Phosphorylation of the exocyst protein Exo84 by TBK1 promotes insulin-stimulated GLUT4 trafficking

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Insulin stimulates glucose uptake through the translocation of the glucose transporter GLUT4 to the plasma membrane. The exocyst complex tethers GLUT4-containing vesicles to the plasma membrane, a process that requires the binding of the G protein (heterotrimeric guanine nucleotide–binding protein) RalA to the exocyst complex. We report that upon activation of RalA, the protein kinase TBK1 phosphorylated the exocyst subunit Exo84. Knockdown of TBK1 blocked insulin-stimulated glucose uptake and GLUT4 translocation; knockout of TBK1 in adipocytes blocked insulin-stimulated glucose uptake; and ectopic overexpression of a kinase-inactive mutant of TBK1 reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The phosphorylation of Exo84 by TBK1 reduced its affinity for RalA and enabled its release from the exocyst. Overexpression of a kinase-inactive mutant of TBK1 blocked the dissociation of the TBK1/RalA/exocyst complex, and treatment of 3T3-L1 adipocytes with specific inhibitors of TBK1 reduced the rate of complex dissociation. Introduction of phosphorylation-mimicking mutant of TBK1 blocked the dissociation of the TBK1/RalA/exocyst complex, and treatment of 3T3-L1 adipocytes by TBK1 reduced its affinity for RalA and enabled its release from the exocyst. Overexpression of a kinase-inactive mutant of TBK1 blocked the dissociation of the TBK1/RalA/exocyst complex, and treatment of 3T3-L1 adipocytes with specific inhibitors of TBK1 reduced the rate of complex dissociation. Introduction of phosphorylation-mimicking or nonphosphorylatable mutant forms of Exo84 blocked insulin-stimulated GLUT4 translocation. Thus, these data indicate that TBK1 controls GLUT4 vesicle engagement and disengagement from the exocyst, suggesting that exocyst components not only constitute a tethering complex for the GLUT4 vesicle but also act as “gatekeepers” controlling vesicle fusion at the plasma membrane.
vesicles and permitted their fusion with the plasma membrane. Thus, TBK1 regulates insulin-stimulated glucose transport, and the dynamic interaction of TBK1, RalA, and the exocyst is required for the efficient delivery of exocytic vesicles.

**RESULTS**

**TBK1 is required for insulin-stimulated GLUT4 translocation**

The activation of nuclear factor κB (NFκB) by high-fat diet induces the expression of the IkB kinase ε (IKKe) and TBK1 in adipose tissue, and these kinases can activate Akt and PDE3B to promote anabolic processes in obesity (28–32). Given the important roles of the IKKs in energy storage, we examined whether these kinases play a direct role in glucose transport. We used small interfering RNA (siRNA) oligos to knock down IKKα in 3T3-L1 adipocytes, followed by measurement of[^14C]-2-deoxyglucose (2-DG) uptake in response to insulin (Fig. 1, A and B). Insulin treatment produced an about seven- to eightfold increase in 2-DG uptake; electroporation with scrambled control siRNA was without effect. Knockdown of IKKα, IKKβ, and IKKe with specific siRNAs had no discernable effect on 2-DG uptake in the basal or insulin-stimulated state. However, knockdown of TBK1 produced an about 50% reduction in insulin-stimulated glucose uptake (Fig. 1A), comparable to what has been reported with Akt knockdown (34), without affecting basal transport activity. The knockdown of TBK1 appeared to be greater than 90% and was accompanied by a modest decrease in the stimulation of phosphorylation of Akt by insulin, whereas the knockdown of the other kinases did not affect Akt phosphorylation (Fig. 1B).

To explore the role of the kinase activity of TBK1 in insulin-stimulated glucose uptake, we measured[^14C]-2-DG uptake in 3T3-L1 adipocytes overexpressing wild-type or a kinase-inactive mutant of TBK1 (K38A) (fig. S1, A and B). Whereas overexpression of wild-type TBK1 was without effect, overexpression of the kinase-inactive mutant produced an about 50% inhibition of insulin-stimulated glucose uptake without affecting basal amounts. In addition, overexpression of wild-type TBK1 increased Akt phosphorylation in the basal and insulin-stimulated state, consistent with previous reports that TBK1 is upstream of Akt (29–31). Overexpression of the catalytically inactive kinase also partially blocked insulin-stimulated Akt phosphorylation without affecting basal activity. Additionally, treatment of cells with the selective but structurally unrelated inhibitors of TBK1 and IKKe, amlexanox or CAY10576 (Cay) (fig. S1, C and D) (35, 36), blocked insulin-stimulated glucose uptake in a dose-dependent manner.

Insulin-stimulated glucose uptake is mainly mediated by GLUT4 (37). Therefore, we examined the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes stably expressing a dual-tagged GLUT4, in which the exofacial loop is tagged with MYC and the cytoplasmic tail is tagged with enhanced green fluorescent protein (eGFP)
As previously described (13, 38, 39), insulin produced a large increase in the translocation of the GLUT4 fusion protein to the plasma membrane, along with the appearance of the exofacial MYC epitope signal. Compared to cells electroporated with scrambled control siRNA, knockdown of TBK1 blocked the appearance of the MYC epitope signal on the plasma membrane in response to insulin, without affecting the localization of the GLUT4 fusion protein in the basal state (Fig. 1, C and D). Although it was difficult to assess the knockdown efficiency of TBK1 in individual cells by immunohistochemistry due to autofluorescence, the knockdown of TBK1 appeared to be about 50%, as assessed by Western blotting, with little effect on the stimulation of Akt phosphorylation by insulin (Fig. 1E).

To explore the relative role of Akt and TBK1 in insulin-stimulated glucose transport, we measured [14C]2-DG uptake in 3T3-L1 adipocytes that were pretreated with Akti-1/2 (Aktki) [a specific inhibitor that targets the PH domain of Akt kinases (40)], the TBK1 inhibitor amlexanox, or the two inhibitors together. Pretreatment of 3T3-L1 adipocytes with Akti or amlexanox produced about 80 and 70% inhibition of insulin-stimulated glucose uptake, respectively. However, insulin-stimulated glucose uptake was completely suppressed by a combination of pretreatment with both inhibitors (Fig. 1F). Insulin-stimulated phosphorylation of Akt was partially inhibited by Akti, amlexanox, and a combination of both inhibitors, but not completely inhibited to the basal state (Fig. 1G). The increase of TBK1 phosphorylation in amlexanox-treated cells is due to the loss of a negative feedback (36).

