Phosphorylation of TXNIP by AKT Mediates Acute Influx of Glucose in Response to Insulin

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SUMMARY

Growth factors, such as insulin, can induce both acute and long-term glucose uptake into cells. Apart from the rapid, insulin-induced fusion of glucose transporter(GLUT)4 storage vesicles with the cell surface that occurs in muscle and adipose tissues, the mechanism behind acute induction has been unclear in other systems. Thioredoxin interacting protein (TXNIP) has been shown to be a negative regulator of cellular glucose uptake. TXNIP is transcriptionally induced by glucose and reduces glucose influx by promoting GLUT1 endocytosis. Here, we report that TXNIP is a direct substrate of protein kinase B (AKT) and is responsible for mediating AKT-dependent acute glucose influx after growth factor stimulation. Furthermore, TXNIP functions as an adaptor for the basal endocytosis of GLUT4 in vivo, its absence allows excess glucose uptake in muscle and adipose tissues, causing hypoglycemia during fasting. Altogether, TXNIP serves as a key node of signal regulation and response for modulating glucose influx through GLUT1 and GLUT4.

Graphical abstract

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SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.041.

AUTHOR CONTRIBUTIONS
N.W. conceived the idea and designed and carried out most of the experiments with the help of A.N.W. and H.D. FDG-PET imaging and analysis were done by A.S.P., E.A.B., and Z.B.M. K.V. and M.H. helped with confocal imaging. J.W. and T.E.M. performed the GLUT4 localization assay. B.Z. did the initial kinase assay. L.C.C. provided intellectual input and guidance.
INTRODUCTION

Protein kinase B (AKT) is an important kinase in the processes of cellular growth and metabolism. Activation of AKT is known to increase glucose uptake into cells for energy storage in muscle and adipose tissues, as well as for energy consumption in cells that are rapidly dividing, such as in cancer. However, the mechanisms of glucose uptake for these two purposes are typically studied separately. For energy storage, uptake is acutely induced by insulin on a timescale of minutes. During this time, activation of AKT downstream of the PI3K (phosphatidylinositol 3-kinase) pathway induces a rapid fusion of glucose transporter (GLUT)4 storage vesicles with the cell surface via phosphorylation of a number of vesicular trafficking proteins, including AKT substrate of 160kDa (AS160), a ras-like in rat brain (Rab) GTPase-activating protein (Eguez et al., 2005; Leto and Saltiel, 2012). On the other hand, the increase in glucose use by rapidly dividing cells has been primarily attributed to a long-term rise in glucose transporter expression, particularly GLUT1 (Elstrom et al., 2004).

GLUT1 and GLUT4 belong to the 14-member facilitative glucose transporter family (Augustin, 2010). While not all members transport glucose, they all share the characteristic that no energy beyond the difference in concentration gradient is required for their metabolite of interest to pass through the transporter. The family is divided into three classes based on their known or predicted topology: class I includes the most well-known glucose transporters, GLUT1 through 4; class II includes the fructose transporter GLUT5 and the urate transporter GLUT9; and class III transporters are less well characterized and studied.

TXNIP (thioredoxin-interacting protein) is an α-arrestin protein that plays a role in regulating glucose and lipid metabolism (Bodnar et al., 2002; Sheth et al., 2005). TXNIP knockout (KO) animals incur hyperlipidemia when fed normally and become hypoglycemic under fasting conditions. TXNIP gene transcription is upregulated by glucose via the transcription complexes carbohydrate-responsive element-binding protein (chREBP)/max-like protein (Mlx) and Mlx-interacting protein (MondoA)/Mlx (Cha-Molstad et al., 2009;
Stoltzman et al., 2008). TXNIP protein negatively regulates glucose uptake in cells via its role as a GLUT1 adaptor to the clathrin-mediated endocytosis machinery (Parikh et al., 2007; Wu et al., 2013). This supports the model in which TXNIP suppresses glucose influx: too much glucose entering the cell induces the TXNIP protein in order to limit glucose uptake. We previously established that AMP-activated kinase (AMPK) stimulates glucose uptake by phosphorylating TXNIP, causing its degradation, thus releasing GLUT1 from endocytosis (Wu et al., 2013). As such, TXNIP serves as a key regulatory point for cells in deciding how to respond to acute energy stress. In addition, TXNIP is reported to be involved in inflammasome function due to its interaction with thioredoxin (Zhou et al., 2010). Since inflammasome is activated by ROS (reactive oxygen species) generated from mitochondria (Zhou et al., 2011), TXNIP may also modulate inflammasome activation via its regulation on glucose influx and subsequent mitochondria activity in response to glucose level changes.

