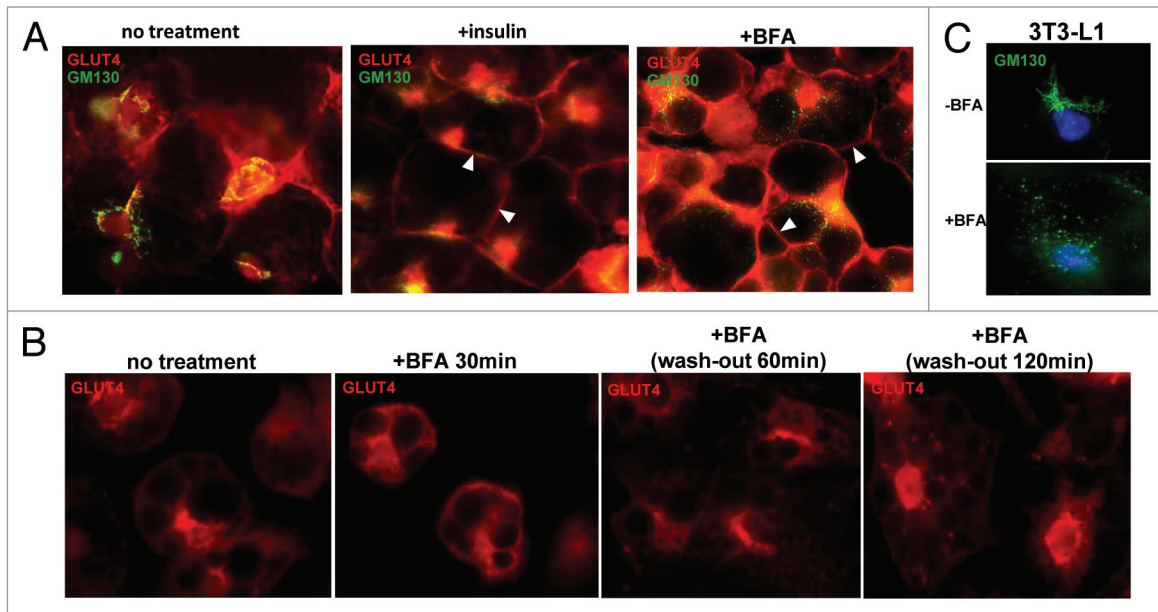
Activation of Akt by BFA (as well as by insulin) occurs through PI3K as demonstrated by complete inhibition in Akt (Ser 473) formation by wortmannin (**Fig. 1C**). Inhibition of PI3K had reduced inhibitory effect on phosphorylation of AS160 (Ser 588), similar to the effect observed in the presence of Akt inhibitors. Although it has been reported that mTOR may contribute to Akt (Ser 473) phosphorylation,<sup>41</sup> inhibiting mTOR with rapamycin did not significantly impact Akt or AS160 phosphorylation in cells treated with either insulin or BFA.

Since PI3K appears to mediate the BFA effect, and PI3K is downstream from IR and IRS-1, we assessed the effect of BFA on IR and IRS-1 phosphorylation. IR and IRS-1 are activated in response to insulin by phosphorylation of tyrosine residues: IR contains multiple phosphorylation sites including tyrosine 1146, 1150 and 1151, while IRS-1 can be phosphorylated on tyrosine 460, 608, 628, 895, 939, 1172 and 1222.<sup>42,43</sup> 3T3-L1 adipocytes were serum starved for 4 h, then untreated or treated with either insulin or BFA for 30 min, solubilized, and the lysates were immunoprecipitated with anti-phospho-tyrosine antibodies. Precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-IR (β-subunit) and anti-IRS-1 antibodies. Both IR and IRS show low levels of basal tyrosine phosphorylation in untreated cells, and significantly increased phosphorylation after insulin or BFA treatment (**Fig. 1D**). Immunoblotting with anti-AS160-phosphoserine 588 antibodies confirmed activation and propagation of insulin signaling. The ability of BFA to activate components of the insulin pathway raised the possibility that BFA may regulate events normally controlled by insulin.

### BFA stimulates redistribution of GLUT4

Previous immunogold labeling studies implicated BFA in the translocation of GLUT4 from intracellular stores to sites underneath the plasma membrane in rat adipocytes.<sup>8</sup> Here, we examined the localization of endogenous GLUT4 in differentiated mouse 3T3-L1 adipocytes in response to a 30 min treatment with insulin or BFA. Both insulin and BFA triggered redistribution of GLUT4 to cell surface (**Fig. 2A**, marked with arrowheads). The surface signal appears similar in insulin- and BFA-treated cells, suggesting that BFA is as effective as insulin in stimulating GLUT4 redistribution. BFA is a reversible inhibitor and can be washed out of cells.<sup>44,45</sup> Thus, we examined whether BFA-stimulated GLUT4 relocation is reversible. As shown in **Figure 2B**, GLUT4 is largely relocated toward cell surface after 30 min of BFA treatment, but starts to return to the perinuclear region 60 min after BFA wash-out, and appears analogous to that of untreated cells 120 min after BFA wash-out.

