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ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation

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Endoplasmic reticulum (ER) stress is emerging as a potential contributor to the onset of type 2 diabetes by making cells insulin-resistant. However, our understanding of the mechanisms by which ER stress affects insulin response remains fragmentary. Here we present evidence that the ER stress pathway acts via a conserved signaling mechanism involving the protein kinase PERK to modulate cellular insulin responsiveness. Insulin signaling via AKT reduces activity of FOXO transcription factors. In some cells, PERK can promote insulin responsiveness. However, we found that PERK also acts oppositely via phosphorylation of FOXO to promote FOXO activity. Inhibition of PERK improves cellular insulin responsiveness at the level of FOXO activity. We suggest that the protein kinase PERK may be a promising pharmacological target for ameliorating insulin resistance.

Newly synthesized proteins in the secretory pathway are folded, processed, and assembled in the endoplasmic reticulum (ER). Misfolded proteins are eliminated via the ER-associated degradation (ERAD) pathway to ensure that only correctly folded proteins exit the ER (Nakatsukasa and Brodsky 2008). When substrates exhaust the regulatory capacity of the ERAD pathway, misfolded proteins accumulate, leading to a stress response that halts protein translation and increases the production of molecular chaperones involved in protein folding (Wu and Kaufman 2006). ER stress signaling is mediated through three parallel pathways, including the protein kinases Ire1 and PERK and the transcription factor ATF6 (Malhi and Kaufman 2011). PERK is best known for its ability to inhibit protein biosynthesis through phosphorylation of eIF2α. Whether it has additional regulatory roles in the ER stress pathway remains to be addressed.

ER stress is associated with metabolic disturbance and obesity. ER stress is activated under conditions of cellular nutrient overload, including lipotoxicity (Malhi and Gores 2008). Elevated ER stress is known to cause insulin resistance. However, the molecular pathways through which ER stress impairs cellular insulin response remain incompletely understood. Regulation of the nuclear localization of the Forkhead transcription factor FOXO is a key output of insulin signaling (for review, see Huang and Tindall 2007). Insulin signaling via phosphatidylinositol-3-kinase (PI3K) leads to elevated AKT activity. AKT-mediated phosphorylation of FOXO creates binding sites for 14-3-3 proteins, which promotes cytoplasmic localization of FOXO, thereby lowering its activity in the nucleus. ER stress has been linked to this pathway via Ire1-mediated activation JNK signaling (Urano et al. 2000; Ozcan et al. 2004). JNK phosphorylates the insulin receptor substrate (IRS) proteins and limits activation of PI3K/AKT signaling in response to insulin. Lower AKT activity leads to elevated nuclear FOXO activity. Here we report a parallel mechanism through which the ER stress pathway acting via the protein kinase PERK overrides insulin-induced suppression of the FOXO activity.

Results

ER stress promotes FOXO activity independent of AKT

To identify new regulators of insulin signaling, we performed an RNAi screen for modifiers of FOXO activity (Zhang et al. 2011). Because insulin signaling via AKT

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leads to inactivation of FOXO family transcription factors, FOXO overexpression can challenge the regulatory capacity of the insulin pathway. This provides a sensitive assay for modulators of pathway activity (Junger et al. 2003; Hietakangas and Cohen 2007). Hrd3 (CG10221), an ERAD protein, was among the FOXO modifiers identified in the RNAi screen. Depletion of Hrd3 enhanced the FOXO overexpression phenotype in the *Drosophila* eye, further reducing eye size ($P < 0.001$) (Fig. 1A; quantification in Supplemental Fig. S1A). On its own, Hrd3 depletion had no effect on eye size (Fig. 1B). Efficacy of the RNAi transgene was verified by PCR (Supplemental Fig. S1B). Enhancement of the FOXO overexpression phenotype was also observed when one copy of the endogenous *Hrd3* gene was removed, providing a genetically independent confirmation of the interaction (Supplemental Fig. S1C).