**TBK1 directly phosphorylates Exo84 both in vitro and in cells**

TBK1 can associate with the Sec5 subunit of the exocyst, in a manner crucial for cancer cell survival (33). A Scansite database search (http://scansite.mit.edu/) to predict potential substrates of TBK1 revealed that Exo84 contains several TBK1 consensus phosphorylation sites (41, 42). Mammalian Exo84 has a Ral binding domain (RBD) and a putative helical domain potentially important for its interaction with other exocyst subunits (fig. S2A) (43–47). TBK1 phosphorylated recombinant glutathione S-transferase (GST)–Exo84, as well as myelin basic protein (MBP) in vitro (fig. S2B). TBK1 directly catalyzed the phosphorylation of both wild-type Exo84 and Exo84 RBD, but not GST itself (Fig. 2A). We also made GST-free Exo84 RBD by cleaving GST from GST-tagged Exo84 RBD to use as a substrate in the kinase assay (fig. S2C). TBK1 fused to maltose-binding protein (MBP-TBK1) increased the phosphorylation of Exo84 in a dose-dependent manner (Fig. 2B). Phosphorylation of Exo84 was detected only with TBK1 but not with IKKβ, IKKe, PKA, Akt, or PKCa in vitro (Fig. 2C).

To determine whether TBK1 can phosphorylate Exo84 in cells, we cotransfected COS-1 cells with MYC-Exo84 and increasing amounts of Flag-tagged wild-type TBK1 or the kinase-inactive mutant K38A. Expression of wild-type TBK1 but not the inactive mutant reduced the electrophoretic mobility of Exo84, suggestive of Exo84 phosphorylation (Fig. 2D). Phosphorylation of Exo84 was detected by blotting.
with antibodies that recognize a 14-3-3 binding motif (pS-14-3-3), as previously reported (32), but not with antibodies that recognize phosphorylated PKC substrates (pS-PKC sub). Expression of wild-type TBK1 increased the phosphorylation of Exo84, which was reduced by treatment with calf intestinal phosphatase (CIP) (Fig. 2E, compare lane 3 to lane 7 in top panel). Expression of IKKe was less effective (Fig. 2E, compare lane 3 to lane 5 in top panel). Although wild-type TBK1 formed a less stable complex with Exo84 than the TBK1 K38A mutant, the interaction of Exo84 with wild-type TBK1 was rescued by treatment with CIP (Fig. 2E, compare lanes 3 and 4 to lanes 7 and 8 in top panel). These data are consistent with our previous findings (32), demonstrating that TBK1 transiently associates with its substrates and subsequently dissociates upon phosphorylation. We also performed a reciprocal immunoprecipitation and detected the interaction of Exo84 with the kinase-inactive K38A mutant but not wild-type TBK1 (fig. S2D). To further test whether the interaction between Exo84 and TBK1 was specific, we incubated several GST–tagged fusion proteins, including the Ral binding protein 1 [RalBP1 (48)] RBD, Exo84 RBD, and wild-type Exo84, with lysates from COS-1 cells expressing wild-type TBK1 or the kinase-inactive K38A mutant. The inactive K38A mutant, but not wild-type TBK1, was preferentially pulled down by GST–Exo84 but not by other fusion proteins, suggesting that the catalytically inactive kinase TBK1 specifically interacted with its substrate Exo84 (fig. S2E). These data suggest that Exo84 is a bona fide substrate of TBK1 both in vitro and in cells and, furthermore, that TBK1 in its inactive state directly interacts with Exo84 and subsequently dissociates upon phosphorylation.

**TBK1 directly interacts with Exo84 through the coiled-coil domain of TBK1 and helical domain of Exo84**

To understand how TBK1 might control the function of the exocyst, we coexpressed various mammalian Exo84 deletion (Del) mutants with wild-type TBK1 and the K38A mutant (Fig. 3A) in COS-1 cells. Kinase-inactive TBK1 preferentially communoprecipitated with wild-type Exo84 and the Del 1 mutant, whereas the interaction of the other Exo84 Del mutants with TBK1 K38A was reduced, suggesting that the helical domain of Exo84 is required for the interaction with TBK1 (Fig. 3B). Several scaffolding proteins interact with TBK1, including TANK, NAP1, and SINTBAD, which play important roles in the activation of the kinase in response to different stimuli (49–51). It is possible that the phosphorylation of Exo84 by TBK1 might require scaffolding proteins, exocyt subunits, or other associated proteins. To test whether the interaction of TBK1 with Exo84 was direct or indirect, we performed an in vitro protein interaction assay. In vitro translated, kinase-inactive TBK1 (Fig. 3A) preferentially communoprecipitated with in vitro translated wild-type Exo84 (fig. S3A), as shown in cells (Figs. 2E and 3B). In vitro translated Exo84 lacking the helical domain (Del 2 and Del 4) showed a substantially reduced interaction with TBK1, whereas in vitro translated Exo84 helical domain (Del 5) interacted with the kinase-inactive mutant. Thus, the helical domain of Exo84 is necessary and sufficient for its interaction with TBK1 in vitro.

TBK1 has three structurally distinct domains: the kinase domain, the ubiquitin-like domain (ULD), and the scaffold and dimerization domain (coiled-coil domain) (52–54). TBK1 recognizes its substrates through the ULD domain, which is proximal to its kinase domain (55). Only full-length wild-type TBK1 coprecipitated with in vitro translated Exo84 or the Del 5 mutant (fig. S3, B and C). Similarly, wild-type Exo84 and the Del 5 mutant coprecipitated only with the full-length wild-type TBK1 in reciprocal immunoprecipitation analysis of in vitro translated proteins with anti-MYC antibodies (fig. S3D). These data suggest that although TBK1 exists in different complexes depending on the cellular context, the kinase directly interacts with Exo84 by binding between the helical domain of Exo84 and the coiled-coil domain of TBK1. TBK1 and the related kinase IKKe both have a kinase domain in the N terminus and share 64% similarity in amino acid sequences (25). It is possible that the differential substrate specificity of these two related kinases might be determined through their C-terminal coiled-coil domain.