BFA is best known as an inhibitor of trafficking at the ER-Golgi interface (by inhibiting the recruitment of the



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**Figure 2.** BFA causes reversible redistribution of GLUT4. Differentiated 3T3-L1 adipocytes were serum starved for 4 h and then treated with insulin or BFA for 30 min (**A, C**) or treated with BFA for 30 min, followed by BFA wash out for 60 or 120 min (**B**). Cells were processed for immunofluorescence to detect GLUT4 (red) and a Golgi marker GM130 (green). Insulin and BFA induce redistribution of GLUT4 to the plasma membrane (arrowheads in **A**). The BFA effect is reversible and GLUT4 returns to intracellular localization after BFA wash-out. BFA also causes reversible fragmentation of the Golgi in adipocytes (**C**). Representative images from at least 3 experiments are shown.

coatmer coat) and rapidly causes disruption of the Golgi ribbon.<sup>29</sup> We assessed BFA effects on Golgi architecture in differentiated 3T3-L1 adipocytes by monitoring the localization of the Golgi marker protein golgin GM130.<sup>46</sup> As shown in **Figure 2C**, instead of a single perinuclear Golgi ribbon seen in untreated cells, BFA causes dispersal of the Golgi into multiple small puncta scattered throughout the cell, analogous to that observed in other cell types.

The BFA-induced relocation of GLUT4 to cell surface was confirmed by subcellular fractionation. Cells were serum starved for 4 h, pretreated or not with Akt inhibitor (MK2206) for 1 h, and then treated with insulin or BFA for 30 min. Cells were disrupted, plasma membrane (PM) fractions were isolated by differential centrifugation and immunoblotted to assess levels of GLUT4. As shown in **Figure 3A**, control cells have low levels of GLUT4 in the PM, while PM fractions from cells treated with insulin or BFA are significantly enriched in GLUT4. In this experiment, IRAP and transferrin receptor (TfR) provide PM markers to show similar recovery during fractionation.

We also assessed whether BFA-induced GLUT4 translocation in 3T3-L1 adipocytes is Akt-dependent. As shown in **Figure 3A**, similar levels of GLUT4 were recovered in PM fractions when insulin and BFA were used in the absence or presence of MK2206. Inhibition of Akt was confirmed by lack of Akt (Ser 473) phosphorylation (**Fig. 3B**; Akt (Ser 473) panel). However, despite complete lack of Akt (Ser 473), significant (albeit diminished) level of AS160 phosphorylation was observed (AS160 (Ser 588) panel), suggesting that within our 3T3-L1 adipocyte system AS160 might be phosphorylated through pathways that are Akt insensitive, possibly involving AMPK.<sup>47</sup>

fractionation studies showing GLUT4 translocation to the PM suggested that BFA may increase glucose uptake. Thus, we measured glucose uptake in differentiated 3T3-L1 cells after serum starvation for 4 h and subsequent 30 min treatment with insulin or BFA. Untreated adipocytes show minimal basal glucose uptake, but treatment with insulin induced robust glucose uptake (**Fig. 3C**). Akt activation is required for the insulin-induced response, as MK2206 significantly decreases glucose uptake. In contrast, BFA treatment minimally increased glucose uptake, in agreement with previous reports.<sup>8,15,48</sup> Thus, despite activating components of the insulin signaling pathway, BFA doesn't reconstitute glucose uptake.

#### BFA stimulates Akt-mediated phosphorylation of the FoxO1 transcription factor

Insulin stimulates Akt-mediated phosphorylation of the forkhead transcription factors, including FoxO1.<sup>49</sup> FoxO1 contains multiple phosphorylation sites including threonine 32, serine 256 and serine 319 that become phosphorylated in response to insulin-stimulated Akt.<sup>49</sup> Because BFA activates Akt we examined whether BFA can promote phosphorylation of endogenous FoxO1 in 3T3-L1 adipocytes. We used antibodies specific for FoxO1 phosphorylated on serine 256 and show in **Figure 4A** that endogenous FoxO1 is phosphorylated on serine 256 in 3T3-L1 adipocytes treated with insulin or BFA, while untreated cells do not contain phosphorylated FoxO1 (the lower band detected by the antibody is FoxO4, which is not strictly regulated by insulin). GAPDH serves as a loading control in this experiment.

To confirm and extend these findings, we examined BFA effects on Flag-tagged FoxO1 exogenously expressed in HeLa

cells. In support, we observed robust phosphorylation of Flag-FoxO1 on serine 256 after insulin or BFA (Fig. 4B). Importantly, the phosphorylation of FoxO1 was significantly decreased in the presence of MK2206, indicating that it is mediated through an Akt-dependent mechanism (Fig. 4B).