In this report, we describe a mechanism whereby growth factors also induced acute glucose uptake into cultured cells through the phosphorylation of TXNIP by AKT at the same site that AMPK phosphorylates. Moreover, we confirm that TXNIP can interact with GLUT4 and play a role in basal GLUT4 endocytosis in vivo. These combined findings suggest that TXNIP acts as a general endocytosis adaptor for class I glucose transporters GLUT1 and GLUT4 and as a node for regulating glucose transport activity in response to various stimuli and stresses.

RESULTS

Growth Factor Stimulation Led to an Upshift in TXNIP Mobility on SDS-PAGE

Because TXNIP mediates acute glucose uptake into cells after AMPK activation, we investigated whether TXNIP is similarly involved in growth factor-induced acute glucose uptake. We stimulated cancer cell lines with growth factors for 15 min—HepG2 cells with HGF (hepatocyte growth factor), SKBr3, and BT474 cells with EGF (epidermal growth factor)—and found that TXNIP showed its characteristic mobility upshift on SDS-PAGE gels, as we had previously observed after AMPK activation (Figure 1A). At the same time, the growth factor treatment stimulates acute glucose uptake into the cells. Using differentiated 3T3 adipocytes as surrogates for adipose tissue, we treated these cells with insulin and observed the same upshift of TXNIP. Only the upshifted TXNIP band reacted toward an antibody against the TXNIP phosphorylated serine 308 (S308) site, the same site AMPK acts upon. We verified the specificity of this antibody toward TXNIP using TXNIP primary MEFs (mouse embryonic fibroblasts).

To confirm that AMPK was not the kinase that phosphorylated TXNIP under these conditions, we treated AMPK WT (wild-type) MEFs and AMPK α1/α2 DKO (double-knockout) MEFs with IGF (insulin growth factor) or 2DG (2-deoxyglucose) (Figure 1C). As expected, 2DG activated AMPK, inducing phosphorylation of TXNIP on S308 in an AMPK-dependent manner. On the other hand, IGF also caused phosphorylation of TXNIP on S308, independent of AMPK. Even though AMPK activation caused more dramatic phosphorylation on TXNIP S308, TXNIP was clearly being phosphorylated on S308 during growth factor stimulation in the absence of AMPK activation.
Since growth factors stimulation and AMPK activation causes the same phosphorylation on TXNIP, we wondered if the effects are additive. We treated HepG2 cells with HGF or 2DG separately and together, indeed HGF and 2DG together induce more complete upshift/S308 phosphorylation of TXNIP (Figure 1D). This means both pathways converge on TXNIP in regulating acute glucose uptake.

Activated AKT Phosphorylated TXNIP at S308 on the Plasma Membrane

Growth factors are known to activate multiple pathways, including MAPK (mitogen-activated protein kinase) and PI3K. Since TXNIP S308 is not followed by a proline, the substrate motif required for kinases in the MAPK pathway, we explored whether any kinase downstream of PI3K was responsible for this phosphorylation. Stimulation by growth factors recruits PI3K to the plasma membrane, leading to accumulation of PI (phosphatidylinositol) (3,4,5)P$_3$ on the membrane, which then recruits AKT. The activation of AKT requires the additional phosphorylation of its own S308 site by phosphoinositide-dependent kinase-1 (PDK1). The other kinase family SGK (serum and glucocorticoid-inducible kinase) is also activated downstream of PI3K and shares a similar substrate motif as AKT (Bruhn et al., 2010).

In an effort to identify the kinase responsible for TXNIP S308 phosphorylation, we first assessed the effect of inhibitors: BKM120 is an inhibitor of PI3K, GSK2334470 is an inhibitor of PDK1, MK2260 and AZD5363 are inhibitors of AKT, and GSK650394 is an inhibitor of SGK. We serum-starved HepG2 cells and treated them with various inhibitors for 1 hr prior to stimulation with HGF for 15 min (Figure 2A). All AKT-pathway inhibitors prevented AKT activation and abolished the TXNIP upshift on the gel, but inhibiting SGK had no notable effect. AKT inhibition was confirmed by monitoring the phosphorylation of its known substrate, forkhead box O1/3 (FOXO1/3), while SGK inhibition was confirmed by the phosphorylation of its substrate, NDRG346 (note that AZD5363 is an active site inhibitor of AKT and does not prevent AKT phosphorylation by PDK1 on S308). We also tested the mammalian target of rapamycin (mTOR) and S6 kinase (S6K) due to their involvement in AKT and AMPK activation using their inhibitors TORIN1 and LY2584702 and found they are not responsible for TXNIP S308 phosphorylation under growth factor stimulation (Figure S2A). These results strongly suggest that AKT is required for TXNIP S308 phosphorylation. However, the TXNIP S308 site does not match the optimal substrate motif required for AKT, namely RxRxxS/T (Manning and Cantley, 2007). Because TXNIP is partially localized to the plasma membrane, we hypothesize that co-localization on the plasma membrane is required for and facilitates activated AKT to phosphorylate TXNIP.