Hrd3 is a component of the ERAD complex (Carvalho et al. 2006; Smith et al. 2011). Dysfunction of the ERAD complex activates the ER stress response and leads to elevated expression of the ER chaperone BiP (Baumeister et al. 2005). BiP mRNA levels increased in Hrd3-depleted cells (Fig. 1C), confirming that the cells were under ER stress. Hrd3 depletion was then used to probe FOXO

activity in S2 cells. FOXO activity is regulated at multiple levels, including nuclear localization (Huang and Tindall 2007). FOXO was predominantly nuclear in cells devoid of growth factors, but upon insulin stimulation, FOXO shifted toward the cytoplasm (Fig. 1D). Depletion of Hrd3 by RNAi limited the insulin-induced increase in cytoplasmic FOXO ($P < 0.001$) (Fig. 1D). The effect of Hrd3 depletion was comparable with that of inhibiting insulin signaling by depleting PI3K (Fig. 1D). Induction of ER stress using tunicamycin mimicked the effect of Hrd3 depletion and limited the insulin-induced shift of FOXO into the cytoplasm (Supplemental Fig. S1D). Tunicamycin treatment also enhanced the FOXO overexpression phenotype in vivo ($P < 0.01$) (Supplemental Fig. S1E). These findings suggest that ER stress can increase FOXO activity by promoting nuclear localization of FOXO.

To ask whether the effect of ER stress on FOXO was mediated by regulation of AKT activity, we examined insulin-induced phosphorylation of AKT in Hrd3-depleted S2 cells. No reduction in the level of AKT phosphorylation was observed, compared with control insulin-stimulated cells (Fig. 1E). Furthermore, tunicamycin-induced ER stress did not reduce the amount of FOXO bound to 14-3-3ε (Fig. 1F). Thus, in S2 cells, ER stress appears to act on
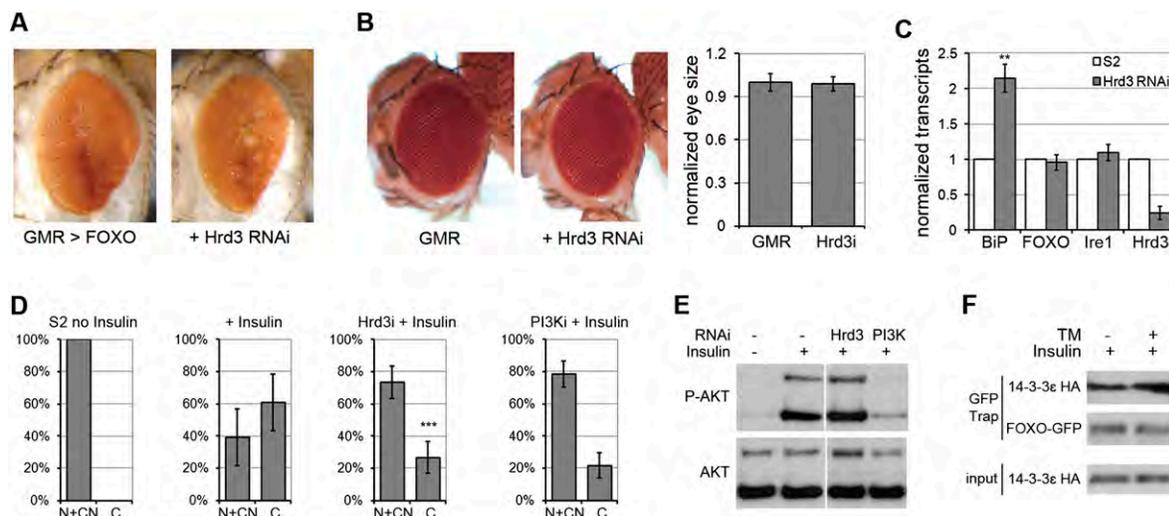
Figure 1. ER stress promotes nuclear localization of FOXO. (*A*) Photomicrographs of *Drosophila* adult eyes expressing UAS-FOXO under GMR-Gal4 control. +Hrd3 indicates coexpression of a UAS-Hrd3^{RNAi} transgene together with UAS-FOXO. Eye size was reduced, on average, by 30%, indicating potentiation of the effects of FOXO (n = 9 eyes of each genotype; $P < 0.001$). Quantification is in Supplemental Figure S1A. (*B*) Adult eyes expressing GMR-Gal4 alone or together with UAS-Hrd3^{RNAi}. Eye size was unchanged. Histogram: average eye area ± SD; $n = 7$. (*C*) S2 cells were treated to deplete Hrd3 transcript by RNAi or with control dsRNA. mRNA levels were measured by quantitative real-time RT–PCR. (*Right*) RNAi reduced Hrd3 transcript by ~80%. Bip transcript increased; (**) $P < 0.01$, Student's t-test. FOXO and Ire1 transcripts were unaffected. Data represent the average of three independent experiments ± SD. (*D*) Quantification of subcellular localization of a FOXO-GFP transgene expressed in *Drosophila* S2 cells. Cells were grown in serum-free medium and deprived of insulin prior to treatment. (*Left* panel) No added insulin. (Second panel) Insulin-treated (10 µg/mL, 30 min). (Third and fourth panels) Insulin-treated 4 d after treatment with dsRNA to deplete Hrd3 or PI3K, as a positive control. (N+CN) Cells with predominantly nuclear localization plus cells with comparable levels in the nucleus and cytoplasm; (C) predominantly cytoplasmic, as described (Zhang et al. 2011). A Fisher's exact test was used to assess the difference in cytoplasmic FOXO comparing insulin-stimulated cells with and without Hrd3 depletion; (***) $P < 0.001$. (*E*) Immunoblots of S2 cells treated with insulin and dsRNAs, as indicated. (*Top* panel) Antibody to S505 phosphorylated *Drosophila* AKT. (*Bottom* panel) Antibody to total *Drosophila* AKT protein. Samples in lanes *1–4* were run on the same gel; intervening lanes were removed between the two sets. (*F*) Immunoblot to monitor the interaction between FOXO-GFP and 14-3-3ε-HA. S2 cells were transfected to express the proteins and treated with insulin with or without tunicamycin to induce ER stress. GFP-tagged FOXO was recovered using GFP trap beads, and blots were probed with anti-GFP and anti-HA to visualize bound 14-3-3.