#### BFA regulates nucleo-cytoplasmic translocation of FoxO1 and represses FoxO1-mediated transcription

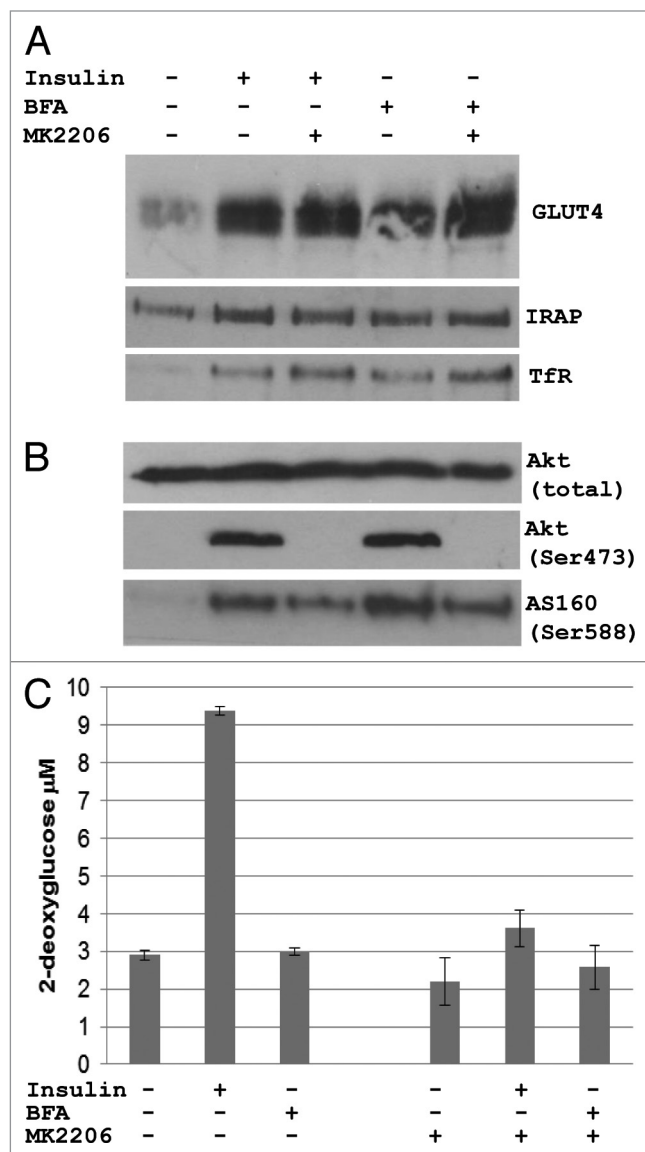
Phosphorylation of FoxO1 leads to decreased binding of FoxO1 to consensus binding sites [T(G/A)TTTT(G/T)] in the promoter region of its target genes and decreases gene transcription.<sup>50</sup> SIRT-1 is a well characterized FoxO1-dependent gene.<sup>21,22</sup> We compared the effects of insulin and BFA on transcription of SIRT-1-Luciferase reporter gene composed of firefly luciferase fused to the 1.5-kb rat SIRT1 promoter region containing a cluster of five FoxO1 core binding repeat motifs (SIRT1-Luc).<sup>21,22</sup> HeLa cells were transfected with SIRT1-Luc, serum starved for 4 h, left untreated or treated with insulin or BFA for 3 h and then luciferase levels were measured. As shown in Figure 5A, the transcription of SIRT1-Luc was significantly decreased in cells treated with insulin or BFA. The levels of inhibition by insulin and BFA were comparable. These functional results suggest that BFA can regulate FoxO1 transcriptional responses.

The insulin-induced decrease in transcription of FoxO1-regulated genes is largely due to the translocation of phosphorylated FoxO1 from nuclei to the cytosol by the 14–3–3 proteins, and the consequent spatial segregation of FoxO1 from its target genes.<sup>17,51,52</sup> Thus, we examined the subcellular localization of FoxO1 in response to BFA. Flag-tagged FoxO1 expressed in 3T3-L1 preadipocytes localizes within the nuclei (Fig. 5B), in agreement with previous reports on localization of endogenous FoxO1.<sup>53</sup> Subsequent treatment of cells with insulin or BFA causes nearly complete relocation of FoxO1 from the nuclei into the cytosol. Analogous relocation in response to insulin or BFA was seen in HeLa cells when GFP-tagged FoxO1 was analyzed (data not shown), confirming the effect of BFA on FoxO1 behavior. The effectiveness of BFA in this experiment was confirmed by the disruption of the Golgi shown by the dispersion of the Golgi marker GM130 from a peri-nuclear ribbon into punctate structures scattered within the cell (lower panels, red signal). These findings suggest that BFA decreases SIRT-1 transcriptional activity by inducing the redistribution of phosphorylated FoxO1 from the nucleus into the cytosol.

## Discussion

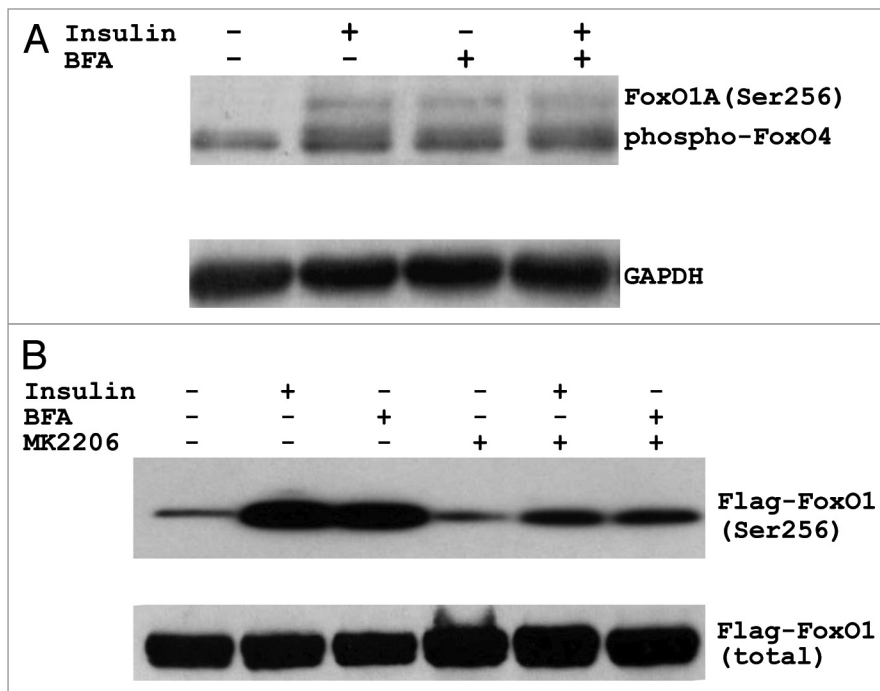
The fungal metabolite BFA has been characterized extensively as a modulator of intracellular trafficking pathways. BFA has been shown to inhibit the catalytic activity of large GDP/GTP exchange factors that activate members of the Arf family of small GTPases necessary for coat recruitment during vesicular traffic.<sup>1,2</sup> BFA is an interfacial inhibitor and structural studies have shown BFA binding within a groove formed by a complex of a GEF bound to its substrate Arf-GDP.<sup>54</sup> However, the complete number and identity of all cellular targets of BFA remain unknown.