Because many endocytic adaptors bind to a phosphatidylinositol lipid marker of the plasma membrane PI(4,5)P$_2$ including β-arrestin (Reider and Wendland, 2011), we reasoned that TXNIP likely interacts with PI(4,5)P$_2$. By threading TXNIP sequence onto arrestin-2, whose complex structure with inositol hexakisphosphate is known (Milano et al., 2006), we identified Lys223 and Arg238 as candidates for lipid interactions and confirmed that simultaneous mutation of both residues to alanine (Ala) abolished TXNIP plasma membrane localization (Figure 2B). This mislocalization of the lysine/Arginine (KR) mutant can also be confirmed with cellular fractionation (Figure S2B). Using this KR mutant (K233A/
R238A) as a control, we carried out in vitro kinase assays with full-length AKT in the presence or absence of liposomes (Figure 2C). Liposomes were made from either a 1:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) alone (control) or that mixture plus P(3,4,5)P3. AKT exhibited significant kinase activity on TXNIP only in the presence of phosphoinositides (PIP3)-containing liposomes, and no AKT kinase activity was found in the presence of the AKT inhibitor AZD5363, implying that AKT recruitment to a PIP3-containing surface is required for TXNIP phosphorylation. On the other hand, the KR mutant remained non-phosphorylated even in the presence of PIP3-containing liposomes, suggesting TXNIP co-localization to the same surface as AKT is also required. Moreover, the S308 on TXNIP was the site of phosphorylation by AKT.

We further confirmed these results in cell lines. We made HepG2 cells that stably expressed HA-TXNIP-WT, HA-TXNIP-S308A, or HA-TXNIP-KR constructs, then treated these cells with HGF after pretreatment with either MG132 (a proteasome inhibitor) or MK2206 (an allosteric AKT inhibitor) (Figure 2D). HA-tagged proteins were immunoprecipitated and probed for phosphorylation on S308. TXNIP-WT protein was phosphorylated after HGF treatment and accumulated if the proteasome was inhibited. This phosphorylation was diminished by MK2206 treatment and was absent for S308A and KR mutants. Similar results were observed in SKBr3 cells stably expressing various HA-tagged TXNIP constructs (Figure 2E).

From the combined evidence above, we conclude that AKT is the kinase that directly phosphorylated TXNIP on S308 following growth factor stimulation.

**Effect of TXNIP Phosphorylation on Glucose Uptake and Phosphoinositide Interaction**

We have previously shown that TXNIP can be phosphorylated on S308 by AMPK under energy stress (Wu et al., 2013). This phosphorylation leads to dissociation of TXNIP from the glucose transporter GLUT1, preventing GLUT1 from being endocytosed and consequently permitting an acute increase in glucose influx. Phosphorylation on the same TXNIP site by AKT is expected to result in an equivalent increase in glucose influx.

First, we verified that TXNIP S308 phosphorylation was necessary for AKT-induced glucose uptake in primary MEFs (Figures 3A and 3B). We isolated primary MEFs from TXNIP KO mice, reconstituted them with either HA-TXNIP WT or HA-TXNIP S308A mutant constructs, and carried out glucose uptake assays using 3H-2DG. We found increased glucose uptake after IGF stimulation, but only in HA-TXNIP WT reconstituted cells in the absence of the AKT inhibitor MK2206. Here, we assume that the major glucose transporter expressed on primary MEFs is GLUT1, based on the BioGPS database (http://www.biogps.org), and that the differential glucose uptake is through TXNIP regulation of GLUT1. AKT plays an important role downstream of insulin signaling in the acute response to elevated blood glucose levels through its regulation of GLUT4 trafficking. In cultured adipocytes, TXNIP overexpression results in the inhibition of glucose uptake, while knocking down TXNIP using siRNA (small interfering RNA) results in increased glucose uptake (Parikh et al., 2007). Here, we found that phosphorylation on TXNIP S308 was a
major event downstream of AKT activation, which is required for insulin-stimulated glucose uptake in cultured adipocytes (Figures 3C and 3D).