FOXO localization by a mechanism independent of AKT. These findings raised the possibility that ER stress might be able to override the regulation of FOXO activity by insulin signaling.

ER stress acts via PERK to regulate FOXO activity

The protein kinases Ire1 and PERK are activated upon ER stress. Activation of ER stress by depletion of Hrd3 led to an increase in Ire1 levels (Supplemental Fig. S2A). However, we did not observe any effect of Ire1 over-expression on FOXO nuclear export (Supplemental Fig. S2B). In an RNAi screen, depletion of Ire1 was reported to modestly reduce FOXO abundance but not to affect nuclear localization (Mattila et al. 2008). We confirmed that depletion of Ire1 by >50% did not affect insulin-induced relocalization of FOXO to the cytoplasm or FOXO mislocalization caused by depletion of Hrd3 (Supplemental Fig. S2C–E). Furthermore, depleting Ire1 by RNAi or removing one copy of the *Ire1* gene did not modify the FOXO overexpression phenotype in vivo (Supplemental Fig. S2F). Thus, it appears unlikely that

the effects of ER stress on FOXO are mediated by Ire1 in *Drosophila*. However, feedback from Ire acting via JNK on IRS might affect FOXO activity in other cellular contexts (Ozcan et al. 2004).

In contrast, depletion of PERK by two independent RNAi lines counteracted the effects of FOXO overexpression, leading to an increase in eye size (Fig. 2A; Supplemental Fig. S3A). PERK depletion had no effect on its own (Fig. 2A). The RNAi transgene gave >80% depletion of the *perk* transcript (Supplemental Fig. S3B). In S2 cells, PERK depletion prevented the effect of ER stress on FOXO localization (Fig. 2B). Again, PERK depletion had no effect on its own. Next, we made use of the larval fat body to examine the effects of ER stress on endogenous FOXO protein, visualized by antibody labeling. ER stress caused by Hrd3 depletion induced nuclear accumulation of FOXO, and this was prevented by simultaneous depletion of PERK (Fig. 2C). Conversely, PERK overexpression in vivo strongly elevated the mRNA level of 4E-BP (Fig. 2D), a well-established transcriptional target of FOXO. This increase was blunted by reducing FOXO activity (Fig. 2D). These observations suggest that PERK contributes signif-