BFA has been shown to mimic insulin action and induce the relocation of GLUT4 from intracellular compartments to cell



**Figure 3.** BFA causes redistribution of GLUT4 but not increase in glucose uptake. (A–B) Differentiated 3T3-L1 adipocytes were serum starved for 4 h, treated with insulin or BFA, or pretreated for 1 h with the Akt inhibitor MK2206 and then treated with insulin or BFA for 30 min. Cells were homogenized and fractionated to isolate plasma membrane fractions (A) and cytosol (B), and fractions were immunoblotted with indicated antibodies. Insulin and BFA increase GLUT4 levels in plasma membranes, and this is independent of Akt activity. IRAP and Tfr act as internal loading controls. The inhibitory effect of MK2206 is confirmed by lack of phospho-Akt (Ser473). Representative blots from at least 3 experiments are shown. (C) Cells treated as in A were subjected to glucose uptake. Only insulin but not BFA stimulates glucose uptake. Insulin action requires Akt activation since MK2206 reduces insulin-induced glucose uptake. Values shown are averages from at least 3 independent experiments.

surface in rat adipocytes.<sup>8</sup> This suggested that BFA may intersect with the insulin-signaling pathway and we tested BFA effects on key components of the insulin pathway. We show that BFA causes phosphorylation of IR, IRS-1, Akt and AS160. Phosphorylation requires PI3K activity because the PI3K inhibitor wortmannin abrogates the BFA-induced phosphorylation of Akt and



**Figure 4.** BFA causes phosphorylation of the FoxO1 transcription factor. **(A)** 3T3-L1 adipocytes serum starved for 4 h were treated with insulin, BFA or both for 30 min. Cells were lysed and the lysates immunoblotted with indicated antibodies. Insulin and BFA alone, or BFA and insulin together cause phosphorylation of FoxO1 (Ser256). The anti-Phospho-FoxO1 antibody also detects FoxO4 (lower band in all lanes). GAPDH provides a loading control. **(B)** HeLa cells transfected with Flag-FoxO1 for 24 h were serum starved for 4 h, pretreated or not with Akt-inhibitor MK2206 for 1 h, and then treated with insulin or BFA for 30 min. Cells were lysed and the lysates immunoblotted with indicated antibodies. Both insulin and BFA induced phosphorylation of Flag-FoxO1(Ser 256). Both effects are Akt-dependent since the Akt inhibitor MK2206 significantly diminished FoxO1 phosphorylation. Flag-FoxO1 total provides loading control. Representative blots from at least 3 experiments are shown.

diminishes the phosphorylation of AS160. We also show that BFA action is propagated through Akt since it was inhibited by Akt inhibitors (MK2206 and Perifosine). BFA effects on the insulin pathway appears specific as BFA does not interface with the mTOR pathway and the mTOR inhibitor rapamycin had no effect on BFA-induced phosphorylation of Akt or AS160.

The molecular mechanism through which BFA causes IR activation remains to be characterized: it is possible that BFA, like insulin, binds to the external domain of IR and causes a conformational shift that initiates signaling; alternatively, BFA could bind to the intracellular tyrosine kinase domains of the receptor, as shown previously for the tropical fungal metabolite L-783 281 that activates IR by directly interacting with the kinase domain.<sup>55</sup> Alternatively, BFA may utilize as yet unknown indirect mechanism for IR activation. Future studies will be needed to identify the biochemical and structural aspects of BFA effect on the insulin receptor.

Our novel finding that BFA activates the insulin pathway led us to assess BFA effects on various insulin-regulated cellular responses. One of key actions of insulin in adipocytes and muscle cells is to increase translocation of GLUT4 from intracellular stores to the plasma membrane and increase glucose uptake.<sup>56</sup> We examined BFA effect on both responses and confirmed previous

reports that BFA causes redistribution of GLUT4 from a perinuclear localization, but that this relocation is accompanied by a minimal increase in glucose uptake.<sup>8,15,48</sup> Thus, our findings indicate that BFA does not induce glucose uptake, despite causing activation of IR. These results support a two-step model in which GLUT4 translocation to cell surface and GLUT4-mediated glucose uptake are separate events that are regulated by distinct molecular mechanisms. It appears that BFA is able to induce the first step of the GLUT4 pathway, but despite activating IR, IRS-1, Akt and AS160, BFA is not able to stimulate glucose uptake. This may be due to BFA inability to induce late plasma membrane events required for fusion of IRVs with the plasma membrane and/or activation of GLUT4. Recent evidence indicate that insulin has substantial effects on the docking and fusion of IRVs with the plasma membrane and also influences the plasma membrane distribution of GLUT4 itself.<sup>57-60</sup> The molecular mechanisms through which insulin causes these events are under investigation, but it appears that BFA can't mimic these actions of insulin. It is also possible that BFA prevents correct formation of GLUT4 vesicles, and that the redistributed GLUT4 may be trafficked in transport intermediates that lack components crucial for docking and fusion with the cell surface.