We began by generating 3T3L1 TXNIP KO clones using CRISPR technology. After differentiating the KO cells into adipocytes, we re-expressed either the HA-TXNIP WT or a S308A mutant in these cells using lentivirus at a similar level as the endogenous TXNIP in WT 3T3L1 adipocytes (Figure S3A). Insulin stimulation led to the phosphorylation of S308 only on the HA-TXNIP WT, not on the mutant, this phosphorylation was abolished by pretreatment with the AKT inhibitor MK2206 (Figure 3D). We carried out glucose uptake assays under these conditions using tritiated 2-deoxyglucose (Figure 3C). In the cells re-expressing WT TXNIP, glucose uptake increased significantly 15 min after insulin stimulation, this increase was partially suppressed by pretreatment with MK2206. In cells re-expressing S308A mutant TXNIP, the increase in glucose uptake after insulin stimulation was significantly less, comparable to the level with MK2206 inhibition. This indicates that TXNIP plays a role in the AKT-responsive part of glucose influx into adipocytes after insulin stimulation. TXNIP’s C-terminal tail also contains other phosphorylation sites, including some that are sensitive to the MAPK pathway (unpublished data and PhosphoSitePlus). Presumably, the glucose influx that cannot be suppressed by MK2206 results from the activation of other pathways by insulin. Both GLUT1 and GLUT4 are expressed in 3T3 adipocytes (Piper et al., 1991), so the glucose uptake results can be due to either or both transporters.

Previously, we noticed that TXNIP could interact with phosphoinositides due to their negative charges. We modified a liposome floatation assay to see if we could detect a difference in phosphoinositide binding between the WT and P308 (S308 phosphorylated) TXNIP protein (Figures 3E and 3F). Liposomes from either control lipids DOPC/DOPS (1:1) or DOPC/DOPS containing various phosphoinositides were generated by freeze-thaw cycles. After incubating the liposomes with TXNIP protein, OptiPrep was mixed in to generate a dense gradient of 36% and loaded as the bottom layer in ultracentrifuge tubes. An equal volume of 31% OptiPrep in buffer was loaded as the middle layer to prevent unbound protein from mixing with the top layer. Finally, 5% of OptiPrep in buffer was loaded as the top layer. On high-speed centrifugation, liposomes ascend to the interface between the 5% and 31% layers. Using this simple method, we could show that TXNIP binds to and floats with liposomes containing phosphoinositides. TXNIP exhibited stronger interaction for PIP2 and PIP3 than for singly phosphorylated inositides, but did not show any preference for any specific phosphorylated site. With this crude assay, we were able to show a dramatic difference between the behavior of WT and P308 proteins (Figure 3G). TXNIP phosphorylated on S308 was generated by an in vitro kinase reaction between TXNIP and AMPK. The inability of P308 to bind to PI(4,5)P2 and PIP3-containing liposomes indicates that activation of AKT will likely cause TXNIP to detach from the plasma membrane, whether it is interacting with a glucose transporter or not. This provides a more definitive way to attenuate TXNIP inhibition on glucose transport. Rather than simply shutting down already-interacting proteins, this loss of binding affinity through phosphorylation prevents formation of new TXNIP-GLUT complexes as well.
TXNIP Regulation of GLUT4

Because all class I glucose facilitative transporters have a high sequence homology (Deng et al., 2014), and given that TXNIP can interact with GLUT1, it would not be surprising if TXNIP can also interact with other class I members. Indeed, we can show that TXNIP interacts with GLUT1 through GLUT4 in a signal-dependent manner using IP (immunoprecipitation) (Figure S3B) and endocytosis assay (Figure S4) (Blot and McGraw, 2008b). We will focus here on GLUT4 due to its importance in metabolic syndrome and diabetes.