**Phosphorylation of Exo84 by TBK1 decreases its interaction with RalA**

Activation of the GLUT4 vesicle–associated small GTPase RalA upon insulin stimulation leads to engagement with its effector proteins, Sec5 and Exo84 (13). However, these complexes must also dissociate to permit GLUT4 vesicles to engage SNARE proteins at the plasma membrane for fusion. Disengagement between RalA and the exocyst requires phosphorylation of Sec5, thus allowing exocytic vesicle cycling events to continue (24). These results prompted us to investigate the role of Exo84 phosphorylation in this process. Exo84 preferentially coprecipitated with the active mutant of RalA (G23V), and wild-type TBK1 overexpression produced an about 50% reduction in this interaction; this reduction was not observed upon overexpression of the K38A kinase-inactive mutant of TBK1 (Fig. 4A). Wild-type TBK1 overexpression resulted in an about 50% reduction in the interaction of active RalA with Exo84, which was not observed after TBK1 K38A overexpression (Fig. 4B). The interaction between active RalA and endogenous Exo84 or Sec5 was significantly decreased only when wild-type TBK1, but not the K38A mutant, was overexpressed (Fig. 4C).
To examine whether the reduction in the interaction of active RalA with Exo84 was due to changes in RalA activity, we evaluated the activity state of RalA by effector pull-down assay using the effector RalBP1 (48). The Flag-RalA G23V mutant preferentially coprecipitated with GST-RalBP1 RBD, whereas overexpression of neither wild-type TBK1 nor the K38A mutant affected this interaction, indicating that TBK1 did not directly affect RalA activity (fig. S4). The interaction of Exo84 with the RalA G23V mutant was decreased by the expression of wild-type TBK1 but not by the inactive K38A mutant (Fig. 4D). Moreover, phosphorylation of Exo84 was enhanced by RalA G23V expression but barely detected after RalA S28N expression.

Active RalB can activate TBK1 by promoting Sec5/TBK1 complex assembly (33). We tested whether RalA activity enhanced the interaction between TBK1 and Exo84. Exo84 preferentially interacted with the kinase-inactive mutant compared to wild-type TBK1, and this interaction between TBK1 and Exo84 was not affected by the activity state of RalA (Fig. 4E), confirming that active RalA does not promote Exo84/TBK1 complex assembly. These data demonstrate that TBK1-mediated phosphorylation of Exo84 decreases the interaction between RalA and Exo84 by targeting the effector.

Insulin does not directly affect the intrinsic kinase activity of TBK1

TBK1 activity is regulated by phosphorylation on Ser172 within the activation loop (57, 58). However, how TBK1 is activated remains uncertain. Expression of a “fast exchange” mutant (F39L) of RalB bearing increased nucleotide dissociation rates that quickly cycles between the GDP- and GTP-bound states of RalB drives TBK1/Sec5 complex assembly, resulting in enhanced TBK1 kinase activity (33). Although active RalA does not promote Exo84/TBK1 complex assembly (Fig. 4E), Exo84 phosphorylation was enhanced by RalA G23V expression but decreased by RalA S28N expression (Fig. 4, D and E). To explore the possibility that active RalA enhances TBK1 kinase activity, active TBK1 was detected by blotting with antibodies that recognize phosphorylation of TBK1 at Ser172. Phosphorylation of TBK1 at Ser172 was not affected by the activity state of RalA (Fig. 4E), suggesting that active RalA does not increase intrinsic TBK1 activity. Overexpression of wild-type TBK1, but not the K38A mutant, increased the phosphorylation of interferon regulatory factor 3 (IRF3), a direct TBK1 substracte (59, 60), as well as the overall phosphorylation detected with antibodies that recognize the 14-3-3 binding motif. However, none of the RalA or RalB mutants affected endogenous TBK1.
activity (Fig. 5A). Because RalA undergoes activation upon insulin stimulation by Akt-dependent phosphorylation and subsequent inhibition of the RGC1/2 complex, we also examined whether insulin affected TBK1 kinase activity. Treatment of 3T3-L1 adipocytes with the Toll-like receptor 4 agonist polynosinic:polycytidylic acid [poly(I:C)] and lipopolysaccharide (LPS) (58, 61, 62) increased phosphorylation of TBK1 at Ser\textsuperscript{172} as previously described (58, 62), but phosphorylation was not detected in adipocytes exposed to insulin (Fig. 5B). Knockdown of TBK1 decreased phosphorylation of its downstream targets, such as Akt and IRF3. Consistent with these results, poly(I:C) and LPS treatment, but not insulin, stimulated the catalytic activity of TBK1, as shown in a direct TBK1 immune complex assay. These studies suggest that insulin does not directly affect intrinsic TBK1 kinase activity but may change the accessibility of its substrate Exo84 because of a change in the interaction of RalA with its exocyst effectors in response to insulin.

**The interaction of Exo84 with RalA and other exocyst subunits depends on TBK1-mediated phosphorylation of Exo84**

To identify target phosphorylation sites for TBK1 on Exo84, liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed on HA immunoprecipitates from COS-1 cells expressing HA-tagged Exo84 and wild-type TBK1 or its K38A mutant. Eleven potential phosphorylation sites were identified with a total coverage of 60.2% of the Exo84 amino acid sequence (Fig. 6A, fig. S5, and data files S1 and S2). Each of the 11 phosphorylation sites was mutated to either alanine or glutamate by site-directed mutagenesis to prevent or mimic phosphorylation of Exo84. We performed a GST pull-down assay using a GST-RalA G23V fusion protein with lysates from COS-1 cells coexpressing wild-type TBK1 and the K38A mutant, along with HA-tagged wild-type, S8A, or S8E Exo84. The S8E mutant had a mobility shift comparable to that caused by phosphorylation of wild-type Exo84 by TBK1 (Fig. 6B). In addition, the RalA fusion protein precipitated about 70% less of the Exo84 S8E mutant but about 40% more of the S8A mutant compared to the wild-type protein. Overexpression of wild-type TBK1 or the K38A mutant reduced or enhanced the binding between RalA and the Exo84 mutants, respectively, whereas overexpression of wild-type TBK1, but not the K38A mutant, resulted in an about 70% reduction in the interaction of active RalA with wild-type Exo84. These data suggest that TBK1-mediated phosphorylation of Exo84 on these eight sites are necessary and sufficient for RalA disengagement because the Exo84 S8A mutant was resistant to phosphorylation-dependent RalA disengagement, whereas the Exo84 S8E mutant exhibited a lower affinity for RalA.