In addition to regulating GLUT4 trafficking, insulin plays an essential role in maintaining metabolic balance in target cells by regulating the transcriptional repertoire through modifying the activity of FoxO transcription factors. Four FoxO family members have been identified (FoxO1, FoxO3a, FoxO4 and FoxO6) and shown to have distinct but overlapping functions.<sup>61</sup> Among them, FoxO1 is a key regulator of glucose metabolism that controls expression of crucial enzymes of gluconeogenesis and of broad spectrum of proteins involved in maintaining body homeostasis including those regulating lipid metabolism, food intake, pancreatic  $\beta$ -cells proliferation, differentiation and stress-resistance, and differentiation of adipocytes, muscle and vascular cells.<sup>16,62</sup> In addition to directly regulating the expression of target genes, FoxO1 also acts indirectly by regulating levels and activity of other transcription factors.<sup>63</sup>

Insulin regulates FoxO1 function through Akt-mediated phosphorylation that causes FoxO1 relocation from the nucleus to the cytosol and results in decreased expression of FoxO1-regulated genes. One of the genes under FoxO1 control is SIRT1, which serves as a sensor of cytosolic NAD<sup>+</sup>/NADH ratio in response to glucose deprivation, and subsequently deacetylates many transcription factors and their regulators, thus negatively or positively affecting target gene expression. SIRT1 contributes

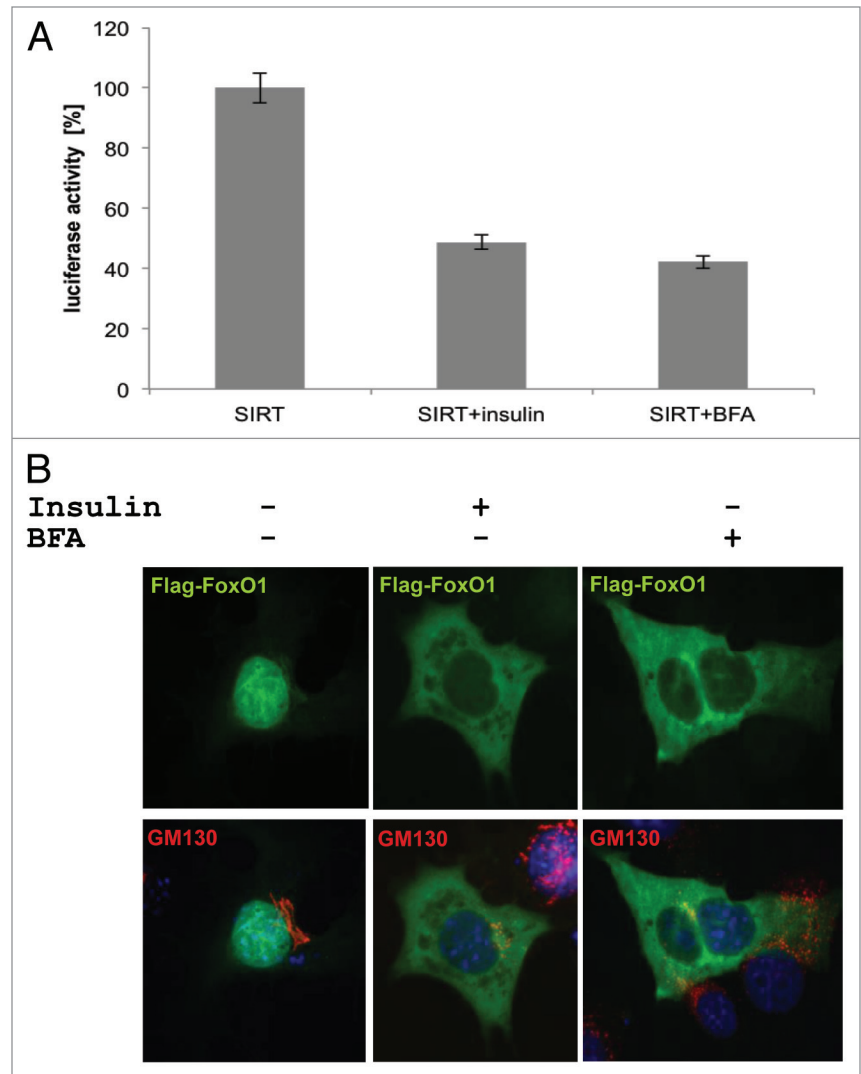
to all crucial cell functions including cell cycle progression, response to stress, apoptosis, autophagy, and metabolism.