GLUT4 is the major glucose transporter in muscle and adipose tissues. Given the quantity of such tissues in the body, they function as the body’s main storage for excess glucose. GLUT4 activity is primarily determined by its location. After a meal, insulin stimulates GLUT4 storage vesicles to fuse with the cell surface membrane, allowing rapid uptake of glucose into the cell through GLUT4 and thus returning the blood glucose level to normal. AKT plays a major role in regulating the steps in the trafficking of GLUT4 vesicles in response to insulin (Leto and Saltiel, 2012). In the absence of insulin, GLUT4 must be kept away from the cell surface to prevent hypoglycemia, this is accomplished by a continuous basal-level endocytosis of GLUT4 that is inhibited during insulin stimulation, followed by a rapid return to normal endocytosis afterward. Both processes involve clathrin-dependent and clathrin-independent pathways (Antonescu et al., 2009; Blot and McGraw, 2006).

For the clathrin-dependent pathway, the N-terminal F5QQI on GLUT4 acts as an adaptor protein AP2 binding signal, even though it is sub-optimal to the AP2 recognition motif YXXΦ. Phenylalanine substitution for tyrosine in the YXXΦ motif yields weaker binding, because the Tyr side chain forms a strong hydrogen bond with an Asp residue in the binding pocket of the μ2 subunit (Owen and Evans, 1998; Piper et al., 1993). The C-terminal LL490 (Al-Hasani et al., 2002; Antonescu et al., 2009; Blot and McGraw, 2006; Govers et al., 2004; Piper et al., 1993) of GLUT4 matches the binding motif [DE]XXX[L/I] to the α-σ2 subunit of AP2 (Kelly et al., 2008), except the acidic residue at the L-4 position is an Arg in GLUT4. Mutating either motif affects GLUT4 endocytosis, but through different mechanisms (Blot and McGraw, 2008a). An F5QQI to A5QQI mutation affects the distribution of GLUT4 in both the basal and return-to-normal states, while mutating LL490 slows the endocytosis of GLUT4 after insulin termination due to defects in cytoplasmic vesicular sorting involving AP1 (Blot and McGraw, 2008a). So far, it is unclear how insulin could inhibit the F5QQI and clathrin-dependent basal GLUT4 endocytosis pathway.

We hypothesize that TXNIP binds to GLUT4 and facilitates its endocytosis under basal conditions. Through its own C-terminal tail interaction to the clathrin endocytosis machinery (Wu et al., 2013), TXNIP may act as a “double catch” together with the GLUT4 F5QQI motif to keep GLUT4 presence on the cell surface low, but this double catch has to be disrupted during insulin stimulation to prevent unwanted GLUT4 endocytosis.

To show that TXNIP affects GLUT4 endocytosis in adipocytes, we measured how fast surface HA-GLUT4 is internalized in the presence and absence of TXNIP in 3T3 adipocytes using a quantitative imaging method as before (Blot and McGraw, 2008b). As expected, the endocytosis rate is faster in WT 3T3 adipocytes than in TXNIP KO clone 3 (Figure 4A). Re-
expressing TXNIP in clone 3 can partially rescue the phenotype. We can also show through IP and endocytosis assay that the interaction between GLUT4 and TXNIP is independent of both F5QQI and LL490 on GLUT4 (Figures S3B and S4).

The consequence of slower GLUT4 basal endocytosis is higher steady-state GLUT4 cell surface distribution in the absence of TXNIP (Figure 4B). Here, we used the reporter HA-GLUT4-GFP construct with the HA-tag inserted into the first extracellular loop of GLUT4 and with GFP fused at the GLUT4 C-terminus. This construct allowed for quantification of cell-surface GLUT4 using a HA-tag antibody relative to the total cellular GLUT4 measured by GFP (Blot and McGraw, 2008b). This difference disappears when the cells are treated with insulin, consistent with our model that insulin stimulation inhibits TXNIP GLUT4 interaction.

Next, we decided to investigate the physiological role TXNIP plays in blood glucose clearance. TXNIPflox/flox mice were purchased from the Jackson laboratory (JAX) and crossed with CMV-Cre mice to generate global KO animals. After overnight fasting to reduce the influence of insulin, we carried out a 18FDG-PET (18F-2-deoxyglucose positron emission tomography) scan with a small amount of 18FDG injected through the tail vein. Consistent with our model, the TXNIP KO animals had visibly higher 18FDG absorption into skeletal muscle than did WT animals (Figure 4C). To quantitate 18FDG absorption, tissues were dissected and their radioactivity was measured in a γ-counter. Indeed, skeletal muscle and adipose tissues absorbed more glucose in the KO mice under starved conditions than in the WT mice (Figures 4D and 4E), and this was not due to increased expression of GLUT4 (Figure 4H). TXNIP KO mice have been reported to be hypoglycemic under starved conditions (Sheth et al., 2005), partly due to reduced gluconeogenesis from the liver in the KO mice (Chutkow et al., 2008). We confirmed the hypoglycemic phenotype during our 18FDG-PET experiment (Figure 4G). The liver of KO animals behaved as expected of hypoglycemic animals, with decreased glucose uptake to conserve glucose availability for organs, such as the brain (Figure 4F). In the absence of TXNIP, GLUT4 cannot be endocytosed efficiently, leaving a higher basal level of GLUT4 on cell surfaces, resulting in excess glucose influx into non-vital skeletal muscle and adipose tissues during fasting conditions. It is likely that this unregulated glucose uptake into muscle and adipose tissue contributes to the hypoglycemic phenotype of fasting TXNIP KO mice.