To understand whether TBK1-dependent phosphorylation of Exo84 influences its interactions with other exocyst subunits, we immunoblotted HA-immunoprecipitates from COS-1 cells expressing HA-tagged wild-type, S8A, or S8E Exo84 for the two other exocyst subunits, Sec5 and Sec8, which interact with Exo84 in the exocyst complex (63). Sec5 and Sec8 interacted more strongly with the S8A mutant than the S8E mutant (Fig. 6C). Overexpression of wild-type TBK1 reduced the binding of wild-type Exo84 but not the mutant forms of the protein to Sec5 and Sec8. Both Exo84 S8A and S8E also underwent less phosphorylation by TBK1 overexpression. Together, these data suggest that phosphorylation of Exo84 by TBK1 induces its disengagement from both RalA and other exocyst components, suggesting that phosphorylation plays a crucial role in the cyclic engagement and disengagement process.

**Phosphorylation of Exo84 is required for insulin-stimulated GLUT4 trafficking**

Insulin-stimulated GLUT4 trafficking requires the assembly and recognition of the exocyst for targeted exocytosis; both of these processes are controlled by small G proteins (8, 11, 13). However, both its size and architecture indicate that once bound to the vesicle, the exocyst may serve as a barrier for SNARE-mediated vesicle fusion (24, 64). Therefore, GLUT4 vesicles must engage with, but subsequently disengage
from the exocyst for efficient docking and fusion with the plasma membrane. To evaluate the functional role of Exo84 phosphorylation in insulin-stimulated GLUT4 trafficking, we examined the effects of Exo84 overexpression on the trafficking of GLUT4 in response to insulin. In control cells and cells overexpressing wild-type Exo84, GLUT4 was detected in the intracellular compartment in the basal state and translocated to the plasma membrane in response to insulin, which was accompanied by the appearance of the exofacial MYC epitope signal. Expression of the Exo84 S8A mutant did not affect the basal localization of GLUT4; it reduced the exofacial MYC epitope signal in response to insulin. Expression of the Exo84 S8E mutant similarly reduced insulin-stimulated GLUT4 translocation. About 74 and 54% of the insulin-stimulated appearance of the MYC epitope signal on the plasma membrane was blocked by the expression of the Exo84 S8A and S8E mutants, respectively (Fig. 7B). In these experiments, the amounts of overexpressed HA-tagged wild-type Exo84, S8A, or S8E mutants were comparable to endogenous amounts of Exo84 and did not affect Akt phosphorylation in the basal or insulin-stimulated states (Fig. 7C). Thus, both the S8A and S8E mutants block GLUT4 exocytosis because of their ability to prevent the assembly or disassembly of the exocyst complex, respectively. These observations reveal that Exo84 phosphorylation is critical for insulin-stimulated GLUT4 trafficking by controlling GLUT4 vesicle engagement and disengagement from the exocyst.

**Exo84 phosphorylation is temporally and spatially regulated in insulin-stimulated GLUT4 trafficking**

Our data indicated that GLUT4 vesicle recognition at the plasma membrane was triggered by RalA activation upon insulin stimulation, but once bound, the phosphorylation of Exo84 and Sec5 releases the GLUT4 vesicle from the exocyst to permit fusion. We asked whether Exo84 phosphorylation was dynamically regulated during this process in response to insulin in 3T3-L1 adipocytes. Endogenous RalA rapidly associated with Exo84 in response to insulin (Fig. 8A and fig. S6A). This effect was maximal at 2 min after insulin stimulation and declined thereafter. Assessment of RalA activity by pull-down assay with a GST-RalBP1 RBD fusion protein revealed that RalA was maximally activated within 2 min and maintained for at least 30 min (Fig. 8B and fig. S6B). Although amlexanox pretreatment did not affect RalA activity, it prevented the decline in RalA-Exo84 binding, suggesting that inhibition of TBK1 specifically delays the rate of dissociation of RalA-Exo84 interaction but does not influence RalA-GTP binding (Fig. 8A and fig. S6A). Phosphorylation of Exo84, as assessed by blotting with antibodies that recognize the 14-3-3 binding motif, peaked 2 min after insulin stimulation and declined after 30 min (Fig. 8A and fig. S6C). It is likely that disruption of the exocyst complex might also be crucial to initiate another round of vesicle fusion. The interaction of Exo84 with Sec8 and Sec5 releases the GLUT4 vesicle from the exocyst to permit fusion. We asked whether Exo84 phosphorylation was dynamically regulated during this process in response to insulin in 3T3-L1 adipocytes. Endogenous RalA rapidly associated with Exo84 in response to insulin (Fig. 8A and fig. S6A). This effect was maximal at 2 min after insulin stimulation and declined thereafter. Assessment of RalA activity by pull-down assay with a GST–RalBP1 RBD fusion protein revealed that RalA was maximally activated within 2 min and maintained for at least 30 min (Fig. 8B and fig. S6B). Although amlexanox pretreatment did not affect RalA activity, it prevented the decline in RalA–Exo84 binding, suggesting that inhibition of TBK1 specifically delays the rate of dissociation of RalA–Exo84 interaction but does not influence RalA–GTP binding (Fig. 8A and fig. S6A). Phosphorylation of Exo84, as assessed by blotting with antibodies that recognize the 14-3-3 binding motif, peaked 2 min after insulin stimulation and declined after 30 min (Fig. 8A and fig. S6C).
the dissociation of the entire complex. TBK1 also associated with Exo84, an interaction that peaked 30 min after insulin stimulation, and amlexanox pretreatment enhanced the basal interaction between TBK1 and Exo84 (Fig. 8A and fig. S6F).