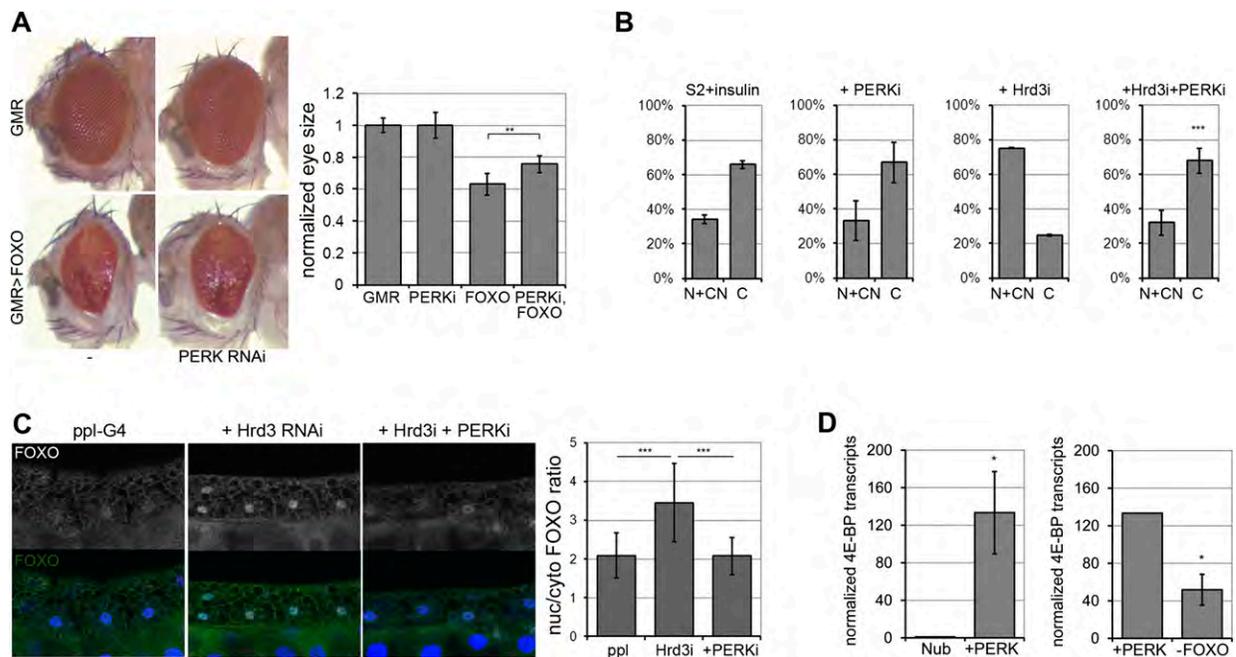
Figure 2. ER stress acts via PERK to regulate FOXO activity. (*A*) Adult eyes from animals expressing GMR-Gal4 alone or with UAS-FOXO. (*Right* panels) Coexpressed UAS-PERK[RNAi]. Histogram: average eye area ± SD; $n \geq 7$. Depletion of PERK offsets the effects of FOXO overexpression, increasing eye area; (**) $P < 0.01$, Student's t-test. (*B*) Quantification of subcellular localization of FOXO-GFP in insulin-stimulated S2 cells. Cells were treated with dsRNA to deplete PERK, Hrd3, or both, as indicated. PERK depletion had no effect but counteracted the effect of Hrd3 depletion. A Fisher's exact test was used to assess the difference in cytoplasmic FOXO comparing Hrd3 depletion and codepletion of Hrd3 and PERK; (***) $P < 0.001$. (*C*) Immunolabeling of endogenous FOXO protein in larval fat body cells. Anti-FOXO is shown in green. Nuclei were labeled with DAPI (blue). Fat body cells are polyploid. *ppl-Gal4* was used to drive UAS-RNAi transgenes for Hrd3 and PERK in fat body cells, as indicated. Hrd3 depletion increased nuclear FOXO (*center*), compared with the *ppl-Gal4* control (*left*). Simultaneous depletion of PERK reduced nuclear FOXO accumulation. The ratio of nuclear to cytoplasmic FOXO label was quantified using DAPI to define the nucleus. (Note that the larger salivary gland cells at the *bottom* do not express the Gal4 driver.) Ratios are shown in the histogram at *right*. (***) $P < 0.001$. (*D*) 4E-BP mRNA level measured by quantitative PCR. (*Left*) RNA from third instar wing discs expressing EP-PERK under Nubbin-Gal4 control. PERK transcript increased 14-fold. (*Right*) FOXO activity was reduced using the dominant-negative alleles $FOXO^{21}/FOXO^{25}$. Data represent the average ± SD for three independent experiments; (*) $P < 0.05$.

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icantly to mediating the effects of ER stress on FOXO activity.

GCN2 is a protein kinase related to PERK. GCN2 and PERK have both been shown to mediate the effects of ER stress by phosphorylation of eIF2α in human cells (Hamanaka et al. 2005). However, unlike PERK, depletion of GCN2 did not potentiate the effects of FOXO over-expression in *Drosophila*.