**DISCUSSION**

We provide evidence that activated AKT induces rapid glucose influx into cells by phosphorylating TXNIP on S308. We also show that TXNIP facilitates GLUT4 endocytosis under basal conditions. In muscle and adipose tissue, TXNIP is required as a double catch for GLUT4 endocytosis in order to prevent hypoglycemia under fasting conditions (Figure S1). Moreover, during insulin stimulation, disruption of the TXNIP–GLUT4 interaction by phosphorylation of TXNIP is necessary to reduce GLUT4 endocytosis. We predict that a mouse carrying a knockin TXNIP S308A mutant will not be able to clear glucose as quickly from the blood after insulin injection as well as the WT can. It remains unclear whether TXNIP is involved in a return-to-normal GLUT4 endocytosis after insulin stimulation ends. This may depend on the duration and amplitude of the insulin signal. Stronger and longer
insulin stimulation may lead to both the degradation of existing TXNIP protein and the inhibition of TXNIP transcription by AKT-dependent FOXO phosphorylation (further discussed below). If there is no TXNIP protein at the end of insulin stimulation, then TXNIP cannot play a role in returning GLUT4 endocytosis to normal. Carefully designed and controlled experiments are required to test this possibility. Our model further predicts that, in muscle, activated AMPK induced by exercise will also phosphorylate TXNIP in order to increase cell-surface GLUT4 for glucose uptake.

Together with our previous data showing that AMPK regulates acute glucose uptake via TXNIP and GLUT1, TXNIP appears to serve as an adaptor and regulatory point for the class I glucose transporters GLUT1 and GLUT4. The use of post-translational modifications of TXNIP permits a cell to adjust its glucose uptake very rapidly in response to the cellular environment and its energetic needs, however, the specifics of this regulation depend on the tissue and cellular context. For example, AKT regulation of GLUT1 function via TXNIP can explain the long-standing question of how PI3K/AKT pathway activation produces immediate glucose uptake in cancer cells. As another example, GLUT1 is highly expressed in the brain where TXNIP expression is very low, rendering the brain’s glucose usage unresponsive to insulin signaling through TXNIP.

There are two other members of the class I glucose facilitator family, GLUT2 and GLUT3, which have predicted 3D structures similar to that of GLUT1. GLUT2 is expressed mainly in liver and pancreas β-islet cells, while GLUT3 is expressed in energetically demanding tissues, such as the brain and testis. Even though we provide some evidence here that TXNIP binds and facilitates GLUT2 and GLUT3 endocytosis, whether TXNIP regulates GLUT2 and GLUT3 functions in vivo remains a question for future studies.

TXNIP is highly dynamic, responding to environmental signals rapidly on both the protein and mRNA levels. The TXNIP protein is regulated on the same residue, S308, by both AMPK and AKT, which are generally thought to regulate opposing pathways. AMPK stimulates catabolic processes to increase the available usable energy source and shuts down anabolic processes to conserve energy under conditions of scarce food, AKT stimulates anabolic processes to encourage food storage and growth. These two apparently different functions converge on one point mechanistically, at the beginning of metabolic change. Specifically, both pathways stimulate glucose uptake into the cell, be it in nutrient-starved cancer cells or in muscle cells after insulin stimulation. Because TXNIP serves as a gatekeeper for glucose transporters, the cell employs this same central molecule to respond to a demand for increased glucose uptake. Beyond S308, there are many other post-translational modification sites on TXNIP’s unstructured C-terminal domain. Additional stimulation or stress signals may also regulate glucose influx via TXNIP, depending on the cellular energy need induced by the specific signal.