To understand the dynamics of the protein interactions of TBK1, Exo84, and RalA in insulin-stimulated GLUT4 trafficking, we investigated whether these proteins reside in the same subcellular compartments. RalA colocalizes with GLUT4 vesicles and translocates to the plasma membrane upon insulin stimulation (13), which also stimulates the assembly of the exocyst complex at the plasma membrane (11, 12). Insulin stimulation enriched GLUT4, RalA, and the exocyst subunits Sec6, Sec8, Sec10, Sec5, and Exo84 in the plasma membrane fractions and reduced the abundance of these proteins in the low-density microsome (LDM) fractions (Fig. 8C). Insulin-regulated aminopeptidase (IRAP) was also enriched in the plasma membrane fractions upon insulin stimulation. In contrast, transferrin receptor (TfR), Rab5, and caveolin were detected in the same fractions basally or after insulin stimulation. In untreated cells, TBK1 protein was detected in most fractions. After treatment with insulin, TBK1 was enriched in the plasma membrane fractions, with a concurrent loss of this protein in the LDM, mirroring GLUT4 trafficking. Phosphorylation of TBK1 at its activating residue Ser172 was also increased in response to insulin in the plasma membrane fractions, although insulin did not stimulate kinase activity, as determined by an immune complex kinase assay (Fig. 5B).

GLUT4 vesicles are targeted to discrete microdomains on the plasma membrane called lipid rafts (12). Caveolin was largely detected in the lipid raft–enriched fractions 3 and 4 of sucrose density gradients (12) and was unaffected by insulin treatment, as previously reported (Fig. 8D) (12). In contrast, TfR was found exclusively at the bottom of the gradient in fractions 7 to 11, where it increased in response to insulin. GLUT4, IRAP, Sec5, Sec8, and Exo84 were detected in both raft (3 and 4) and nonraft (10 and 11) fractions in untreated cells. However, these proteins were enriched only in the lipid raft fractions in cells treated with insulin. Insulin also triggered changes in the phosphorylation and localization of TBK1, and the effect on phosphorylation was more pronounced. Together, these data suggest that Exo84 phosphorylation by TBK1 terminates its interaction with RalA and is thus important for insulin-stimulated GLUT4 trafficking to control disengagement of RalA and disassociation of the exocyst.

**DISCUSSION**

Although there has been substantial progress in the identification of insulin signaling pathways leading to GLUT4 translocation and glucose uptake, the role of TBK1 in these processes remains unclear. TBK1 deficiency attenuates insulin-stimulated glucose uptake in isolated mature adipocytes from epididymal fat. The data described above demonstrated that TBK1 phosphorylated the exocyst subunit Exo84 and regulated GLUT4 trafficking in 3T3-L1 adipocytes. Insulin increases glucose uptake through translocation of GLUT4 in both adipocytes and myocytes. Because TBK1-null mice are not viable, we bred TBK1 flox (TBK1 FL) mice to adiponectin-cre mice to generate adipocyte-specific TBK1 knockout (TBK1 AKO) mice. Adipocytes were isolated from epididymal fat of TBK1 FL and TBK1 AKO mice and subjected to assay of insulin-stimulated glucose uptake. Treatment of adipocytes with insulin significantly increased glucose uptake in TBK1 FL adipocytes but not in TBK1 AKO adipocytes (Fig. 9A). Insulin-stimulated phosphorylation of Akt was modestly, but not completely, attenuated by TBK1 deficiency (Fig. 9B). These data indicate that TBK1 is required for insulin-stimulated glucose uptake through an Akt-independent process in this physiological setting.
uptake (8), our understanding of the mechanisms by which these upstream signaling pathways converge on intracellular GLUT4 storage vesicles for translocation to the plasma membrane remains incomplete. Insulin increases the activity of numerous protein kinases in adipocytes, and some of these are required for the actions of insulin on glucose transport (3, 4). We report here another protein kinase, TBK1, which appears to play a critical role in this process and is required for insulin-stimulated glucose transport and GLUT4 translocation to and insertion in the plasma membrane.

As a noncanonical member of the IKK family of kinases, TBK1 was initially identified through its sequence homology with IKKα and IKKβ, key regulators of the NFκB transcription pathway (49, 66). However, NFκB is not activated in mouse embryonic fibroblasts from IKKα and IKKβ double-knockout mice, whereas TBK1 is dispensable, suggesting that TBK1 does not play an important role in the activation of the NFκB pathway (59, 61, 67–72). TBK1 has also been implicated in the regulation of the production of type I interferons through phosphorylation of IRFs (59, 61, 68, 69, 71). However, TBK1 can counteract inflammation because it inhibits the canonical IKKs, in the process preventing the overproduction of inflammatory mediators (32, 58). In this regard, both IKKε and TBK1 are induced as a consequence of chronic, low-grade inflammation that is associated with obesity (28, 73). Because both kinases contain NFκB regulatory sites in their promoter regions, they are thought to be induced upon NFκB activation and, in turn, play a part in a feedback pathway that attenuates the extent to which inflammatory signals are effective (73, 74). At the same time, TBK1 can promote energy storage by co-opting insulin targets to repress lipolysis and thermogenesis while increasing lipogenesis (30–32, 36). Other alternative roles for TBK1 have been described outside of inflammatory signaling pathways, including interaction with Rab8b to direct maturation of autophagosomes into lytic bacterial organelles (75); phosphorylation of the autophagic adaptor protein, optineurin, thereby allowing specific recruitment of cargo molecules for the autophagic clearance of pathogens (76); and regulation of recycling endosome distribution through phosphorylation of Rab11 effector proteins (77, 78).

Fig. 8. Temporal and spatial dynamics of the TBK1/exocyst/RalA complex in insulin-stimulated GLUT4 trafficking. (A) Exo84 immunoprecipitates from 3T3-L1 adipocytes pretreated or not with amlexanox (50 μM) before being treated with or without insulin (10 nM). As a control, goat serum (GS) was used for immunoprecipitation. n = 3 experiments. (B) Cell lysates from 3T3-L1 adipocytes treated with or without insulin (10 nM) in the presence or absence of pretreatment with amlexanox (50 μM) were incubated with GST-RalBP1 RBD beads, subjected to GST pull-down assays, and analyzed by immunoblot. The amounts of GST fusion proteins used in the precipitation.