PERK-mediated FOXO phosphorylation induced by ER stress

Drosophila FOXO-GFP was purified from ER-stressed cells and analyzed by mass spectrometry for phosphory-lated peptides (Supplemental Table S1). To study the contribution of these potential phosphorylation sites in mediating the effects of ER stress on FOXO activity, we prepared serine-to-alanine substitution mutants for several of the candidate sites (Supplemental Fig. S4; Supplemental Table S1). None of the mutants tested compromised the ability of insulin stimulation to induce cytoplasmic localization of FOXO. Nuclear localization of the FOXO S243A mutant was comparable with that of unmodified FOXO in response to insulin (Fig. 3A). How-ever, S243A FOXO was refractory to ER stress-induced nuclear localization compared with the control protein (Fig. 3B). Flies expressing FOXO S243A in the eye were insensitive to the effect of Hrd3 depletion, compared with

flies expressing intact FOXO (Fig. 3C). To further assess the role of S243 in mediating the effects of PERK on FOXO activity, we coexpressed the two forms of FOXO with PERK in vivo. PERK overexpression had much less effect on eye size when coexpressed with FOXO S243A than with intact FOXO (Fig. 3D). Mutations at the other five sites tested were less effective in reducing the effects of ER stress on FOXO localization or activity (Supple-mental Fig. S4). Taken together, these findings suggest that S243 is a key site through which PERK regulates FOXO localization.

Conservation of PERK/FOXO regulation in human cells

To investigate whether PERK regulates FOXO in human cells, we made use of a luciferase reporter containing the human IRS-2 promoter as a readout of FOXO1 activity (Puig and Tjian 2005). H1299 cells lack endogenous FOXO1 but express FOXO3 (Zhao et al. 2010). Expression of FOXO1 in H1299 cells increased reporter expression. Expression of human PERK alone had little effect, but PERK potentiated the effect of FOXO1 (Fig. 4A). Compa-rable results were obtained in H1299 cells transfected to express PERK, FOXO3, and a luciferase reporter contain-ing four synthetic FOXO3 sites (4FRE) (Teleman et al. 2008) and in MCF7 cells transfected to express PERK with FOXO1 or FOXO3 (Supplemental Fig. S5A–C).
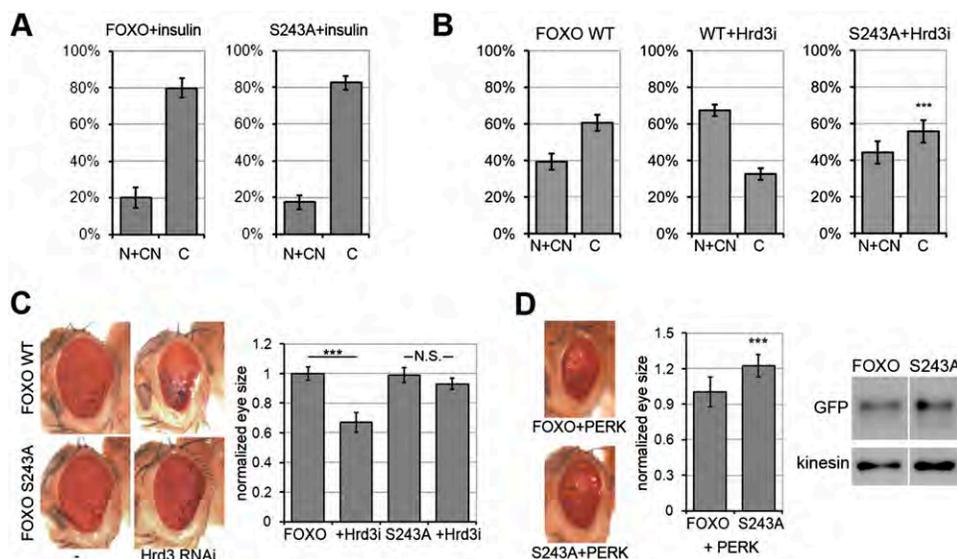
Figure 3. Regulation of FOXO activity by PERK-dependent phosphorylation. (*A*) Localization FOXO-GFP in S2 cells in response to insulin. (*Left*) Insulin stimulated with intact FOXO-GFP. (*Right*) S243A-FOXO-GFP. Data represent the average ± SD for three independent experiments. (*B*) Localization of S243A mutant FOXO-GFP in response to ER stress. (*Left*) Control with intact FOXO-GFP. (*Middle*) Hrd3 depletion shifted FOXO toward the nucleus. (*Right*) S243A FOXO was refractory to Hrd3 depletion. Data represent the average ± SD for three independent experiments. (***) $P < 0.001$ comparing C between FOXO and S243A FOXO in Hrd3-depleted cells (Fisher's exact test). (*C*) Effects of ER stress on eye size in animals expressing UAS-FOXO versus UAS-FOXO-S243A with GMR-Gal4. (*Right*) Eyes coexpressing UAS-Hrd3[RNAi] to induce ER stress. Histogram: average area ± SD; $n \geq 9$ eyes. Depletion of Hrd3 potentiated the effect of FOXO ([***] $P < 0.001$) but did not have a significant effect on S243A (NS). The difference between the two +Hrd3 samples was significant ($P < 0.001$). (*D*) Effects of PERK overexpression on eye size in animals expressing UAS-FOXO versus UAS-FOXO-S243A with GMR-Gal4. (*Left*) PERK overexpression reduced the size of eyes expressing UAS-FOXO more than those expressing UAS-FOXO-S243A. Histogram: average area ± SD, $n = 10$ eyes; (***) $P < 0.001$. (*Right*) Immunoblots showing FOXO-GFP protein visualized with anti-GFP. Anti-kinesin was used to monitor loading.
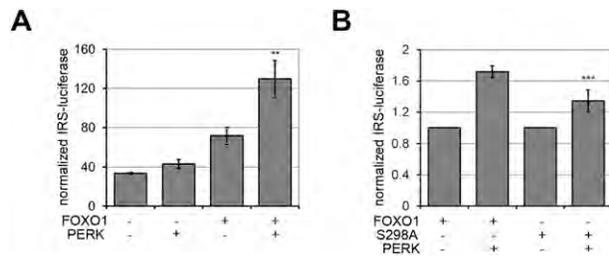
Figure 4. Human PERK regulates FOXO activity. (A,B) Luciferase reporter assays for human FOXO1 activity. H1299 cells were transfected to express a FOXO1-responsive luciferase reporter derived from the IRS-2 gene. Cells were cotransfected to express intact human FOXO1 or S298A mutant FOXO1 and PERK as indicated. Data represent the average ± SD for three or more independent experiments. (A) PERK potentiated the effects of FOXO1; (**) $P < 0.01$, Student's t-test comparing FOXO1 with and without PERK. (B) S298A mutant FOXO1 was refractory to the potentiating effects of PERK; (***) $P < 0.001$ comparing intact and S298A FOXO1 with PERK, Student's t-test.