Analogous to TXNIP’s regulation on the post-translational level, TXNIP is regulated robustly on the transcriptional level by at least two seemingly opposing transcription factors: chREBP/Mlx (or MondoA/Mlx) and FOXO (Cha-Molstad et al., 2009; Hong et al., 2016; Stoltzman et al., 2008; Yu et al., 2009). chREBP/Mlx stimulates TXNIP under nutrient-rich conditions in response to an influx of glucose, decreasing further uptake. FOXO
significantly stimulates TXNIP when growth signals are absent to limit glucose influx. The activation of AKT by growth factors leads to FOXO phosphorylation and its subsequent sequestration in the cytoplasm by 14-3-3 (Tzivion et al., 2011). We have consistently observed increases in TXNIP protein expression in the absence of serum in cultured cells and in several tissues of fasted animals (Figure 4H). In both cases, TXNIP is produced to slow the rate at which glucose enters the cell.

The need for cells to appropriately respond to opposite signals places a high demand on the speed at which TXNIP mRNA and protein are synthesized and degraded. Consistent with the short half-life observed for both TXNIP protein and mRNA, a quick turnover is required to ensure that the cell is ready to rapidly respond to a large dynamic range of stress or stimulation. If TXNIP is dysregulated, the cell may survive under normal conditions, but we predict it will not withstand stress as easily nor mount as robust a response to stimulation as readily if a change in glucose uptake is required. This is notably reflected in the metabolic defects during the fed/fasting transition of TXNIP KO mice. Overall, TXNIP provides a fundamental integration point for both short-term and long-term metabolic and signaling information, permitting the appropriate types and levels of cellular response to glucose availability and demand.

**EXPERIMENTAL PROCEDURES**

Please see more details in the Supplemental Information.

**Cell Culture**

3T3L1 cells were purchased from ZenBio and were maintained and differentiated according to the company’s protocol. 3T3L1 KO clones were made with guide RNA sequence corresponding to 5′-TTACCCGAGTCAAAGCCGT CAGG. HepG2, SKBr3, and BT474 cell lines were purchased from ATCC and cultured according ATCC directions. Primary MEFs were isolated from E12.5 (embryonic day) embryos and cultured in DMEM/10% FBS (fetal bovine serum).

**Animal Work**

Mice were maintained inside a barrier facility according to the institute’s regulations for animal care and handling (IACUC [Institutional Animal Care and Use Committee] PIL-16-02-003, 14-02-008, and 16-08-027). Strains TXNIP<sup>flox/flox</sup> (016847) and CMV-cre (006054) were purchased from the Jackson laboratory. For experiments, sex-matched littermates between 2 and 3 months old were used.

**Statistical Analysis**

p values were calculated using Student’s paired t test with two tails except for the biodistribution studies.

Experiments exploring differences in the biodistribution of 2DG-2-(18F)fluoro-D-glucose (18F-FDG) and glucose levels were analyzed via linear mixed-effects models with a random intercept for littermates in R v3.2.2 (https://www.r-project.org/). All models were originally...
fit including a two-way interaction between sex and genotype. If this interaction was not found to be significant, it was removed from the model, and the model was re-fit with sex and genotype as fixed effects. The p values produced from these tests were false discovery rate-corrected to adjust for multiple testing on the same animals.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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In Brief

Waldhart et al. find that TXNIP, an α-arrestin protein, is an adaptor for endocytosis of the glucose transporter GLUT4. Growth factor stimulation induces acute glucose uptake into cells by activating AKT-mediated TXNIP phosphorylation, forcing TXNIP to dissociate from the transporters, thus inhibiting their endocytosis.
Highlights