We assessed the ability of BFA to induce FoxO1 phosphorylation, influence FoxO1 localization and regulate SIRT-1 transcription. We show that BFA causes phosphorylation of endogenous FoxO1 in 3T3-L1 adipocytes and exogenously expressed FoxO1 in HeLa cells. BFA-induced FoxO1 phosphorylation is inhibited by Akt inhibitors, emphasizing the requirement for Akt activity in the process. We also document that BFA causes the efficient redistribution of FoxO1 from nuclei to the cytosol. Furthermore, BFA causes decreased expression of a FoxO1-responsive SIRT-1-luciferase reporter gene. Because SIRT1-dependent cell functions appear to be at some level disturbed in insulin resistant medical conditions including type2 diabetes, the ability to regulate FoxO1 activity (and consequently its downstream effector target genes such as SIRT-1) by molecules and/or drugs independently of insulin may significantly improve whole body homeostasis in metabolic syndrome patients. FoxO1 has been considered a therapeutic target in metabolic diseases for many years.<sup>64,65</sup> However, progress has been slow due to difficulties in identifying efficient controllers of this transcription factor. Our findings that BFA can regulate FoxO1 function in an Akt-dependent manner may provide a starting point in characterizing BFA and its derivatives as possible therapeutic means to support therapy for type 2 diabetes and other insulin-resistant metabolic conditions. Thus, while BFA does not stimulate glucose uptake and will be ineffective in treating hyperglycemic states, the ability of BFA to mimic insulin action in FoxO1-mediated transcription suggests a new utility of BFA as a therapeutic means to support therapy for type2 diabetes and other insulin-resistant metabolic conditions.

Our findings document novel effects of BFA in cellular homeostasis: in addition to the well-understood inhibition of coat recruitment and trafficking, we show that BFA activates key cellular signaling pathway and thereby regulates numerous cellular processes. Herein, we show that BFA controls transcriptional regulation, but it is extremely likely that BFA also activates additional cellular responses. It remains to be determined whether BFA targets proteins other than Arf GEFs or whether the effects on signaling are the result of inhibiting the Arf GEFs. Inhibition of the GBF1 Arf GEF induces ER stress,<sup>66</sup> and ER stress has been shown to correlate with the phosphorylation of Akt on Ser473 through a PERK (PKR-like kinase)- and PI3K-mediated mechanism.<sup>67</sup> Thus, it is possible

that the BFA response results indirectly from the inhibition of Arf GEF activity. Alternatively, BFA may affect other cellular components, for example kinases. This has been shown for the drug AG1478, a tyrosinase inhibitor that inhibits GBF1,<sup>28</sup> and also potentially inhibits the EGF receptor tyrosine kinase.<sup>68-71</sup>

It is also noteworthy that the activation of the insulin receptor pathway occurred within 30 min of treatment, a time scale similar to that used in numerous studies exploring BFA effects on trafficking and organellar morphology (ex:<sup>30,32,34,72-77</sup>). In addition to BFA, other drugs such as LG186,<sup>3</sup> Golgicide,<sup>78</sup> AMF-26,<sup>79</sup> AG1478,<sup>80</sup> and exo2<sup>81</sup> have been shown to inhibit secretion



**Figure 5.** BFA causes nuclear exclusion of the FoxO1 transcription factor and decreases transcription of FoxO1-regulated genes. **(A)** HeLa cells were co-transfected with SIRT1-luciferase and Renilla Luciferase for 24 h, serum starved for 4 h, and then treated with insulin or BFA for 3 h. Cells were lysed and luciferase activity was measured. The data were normalized to luciferase activity in control untreated cells. Both, insulin and BFA reduce transcription of the SIRT-1 reporter. Shown are averages of at least 3 experiments. **(B)** 3T3-L1 preadipocytes were transfected with Flag-FoxO1 for 24 h, serum starved for 4h, and then treated with insulin or BFA for 30 min. Cells were processed for immunofluorescence to detect FoxO1 (green), the GM130 Golgi marker (red), and counterstained with Hoechst to detect nuclei (blue). Both insulin and BFA induce FoxO1 relocation from nuclei into the cytoplasm. Representative images from at least 3 experiments are shown.

and/or affect Golgi morphology. However, whether these drugs, like BFA, also activate multiple cellular targets remains unknown.

In summary, our studies strongly suggest that cellular phenotypes observed in studies using BFA (and perhaps other drugs) likely represent compound responses and may only partially reflect inhibition of large GEFs and Arf inactivation. Hence, it might be more prudent to use siRNA-based depletion of a protein known to be essential for a specific process than a drug with multiple targets. It is perhaps telling that treating cells with BFA leads to phenotypes that qualitatively differ from those caused by depletion of BFA-sensitive GEFs: BFA treatment causes the complete collapse of the Golgi and relocation of Golgi proteins to the ER while depletion of GBF1 causes extensive tubulation of the Golgi with Golgi proteins remaining in tubules or in ER exit sites/ERGIC.<sup>3,29,82,83</sup> Similarly, BFA causes the collapse of recycling endosomes within the peri-centrosomal region, while depletion of BIG1/2 causes extensive tubulation without collapse.<sup>84-89</sup> The additional phenotypes might be due to BFA effects on signaling pathways, in agreement with growing evidence that numerous signaling pathways modulate Golgi structure and function.<sup>90-93</sup>

## Experimental Procedures

### Antibodies

Rabbit, polyclonal anti-Akt rabbit polyclonal anti-phospho-Akt (Ser 473), rabbit polyclonal anti-phospho-AS160 (Ser 588), rabbit polyclonal anti-phospho-AS160 (Thr 642) AS160, rabbit polyclonal anti-FoxO1 (Ser 256 Fox) were from Cell Signaling Technology, Inc. (Danvers, MA). Goat polyclonal anti-GLUT4(C-20) (used for western blot), rabbit polyclonal anti-IR( $\beta$ )(C-19) rabbit polyclonal anti-IRAP, rabbit polyclonal anti-IRS1(A-19), mouse monoclonal anti-phosphotyrosine (PY20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-GAPDH, rabbit polyclonal anti-GLUT4 (used for immuno-fluorescence) antibody were from Abcam (Cambridge, MA). Mouse monoclonal anti-transferrin receptor antibody was from Invitrogen (Madison, WI). Mouse monoclonal anti-GM130 was from BD Transduction Laboratories (Mississauga, ON). Mouse monoclonal anti-Flag antibody was from Sigma-Aldrich (St. Louis, MO).