To identify potential PERK phosphorylation sites on human FOXO1, we performed an in vitro kinase assay using purified FOXO1 and PERK proteins, followed by mass spectrometry. Five phospho-sites were detected (Supplemental Table S2). Mutation of FOXO1 S261 to alanine had no effect on PERK-induced FOXO1 activity (Supplemental Fig. S5D). The other four sites—S298, S301, S303, and S311—were clustered in a single peptide that corresponds to the region of *Drosophila* FOXO containing S243.

To assess the contribution of ER stress to phosphorylation of these sites, we performed SILAC (stable isotope labeling by amino acids in cell culture) mass spectrometry on samples of human FOXO1 immunopurified from control MDA-MB231 cells and cells treated with tunicamycin. Six peptides showed increased phosphorylation on serine or threonine residues in the ER-stressed samples (Supplemental Tables S3, S4). Phosphorylation of the peptide containing S298, S301, S303, and S311 was enriched 1.5-fold in FOXO1 from ER-stressed cells. Phosphorylation was observed on S298 alone or on S298 in combination with either S301 or S303. These sites were also phosphorylated by PERK in vitro. The corresponding peptide was also phosphorylated in FOXO1 and FOXO3 in ER-stressed H1299 cells (Supplemental Table S5). We prepared alanine substitution mutants for S298, S301, and S303 to assess the contribution of these sites to mediating the effects of PERK on FOXO1. The S298A mutation reduced the responsiveness of FOXO1 to PERK (Fig. 4B), but mutation of S301 or S303 had little or no effect (Supplemental Fig. S5D). The observation that S298A accounts for approximately half of the effect of PERK on FOXO1 suggests that other phospho-sites also contribute to mediating the effects of ER stress on FOXO1 in vivo.

Previous reports have identified other phosphorylation sites through which FOXO activity can be regulated. Phosphorylation of FOXO1 on S249 by CDK1 has been reported to reduce interaction with 14-3-3, thereby increasing FOXO activity (Yuan et al. 2008). However,

CDK2-mediated phosphorylation of FOXO1 at S249 was reported to increase cytoplasmic localization of FOXO and reduce its activity (Huang et al. 2006). Thus, the role of S249 remains unclear. MST1 has also been reported to phosphorylate FOXO1 and FOXO3 on several residues, among which phosphorylation of S207 in FOXO3 was found to reduce interaction with 14-3-3 (Lehtinen et al. 2006). The MST1 target site in FOXO1 is inside the conserved DNA-binding domain and corresponds to S147 in *Drosophila* FOXO. Mutation of *Drosophila* FOXO to S147A did not have a significant effect on nuclear accumulation in response to ER stress (Supplemental Fig. S4D).