- TXNIP is a substrate of AKT
- TXNIP facilitates GLUT4 endocytosis
- Growth factors induce acute glucose uptake in cells via regulating TXNIP
Figure 1. Growth Factor Caused Upshift in TXNIP Mobility on SDS-PAGE
(A) HepG2, SKBr3, and BT474 cells were serum-starved for 4 hr and then treated with the indicated growth factor for 15 min. Glucose uptake under the same conditions was measured using $^3$H-2-deoxyglucose (2DG) (n = 3, mean ± SEM).
(B) Differentiated 3T3L1 adipocytes were treated with insulin, and primary TXNIP WT and KO MEFs were treated with IGF for 15 min.
(C) The AMPK WT and double-knockout (α1 and α2) MEFs were treated with either IGF or 2DG for 15 min after serum starvation. The upshift detected by total TXNIP antibody correlated with signals from the pTXNIP S308 antibody. While AMPK activation-induced TXNIP 308 phosphorylation can only be seen in WT MEFs, IGF-induced phosphorylation can be seen in both cell lines.
(D) HepG2 cells were treated with HGF, 2DG, or HGF and 2DG for 15 min and probed with various antibodies.
Figure 2. AKT Phosphorylated TXNIP at S308 on the Plasma Membrane
(A) HepG2 cells were pretreated for 1 hr with various inhibitors before a 15 min stimulation by HGF: PI3K inhibitor BKM120 (1 μM), PDK1 inhibitor GSK2334470 (3μM), AKT inhibitors MD2206 (1 μM) and AZD5363 (1 μM), or SGK inhibitor GSK650394 (10 μM).
(B) Live-cell confocal imaging of HepG2 cells stably express GFP-TXNIP WT or K233A/R238A mutant.
(C) In vitro kinase assay using commercial GST-AKT (full-length) and His-TXNIP from bacteria. AKT phosphorylated TXNIP on S308 only in the presence of PIP3-containing liposomes without the AKT inhibitor AZD5363 (AZD).
(D) HepG2 cells stably expressing HA-TXNIP WT, S308A, or K233A/R238A mutant were treated with HGF with or without MG132 (proteasome inhibitor) or MK2206 pretreatment.
(E) SkBr3 cells stably expressing HA-TXNIP WT, S308A, or K233A/R238A mutant were treated with EGF with or without MK2206 pretreatment.
Figure 3. TXNIP Mediated AKT Activation-Induced Glucose Uptake in Cells

(A) Glucose uptake in TXNIP KO primary MEFs re-expressing HA-TXNIP WT or S308A mutant. Cells were either pretreated with control DMSO or 1 μM MK2206 for 1 hr, then treated with IGF for 15 min before adding ³H-2DG for 15 more min. Fold changes in WT and S308A cells were normalized to their respective controls (n = 3, mean ± SEM).

(B) Western blots corresponding to the cells used in (A).

(C) A TXNIP KO clone of 3T3L1 cells, generated using CRISPR technology, was differentiated into adipocytes and infected with virus to re-express HA-TXNIP WT or S308A. The glucose influx was measured by ³H-2DG uptake as in (A) (n = 3, mean ± SEM).

(D) Western blots corresponding to cells used in (C).

(E) Schematic of the liposome floatation assay and TXNIP western blot to show the top two fractions containing TXNIP floated with liposomes. An oversaturating amount of protein was used for these assays.
(F) Top two fractions of liposome float assay testing the interaction between WT TXNIP protein and various phosphoinositide-containing liposomes.

(G) Differential interaction between WT, P308 (protein phosphorylated on S308), and S308A mutant TXNIP with Pl(4,5)P₂- and PIP₃-containing liposomes. The P308 protein shows characteristic upshift in the blots.
Figure 4. TXNIP Regulation of GLUT4

(A) GLUT4 endocytosis assay in WT 3T3 adipocytes (n= 3), TXNIP KO adipocytes (n= 3), and KO adipocytes transfected with TXNIP (n= 4). Rate lines ± SEM. The data were scaled to 15 min for KO. Internalization index (proportional to internalization rate constant) was calculated from the slope of rate lines. Averages ± SEM are shown.

(B) Ratio of cell-surface GLUT4 to total-cellular GLUT4 in differentiated WT 3T3L1 and TXNIP KO adipocytes using the HA-GLUT4-GFP reporter under basal or 1 nM insulin.

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stimulated (30 min) conditions (n = 9, means ± SEM). p value calculated using Student’s t test for (A) and (B).

(C–F) Representative combined PET and CT (computed tomography) images of a WT and a KO mouse are normalized for visual comparison and displayed on identical color maps in Hounsfield units (HU) for CT data and standard uptake value (SUV) for PET data. The distribution of $^{18}$F-FDG uptake in skeletal muscle tissue (quadriceps femoris muscle) (D), epididymal white adipose tissue (E), and liver (F) are shown. The data are presented as percent injected dose per gram (percent ID/g horizontal lines and the error bars represent the mean ± SEM).

(G) Blood glucose level after overnight fasting. For (D)–(G), data were analyzed via linear mixed-effects models with random intercepts for littermates (n = 6). All p values were false discovery rate (FDR)-corrected for multiple testing, and their respective FDR q-values are shown.

(H) Western blot of skeletal muscle showing no change in GLUT4 expression between WT and KO animals before and after fasting.