Anti-rabbit and anti-mouse antibodies conjugated with HRP were from Pierce/Thermo Fisher Scientific Inc. (Rockford, IL), anti-goat conjugated with HRP antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit Alexa 488 or Alexa 594 and anti-mouse Alexa 488 or Alexa 594 were from Invitrogen (Madison, WI).

### Plasmids

Construct encoding FoxO1-Flag was kindly provided by Dr. K. Kandror (Boston University). Construct encoding Luc-SIRT1 (pGL3-rSIRT1 promoter 1.5kb luciferase) was a generous gift from Dr. RW Alexander (Emory University).

### Reagents

MK2206 and Perifosine were from Selleckchem (Houston, TX). MK2206 was used at 20  $\mu$ M (stock in DMSO) and perifosine was used at 20  $\mu$ M (stock in ethanol). Wortmannin, was from Sigma (St. Louis, MO) and was used at 1 mM,

rapamycin was from Sigma-Aldrich (St. Louis, MO) and was used at 100 nM, BFA was from Cell Signaling Technology (Danvers, MA) and was used at 5  $\mu$ g/ml (from a 1000X stock in ethanol), insulin was from Sigma-Aldrich (St. Louis, MO) and was used at 100 nM. Hoechst was from Invitrogen (Madison, WI) and was used at 0.5 mg/ml.

### Mammalian cell culture, differentiation and transfection

Mouse 3T3-L1 preadipocytes (embryonic fibroblasts) were purchased from American Type Cell Culture collection (Manassas, VA). Cells were maintained in preadipocyte medium (DMEM, high glucose 4.5g/L, 10% bovine calf serum (BCS), penicillin, streptomycin, amphotericin B) at 37 °C, with 5% CO<sub>2</sub> in humidified incubator. For differentiation into adipocytes, cells were grown until 100% confluent and then incubated additional 48 h when medium was replaced with differentiation medium (DMEM/Ham's F-12(1:1, v/v), 15 mM HEPES pH 7.4, 10% fetal bovine serum (FBS), containing biotin, pantothenate, human insulin, dexamethasone, 100 units/ml penicillin and 100 mg/ml streptomycin, amphotericin B, isobutylmethylxanthine and PPAR $\gamma$  agonist). Cells were maintained in this medium for 4 days. Subsequently, the differentiation medium was replaced with maintenance medium (DMEM/Ham's F-12(1:1, v/v), 15mM HEPES pH 7.4, 10% fetal bovine serum (FBS), biotin, pantothenate, dexamethasone, 100 units/ml penicillin and 100 mg/ml streptomycin, amphotericin B). All experiments were performed on cells in passage 7–9 and after 8–10 d post differentiation.

HeLa cells were grown in Minimum Essential Medium (MEM supplemented with glucose and glutamine supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin and 1 mM sodium pyruvate. All these reagents were purchased from Cellgro (Manassas, VA). Cells were grown at 37 °C in 5% CO<sub>2</sub> in 6 well dishes till ~75% confluent and then were transfected using Mirus TransIT-LT1 Transfection Reagent (Mirus Bio Corp., Madison, WI) according to manufacturer's protocol. In some cases, cells were grown on glass coverslips to ~75% confluence and then transfected. After transfection, cells were grown overnight and processed for immunofluorescence.

### Immunofluorescence microscopy

Cells were washed in phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde for 10 min, and quenched with 10mM ammonium chloride. Cells were permeabilized with 0.1% Triton X-100 in PBS for 7 min. The coverslips were then washed with PBS and blocked in, 2.5% goat serum, 0.2% Tween 20 in for 5 min followed by blocking in PBS, and in 0.25% fish skin gelatin, and 0.2% Tween 20 in PBS. Cells were incubated with primary antibody diluted in fish skin gelatin for 1 h at room temperature. Coverslips were washed with PBS- 0.2% Tween20, blocked like above 20 and incubated with secondary antibodies diluted in goat serum for 45 min. Coverslips were washed as described above and mounted on slides in ProLong Gold antifade reagent (Invitrogen, Madison, WI). Fluorescence images were captured on Perkin Elmer ERS 6FE spinning disk confocal microscope and images were processed and analyzed in Volocity version 5.3 software (Perkin Elmer, Shelton, CT).



### SDS-PAGE and immunoblotting

Cells were lysed with RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, containing complete proteases inhibitors Cocktail, EDTA-free (Santa Cruz, CA). Lysates were resolved on 8% SDS-PAGE and transferred to NitroPure nitrocellulose (NC) membrane (Micron Separations, Westborough, MA) by wet transfer for 90 min at 100V at room temperature. In most cases, the same NC was cut into sections and probed with the indicated antibodies as in.<sup>94</sup>