Opposing activity of AKT and PERK on FOXO

Insulin signaling via PI3K activates AKT and increases phosphorylation of FOXO, creating 14-3-3-binding sites, which promotes cytoplasmic accumulation of FOXO (for review, see Huang and Tindall 2007). Consequently, FOXO activity is expected to be higher under conditions of low insulin pathway activity. Conversely, our findings suggest that PERK potentiates FOXO activity. S298 is not among the FOXO1 sites phosphorylated by AKT, which include T24, S256, and S319 (Greer and Brunet 2005). This raised the possibility that PERK-mediated phosphorylation of FOXO might be able to counteract the effects of AKT, in effect opposing the effects of the insulin pathway.

To test this possibility, we used a cellular assay to model insulin resistance and monitored the effects of PERK depletion on FOXO activity. H1299 cells were serum-starved to remove insulin and treated with the PI3K inhibitor LY294002 to further reduce AKT activity. Under these conditions, endogenous FOXO3 activity increased, as monitored using a FOXO3 luciferase reporter (Fig. 5A), while simultaneous depletion of PERK by siRNA treatment lowered FOXO3 activity (Fig. 5B). Next, we examined the effects of reduced PERK activity on endogenous FOXO1 and FOXO3 targets in HEPG2 liver cells (which express both FOXO1 and FOXO3). HEPG2 cells were deprived of serum and treated with the PI3K inhibitor to lower AKT activity. Several FOXO targets showed a consistent pattern of increased expression under these conditions (Medema et al. 2000; Nakae et al. 2003; Martinez-Gac et al. 2004; Puig and Tjian 2005), and this increase too was offset by depletion of PERK (Fig. 5C). Thus, endogenous PERK activity contributes to FOXO activation under conditions in which the inhibitory effects of AKT are removed. These observations support the idea that PERK acts in opposition to AKT in the regulation of FOXO activity.

Feedback regulation linking the ER stress and insulin pathways

We noted that the insulin receptor (INSR) was among the FOXO1 targets up-regulated in HEPG2 cells under low AKT activity and that this was offset by depletion of PERK activity (Fig. 5C, INSR). This suggested that PERK, acting via FOXO1, could promote cellular insulin responsiveness by increasing INSR expression. If so, depletion of PERK should lower insulin signaling and

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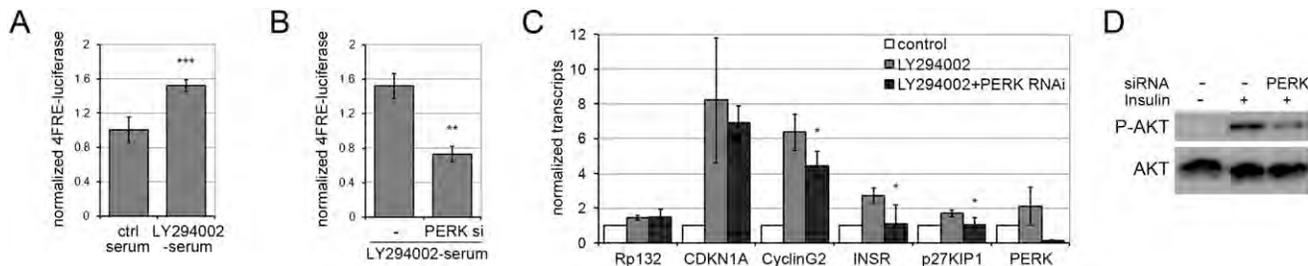
Figure 5. Depletion of human PERK limits FOXO activity. (*A*) Luciferase reporter assays for endogenous FOXO3 activity in H1299 cells. H1299 cells were transfected to express a FOXO3-responsive luciferase reporter containing four synthetic FOXO3-binding sites (4FRE). Cells were serum-starved to reduce insulin signaling and treated with the PI3K inhibitor LY294002 to further reduce AKT activity. Data represent the average ± SD for six independent experiments. (***) $P < 0.001$. (*B*) FOXO3 Luciferase assays as in *A*, except that cells were treated with siRNA to deplete endogenous PERK or were left untreated. Data represent the average ± SD for three independent experiments. (**) $P < 0.01$. (*C*) Normalized mRNA levels of FOXO1 targets measured by quantitative PCR. HEPG2 cells were serum-starved to reduce insulin signaling and treated with the PI3K inhibitor LY294002 to further reduce AKT activity. Four of the 14 FOXO targets tested showed a consistent increase in mRNA levels (gray bars) compared with control cells in normal medium (open bars). (Black bars) PERK siRNA treatment. (*) $P < 0.05$ with/without PERK siRNA treatment. Efficacy of PERK siRNA treatment is shown at the far *right*. (*D*) Immunoblot of HepG2 cells treated with insulin and with siRNA to deplete endogenous PERK. (*Top* panel) Probed with antibody to S473 phosphorylated human AKT. (*Bottom* panel) Probed with antibody to total AKT protein.