The data presented here suggest a new, specific substrate for TBK1 that also indicates an anabolic role for the kinase in insulin-stimulated glucose transport and GLUT4 translocation. Experiments with inhibitors or siRNA knockdown indicate that although TBK1 is an upstream kinase for Akt (30, 31), its effect on glucose uptake is not regulated solely through Akt. Here, we present evidence that direct phosphorylation of the exocyst subunit Exo84 by TBK1 dissociated the effector protein from RaA and the rest of the exocyst subunits, in the process ensuring that GLUT4 trafficking is not arrested at the plasma membrane.

The regulation of GLUT4 by insulin involves a complicated process of regulated recycling by coordinating the sorting, tethering, docking, and fusion of exocytotic vesicles (8). In untreated cells, GLUT4 resides in specialized storage vesicles in intracellular compartments, which are transported along cytoskeletal tracks to the plasma membrane. Although GLUT4 vesicle fusion is catalyzed by SNARE proteins present on the vesicle and target membranes, efficient control of the process requires that the vesicle is first targeted to discrete microdomains in the plasma membrane that are enriched in SNARE proteins.
The data presented here lead us to propose that after activation of RalA by insulin, the G protein binds to both Sec5 and Exo84, thus engaging and directing GLUT4 vesicles to regions on the plasma membrane where fusion machineries are enriched. In addition, direct phosphorylation of the exocyst subunit Exo84 by TBK1 disengages the effector protein from RalA and the rest of the exocyst subunits, in the plasma membrane where fusion machineries are enriched. Although it is somewhat unexpected that a protein kinase generally associated with inflammatory responses is used for an anabolic program such as insulin-stimulated glucose uptake, the idea is not unprecedented. TBK1 induction in obesity is associated with continuous energy storage (28, 36), and along with IKKe, TBK1 phosphorylates and activates the phosphodiesterase PDE3B, leading to decreased lipolysis and thermogenesis (32). Moreover, cells in which TBK1 has been inhibited or knocked out are hypersensitive to inflammatory signals (36, 58, 80), suggesting that the kinase may also play a feedback role in countering catabolic stimuli. Future studies will explore the physiological role of the kinase in the regulation of energy homeostasis.

MATERIALS AND METHODS
Materials and reagents
All chemicals were obtained from Sigma-Aldrich unless stated otherwise. 2-Deoxy-D-[14C]glucose and [γ-32P]ATP were obtained from PerkinElmer Life Sciences. Stealth duplex siRNAs were obtained from Life Technologies, and their sequences are as follows: 5′-AGAACACAGUGUAUAACUCCCCACAGG-3′ and 5′-CCUCUGGGAGUUUUAACACUGUUCU-3′ in combination with...
5′-GAUAAGUCGGAAGUGUGAGAGA-3′ and 5′-UUCUCAUCCACACUCCUCGAGUAAUC-3′ against mouse Tbk1; 5′-UGGACAGUGCGAAGAGGAGG-3′ and 5′-CCUCAACUCUGUUCAGCAUGCUCA-3′ in combination with 5′-AGGCUAUAUCAGGAGAUC-3′ and 5′-AGUACUCCAGAUGGCAGUUCUC-3′ against mouse Ikkb; 5′-UGGACACCAAGUUGGUAAC-3′ and 5′-AGCAUGUGCGAAUACAA-3′ in combination with 5′-CAGACAGCAAUGGUGGCGCA-3′ and 5′-AGGCUAUAUCAGGAGAUC-3′ against mouse Ikkb.

Cell culture and transfection
3T3-L1 fibroblasts (American Type Culture Collection) were cultured and differentiated as described previously (81). Cells were routinely used within 10 days after completion of the differentiation process; only cultures in which >90% of cells displayed adipocyte morphology were used. 3T3-L1 adipocytes were transfected with 200 μg of plasmids or siRNAs by electroporation as described previously (19). Electroporation of mature adipocytes was done within 1 to 4 days after differentiation at 160 kV, 950 μF. Before electroporation, adipocytes were trypsinized and suspended in calcium (+) and magnesium (+) Dulbecco’s phosphate-buffered saline (DPBS). Electroporation with siRNAs to knock down the gene of interest was continued for 4 days after electroporation. 3T3-L1 adipocytes were starved in reduced serum (0.5% FBS) medium for 12 hours. After serum starvation, cells were pre-treated for 1 hour with amlexanox or Cay at the given concentrations and stimulated with 10 nM insulin for indicated time.

Antibodies
Anti-Flag antibody was obtained from Sigma, and anti-HA, anti-MYC, anti-GST, anti-Exo84, and anti-Sec10 antibodies were obtained from Santa Cruz Biotechnology. Anti-IKKα, anti-TBK1, anti-phospho-TBK1 (Ser172), anti-Akt, anti-phospho-Akt (Ser473), anti-IKKβ, anti-IKKα, anti-PARPγ, anti-C/EBPβ, anti-C/EBPδ, anti-perilipin, anti-phospho-PKC substrate, anti-phospho (Ser) 14-3-3 binding motif (R-X-Y/F-X-pS), anti-IRF3, anti-phospho-IRF3 (Ser396), anti-IRAP, anti-histone 3, and anti-Rab5 antibodies were purchased from Cell Signaling Technology. Anti-Ralα, anti-Sec8, and anti-caveolin1 antibodies were purchased from BD Biosciences. Anti-GLUT4 was purchased from Enzo Life Sciences.

Plasmids and mutagenesis
The Flag-tagged human TBK1 wild-type, TBK1 K38A, IKKe wild-type, and IKKe K38A complementary DNAs (cDNAs) were provided by T. Maniatis (Columbia University). The rat Exo84 cDNA was provided by Y. Shimaoka (University of Michigan). The Flag-tagged human TBK1 wild-type, TBK1 K38A, and TBK1 1–301 (wild-type), and TBK1 1–301 (KM) cDNAs were provided by I. Dikic (Goethe University, Germany). Site-directed mutagenesis (QuikChange, Agilent Technologies) was used to generate the phosphomimetic and phosphorylation deficient mutants of Exo84 as well as the truncation mutants (Exo84 Del 2 and Del 3). Del 1, Del 4, and Del 5 mutants of Exo84 were cloned from rat Exo84 wild-type cDNA by polymerase chain reaction and subcloned into pKH3 vector. Flag-tagged RalA constructs (wild-type, G23V, S28N, and F39L) have been previously described (13).