### Immunoprecipitation

For immunoprecipitating with anti-phosphotyrosine antibodies 3T3-L1 preadipocytes were grown on 10 cm plates and differentiated into adipocytes as described above. Cells were washed 3 times in PBS, serum starved for four hours and left untreated or treated with insulin or BFA for 30 min. Then, Cells were washed 3 times in PBS and lysed in 500  $\mu$ l of lysis buffer (25 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol), supplemented with proteases and phosphatases inhibitors cocktail EDTA-free (Pierce, ThermoScientific Rockford, IL), passed through syringe with 26 $\frac{1}{2}$  needle and centrifuged for 10 min at 18,000  $\times$  g, 4 °C. Post-nuclear supernatants were pre-cleared with 10ml protein-G-agarose beads (50% slurry from Roche, Indianapolis, IN) over night, at 4 °C with rotation, and then centrifuged for 2 min at 1,000 rpm (110xg). Anti-phosphotyrosine antibodies (1 $\mu$ g/mg of total protein) were added to precleared supernatant and incubated overnight at 4 °C with rotation, followed by adding 30ml of 50% slurry of protein-G-agarose beads and incubating overnight at 4 °C with rotation. Beads were washed 3 times in 0.5 ml lysis buffer by centrifugation for 2 min at 1,300 rpm. Immunoprecipitated proteins were released from beads in loading buffer, and resolved in 8% SDS-PAGE blotted with anti-IR( $\beta$ ) and anti-IRS-1 antibodies (respectively).

### Cell fractionation

3T3-L1 preadipocytes were grown and differentiated into adipocytes in 15 cm plates, serum starved for 4 h, pre-treated for 1 h with Akt inhibitor MK2206 (20  $\mu$ M) where indicated and then treated for 30 min with insulin (100 nM) or BFA (5  $\mu$ g/ml). Cells were washed and vigorously scrapped from plate three times with ice-cold PBS, and 2 ml and re-suspended in HES buffer (20 mM HEPES, 1 mM EDTA, 255 mM sucrose, pH7.4, containing protease inhibitors cocktail), was added to each plate. Cells were scrapped and passed 12 times through a 23 $\frac{1}{2}$  gauge needle. Disrupted cells were centrifuged for 20 min at 19,000  $\times$  g in TI75 rotor. Pellets were resuspended in 5 ml HES buffer and again centrifuged for 20 min at 19,000xg in TI75 rotor. Pellets were resuspended in 5ml HES buffer and layered onto 6.3ml 38.5% (1.12M) sucrose cushion in HES buffer and centrifuged for 60 min at 100,000  $\times$  g in SW41 swinging rotor. Plasma membrane fractions, were collected from interface. All steps were performed in 4 °C.

### Glucose uptake

3T3-L1 preadipocytes were grown and differentiated in 6- or 24-well plates. Glucose uptake in fully differentiated adipocytes

was performed using enzymatic method as described.<sup>95</sup> Briefly, adipocytes were washed 3 times in PBS and incubated in were serum starved starvation medium (DMEM F12 50/50 supplemented only 100 units/ml penicillin and 100 mg/ml streptomycin) for 4 h, and pre-treated for 1 h with MK2206 (Akt-inhibitor) where indicated. Then, cells were washed 3 times in (Krebs-Ringer-HEPES), containing 0.1% bovine serum albumin (BSA) KHR: (50mM HEPES, 137mM NaCl, 4.7mM KCl, 1.85mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub> and 0.1% BSA, pH 7.4) buffer and treated for 30 min with insulin (100nM) or BFA (5 $\mu$ g/ml). Then, 2-deoxyglucose (Sigma-Aldrich, St. Louis, MO) was added to final concentration of 2mM, cells were incubated for 20 min, and washed three times in KHR buffer. Then cells were lysed by adding 150 $\mu$ l of 100mM NaOH to each well of a 24-well plate and incubating at 72 °C for 30 min. Then, 150 $\mu$ l of 100mM HCl was added to neutralize NaOH, followed by adding 300 $\mu$ l of TEA buffer (200mM triethanolamine hydrochloride containing 200mM KCl, pH 8.1). For the enzymatic reaction, 25 $\mu$ l of each sample was placed in 96-well plate, followed by adding 200 $\mu$ l of the assay solution (50mM TEA buffer, 50mM KCl, pH 8.1, 0.02% BSA, 0.1 mM NADP, 0.2 units/ml Diaphorase from *Clostridium kluyveri*, 10mM Resazurin sodium salt and 15 units/ml *L. mesenteroides* G6PDH) and incubation for 45 min at 37 °C. The fluorescence was read in Biotek Synergy 2 Fluorometer. Each experiment was repeated four times. The concentrations of 2-deoxyglucose taken up and standard deviations are shown. For the standard curve, following concentrations of 2-deoxy-D-glucose-6-phosphate sodium salt (Santa Cruz Biotechnology) in 50mM TEA, pH 8.1 were prepared: 1.875, 3.75, 7.5, 15 and 0-30mM and 25 $\mu$ l of each was used for the enzymatic reaction, as described above.

### Luciferase assay

HeLa cells were grown in 6-well plates and co-transfected in triplicate with pGL3-SIRT-luciferase and internal control Renilla luciferase vector pRL/CMV and empty pGL2-vector. After 24 hours, cells were serum starved for 4 h, washed in PBS and treated with insulin or BFA for 3hours. Cells were washed in PBS and lysed in 500 $\mu$ l of lysis buffer (25mM Triphosphate, pH7.8, 2mM DTT, 2mM diaminocyclohexanetetra-acetic acid (DCTA), 10% glycerol, 1% Triton X-100), by gently shaking for 15 min at room temperature. Cell lysates were analyzed for luciferase activity using Dual-Luciferase Reporter Assay (Promega, Madison, WI), according to manufacturer's protocol and according to Harms K.L. et al.<sup>96</sup> All required reagents were provided in Dual-Luciferase Reporter assay kit. Percentage of basal luciferase activity and standard deviations are shown.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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