Immunoprecipitation and immunoblotting
For immunoprecipitation, cells were washed once with ice-cold PBS before lysis with immunoprecipitation buffer [100 mM tris-HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 10 mM NaF, and 5 mM MgCl2] supplemented with an EDTA-free protease inhibitor tablet (Roche). Lysates were cleared by centrifugation at 13,000g for 15 min and then incubated with 2 μg of antibody for 1 mg of protein for 4 hours to overnight at 4°C. Immunoprecipitates were adsorbed on Protein A/G plus agarose (Santa Cruz Biotechnology) for 1.5 to 2 hours, washed three times in lysis buffer, and eluted in 1.5× SDS sample buffer [240 mM tris-HCl (pH 6.8), 40% glycerol, 8% SDS, 0.04% bromophenol blue, and 5% β-mercaptoethanol] by boiling the samples for 5 min at 95°C. For regular cell lysis, cells were washed once with ice-cold PBS before lysis with SDS buffer [100 mM tris-HCl (pH 8.0), 130 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate, 1 mM Na3VO4, and 10 mM NaF] supplemented with an EDTA-free protease inhibitor tablet (Roche), followed by sonication. The crude lysates were centrifuged at 13,000g for 15 min, and protein concentration was determined using Protein Assay Reagent (Bio-Rad). Samples were diluted in 4× SDS sample buffer and boiled for 5 min at 95°C. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Life Technologies) and transferred to nitrocellulose membranes (Bio-Rad). Individual proteins were detected with specific antibodies and visualized on x-ray film using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and Western Lightning Enhanced Chemiluminescence Substrate (PerkinElmer Life Sciences). Where necessary, blots were incubated in stripping buffer [50 mM tris-HCl (pH 6.8), 150 mM NaCl,
110 mM β-mercaptoethanol, and 2% SDS] and rephrased with the specific antibodies.

**CIP dephosphorylation**

CIP was obtained from New England Biolabs. Immunoprecipitates were incubated for 1 hour at 37°C in a 100-μl reaction containing 50 mM tris (pH 7.5), 150 mM NaCl, 1% NP-40, EDTA-free protease inhibitor tablet, and 5 μl of CIP.

**In vitro kinase assay**

In vitro kinase assays were performed by incubating purified kinase (IKKe, TBK1, IKKβ, PKA Cα, PKCα, or PKBβ) in kinase buffer containing 25 mM tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol (DTT), and 50 μM ATP for 30 min at 30°C in the presence of 0.5 μCi [γ-³²P]ATP and 1 μg of MBP per sample as a substrate. For PKCα, a PKC lipid activator (EMD Millipore) was added. Purified GST, GST–Exo84 wild-type, GST–Exo84 RBD, or GST-free Exo84 RBD were also used as a substrate. GST-free Exo84 RBD was made from GST–Exo84 RBD by cleaving GST with thrombin. The purification of these proteins is described below. The kinase reaction was stopped by adding 4X SDS sample buffer and boiling for 5 min at 95°C. Supernatants were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography.

**TBK1 immune complex kinase assay**

Cells were washed once with ice-cold PBS and lysed with lysis buffer containing 50 mM tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 25 mM β-glycerophosphate, 1 mM Na₃VO₄, 10% glycerol, 1% Triton X-100, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride supplemented with an EDTA-free protease inhibitor tablet (Roche). Lysates were cleared by centrifugation at 13,000g for 15 min and were then incubated with GST fusion proteins bound to glutathione beads (GE Healthcare Life Sciences) for 40 min at 4°C. Beads were washed three times with 1 ml of pull-down lysis buffer and then resuspended in 1.5× SDS sample buffer.

**In vitro translation and coimmunoprecipitation**

Vector constructs (1 μg) as indicated were used in the TnT T7 or SP6 Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer’s protocol. Before coimmunoprecipitation, all in vitro translated proteins were run on SDS-PAGE and then transferred to nitrocellulose. In vitro translation of epitope-tagged proteins was confirmed by immunoblotting with the specific antibodies as indicated and quantified using ImageJ. The same amount of proteins was resuspended in a buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM EDTA, 5 mM DTT, and 0.1% Triton X-100 supplemented with a protease inhibitor tablet and combined with each other for 1 hour at room temperature. The solutions were then centrifuged at 13,000g for 20 min at 4°C. After centrifugation, the supernatant was incubated with anti-MYC or anti-HA antibodies for each immunoprecipitation for 4 hours at 4°C. The resulting immunocomplexes were collected on Protein A/G plus agarose (Santa Cruz Biotechnology) by incubation for 1 hour at 4°C. The immunoprecipitates were washed three times with a buffer and eluted in 1.5× SDS sample buffer by boiling the samples for 5 min at 95°C. Samples were run on SDS-PAGE and transferred to nitrocellulose membrane. Proteins were analyzed by immunoblotting with appropriate antibodies as indicated.

**Protein purification and pull-down experiment**

GST fusion proteins were expressed in Rosetta (DE3)pLysS competent cells (Novagen). GST proteins were induced by 50 to 100 mM isopropyl β-D-1-thiogalactopyranoside for 16 hours at 25°C. Cells were pelleted by centrifugation at 4000g for 10 min. Cells were lysed with lysis buffer containing 50 mM tris (pH 7.5), 0.5 mM EDTA, and 0.3 M NaCl supplemented with an EDTA-free protease inhibitor tablet (Roche). Lysates were mixed with 1:10 dilution of lysozyme (10 mg/ml) and 4 mM DTT and then incubated on ice for 15 min. NP-40 (0.2%) was added to lysates, and lysis was further performed by freeze-thaw cycle. To get soluble proteins, lysates were mixed with a buffer containing 1.5 M NaCl, 12 mM MgCl₂, and 1:1000 dilution of deoxyribonuclease I (10 mg/ml) (Roche) supplemented with an EDTA-free protease inhibitor tablet (Roche). Lysates were passed through a syringe with an 18-gauge needle (BD Biosciences) every 10 to 15 min for 2 hours at 4°C. Soluble proteins were collected by centrifugation at 15,000g for 20 min at 4°C. Supernatants were incubated with glutathione Sepharose beads (GE Healthcare Life Sciences) and washed four times with PBS (pH 8.0). Proteins immobilized on beads were stored at −80°C in PBS containing 10% glycerol and 10 mM DTT. Sometimes, proteins were eluted from glutathione beads by washing the beads with 10 mM glutathione in PBS (pH 8.0). Purified GST-tagged Exo84 RBD proteins were cleaved with thrombin (GE Healthcare Life Sciences) according to the manufacturer’s protocol. For pull-down experiment, cells were washed once with ice-cold PBS and then lysed in pull-down buffer [100 mM tris (pH 7.5), 130 mM NaCl, 1% NP-40, 10% glycerol, 1 mM Na₃VO₄, 10 mM NaF, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT] supplemented with a protease inhibitor tablet (Roche). Lysates were cleared by centrifugation at 13,000g for 15 min and were then incubated with GST fusion proteins bound to glutathione beads (GE Healthcare Life Sciences) for 40 min at 4°C. Beads were washed three times with 1 ml of pull-down lysis buffer and then resuspended in 1.5× SDS sample buffer.

**Subcellular fractionation**

3T3-L1 adipocytes that were 10 days post-differentiated were stimulated as indicated, washed twice in ice-cold PBS (pH 7.5), and then lysed in HE buffer [20 mM HEPES (pH 7.5), 1 mM EDTA, and 250 mM sucrose supplemented with complete EDTA-free protease inhibitor tablets] on ice by scraping with cell lifters (Corning Life Sciences). Samples were transferred and homogenized in a glass Potter homogenizer (Wheaton) for 20 strokes on ice. Fractionation was performed in an NVT 90 rotor (Beckman Coulter) by differential centrifugation using ultracentrifugation with established protocols as previously described (13, 65). Equal amounts of proteins, as determined with Protein Assay Reagent (Bio-Rad), were subjected to SDS-PAGE, followed by immunoblotting analysis using various antibodies as indicated. Plasma membrane fractions were further fractionated by sucrose gradient centrifugation as described below.

**Sucrose gradient centrifugation**

3T3-L1 adipocytes were treated and homogenized same as where subcellular fractionation was performed. Sucrose gradient centrifugation...
was performed as described previously (12). Briefly, the total plasma membrane fraction was solubilized by resuspending the pellet in MBS buffer [20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5) and 150 mM NaCl] with 0.2% Triton X-100. Samples were homogenized in a glass Potter homogenizer (Wheaton) for 10 strokes on ice. Samples (0.4 ml) were mixed with 0.4 ml of 80% (v/v) sucrose and overlaid with 3.5 ml of 30% sucrose and 1 ml of 5% sucrose. The gradient was centrifuged at 35,000 rpm for 19 hours in an SW55 Ti rotor (Beckman Coulter), and 440-μl fractions were collected from the top of each gradient. Each fraction was run on SDS-PAGE, followed by immunoblotting analysis using various antibodies as indicated.

Glucose uptake assay
3T3-L1 adipocytes grown in 12-well plates were subjected to a glucose uptake assay as described previously (12). After serum starvation, cells were incubated with or without 10 nM insulin for 30 min in Krebs-Ringer buffer (12). For inhibitor studies, cells were pretreated for 1 hour with amlexanox or Cay at the given concentrations. The amount of insulin-stimulated glucose uptake was calculated as a percentage by normalizing the ratio of insulin-stimulated 2-DG uptake in each condition to the control.

GLUT4 translocation assay
GLUT4 translocation was measured as described previously (12).

Immunoprecipitation and immunoblotting
For immunoprecipitation, cell lysates were incubated with antibody for 4 hours to overnight at 4°C. Immunoprecipitates were adsorbed on Protein A/G plus agarose (Santa Cruz Biotechnology) for 1.5 to 2 hours, washed three times in lysis buffer, and eluted in 1.5× SDS sample buffer. Proteins were resolved by SDS-PAGE (Life Technologies) and transferred to nitrocellulose membranes (Bio-Rad). Individual proteins were detected with the specific antibodies and visualized on x-ray film using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and Western Lightning Enhanced Chemiluminescence (PerkinElmer Life Sciences).

Mass spectrometry
In-gel digestion followed by LC-MS/MS analysis was carried out by the mass spectrometry–based proteomics resource in the Department of Pathology, University of Michigan, as described previously (32).

Mice and ex vivo glucose uptake assay
Adipocyte-specific TBK1-deficient mice were generated by breeding adiponectin-cre mice to TBK1 FL mice. Epididymal fat was isolated from male 14- to 16-week-old TBK1 FL and adipocyte-specific TBK1 AKO mice. Fat pads were minced and digested with collagenase (1 mg/ml) in PBS at a 37°C shaking water bath. Cells were filtered through 100- and 44-μm filters. Cells were washed three times in lysis buffer, and eluted in 1.5× SDS sample buffer. Proteins were resolved by SDS-PAGE (Life Technologies) and transferred to nitrocellulose membranes (Bio-Rad). Immuno-precipitates were adsorbed on Protein A/G plus agarose (Santa Cruz Biotechnology) for 1.5 to 2 hours, washed three times in lysis buffer, and eluted in 1.5× SDS sample buffer. Proteins were resolved by SDS-PAGE (Life Technologies) and transferred to nitrocellulose membranes (Bio-Rad). Individual proteins were detected with the specific antibodies and visualized on x-ray film using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and Western Lightning Enhanced Chemiluminescence (PerkinElmer Life Sciences).

Mass spectrometry
In-gel digestion followed by LC-MS/MS analysis was carried out by the mass spectrometry–based proteomics resource in the Department of Pathology, University of Michigan, as described previously (32).

Statistical analyses
Averaged values are presented as means ± SEM. When comparing two groups, we performed Student’s t test or Mann-Whitney U test to determine statistical significance. When more than two groups and two factors were investigated, we first performed a Kruskal-Wallis test to establish that not all groups were equal. After a statistically significant Kruskal-Wallis test result, we performed between-group comparisons using the Tukey post hoc analysis for comparisons of all means and Sidak for comparisons of within-factor main effect means. Statistical tests were performed using GraphPad Prism version 6.

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