AKT activity. We confirmed that this takes place in PERK-depleted HepG2 cells (Fig. 5D).

Discussion

Regulatory interplay between ER stress and the insulin pathway operates at multiple levels, suggesting that fine-tuning this balance is of some importance to cellular metabolism. In this study, we presented evidence that PERK can act directly on FOXO to increase FOXO activity. PERK can also act indirectly via AKT to lower FOXO activity. This relationship has the topology of an incoherent feed-forward motif (Alon 2007). Such motifs provide stability in the output signaling systems, allowing initial activation followed by inhibition to limit the response.

ER stress acts by at least three independent routes to control AKT activity. PERK acts via FOXO to up-regulate INSR levels, which increase AKT activity. PERK has recently been reported to promote AKT activity by acting as a lipid kinase via DAG (Bobrovnikova-Marjon et al. 2012). Regulatory feedback between ER stress and insulin signaling also operates at another level. ER stress has been shown to act via Ire1 and JNK to phosphorylate the IRS and thereby reduce insulin signaling and Akt activation (Ozcan et al. 2004). Thus, at least three distinct mechanisms link activity of the ER stress pathway to control of insulin pathway activity. We note that the SILAC data showed increased phosphorylation of three AKT sites (T24, S256, and S319) in addition to increased phosphorylation of the PERK site S298 in cells subjected to ER stress.

Additionally, AKT feeds back to limit PERK activity. Increased AKT activity has been reported to lower PERK activity (Mounir et al. 2011). We also noted that PERK mRNA levels were increased in cells with low AKT activity (Fig. 5C). Inhibitory feedback from AKT to PERK would reinforce the dampening effect of AKT on the output of the ER stress pathway and also provides a means for insulin or growth factor signaling via AKT to modu-

late the effects of ER stress mediated by PERK. The extent to which these feedback systems operate may depend on cellular and physiological context.

Genetic models of obesity, such as the ob/ob mouse, as well as diet-induced obesity have been found to induce ER stress (Ozcan et al. 2004). Obesity-induced ER stress, acting via PERK on FOXO, would enhance the effects of obesity-induced insulin resistance, in which Ire1 lowers insulin responsiveness. Obesity-induced ER stress should also synergize with other causes of insulin resistance to exacerbate the cellular metabolic imbalance. Therefore, regulation of FOXO activity emerges as a nodal point on which the effects of obesity on cellular insulin responsiveness converge. Chronic elevation of FOXO activity may contribute to disease progression. In this context, it is noteworthy that reducing FOXO1 activity by removing one copy of the FOXO1 gene has been shown to improve insulin sensitivity in genetic and diet-induced models of insulin resistance in mice (Nakae et al. 2002; Kim et al. 2009).

This study raises the possibility that pharmacological inhibition of PERK could provide a useful therapeutic approach for combating the effects of ER stress in progression to diabetes. Use of chemical chaperones to overcome ER stress has been reported to decrease insulin and glucose levels and improve glucose and insulin tolerance in insulin-resistant ob/ob mice (Ozcan et al. 2006). A similar finding has been reported using the chemical chaperone sodium phenylbutyrate in obese diabetic humans (Xiao et al. 2011). The ER stress pathway may provide a fruitful source of targets for new therapeutics to treat diabetes and metabolic syndrome.

Materials and methods

Fly genetics

Df(3R)Exel9013, *Df(3R)BSC636*, *UAS-RNAi-PERK (BL)*, and *EP-PERK* flies were obtained from the Bloomington Stock Center.

UAS-RNAi-Hrd3, *UAS-RNAi-Ire1*, *UAS-RNAi-PERK*, and *UAS-RNAi-Ire1* were from the Vienna *Drosophila* RNAi Center.

S2 cell culture and treatments

S2 cells were grown at 25°C in SFM (Gibco) supplemented with L-glutamine. dsRNA was prepared using MegascriptT7 (Ambion) with the following templates: Hrd3 #1, nucleotides 1801–2200 of Hrd3 coding sequence (FBpp0083858); Hrd3 #2, nucleotides 2800–3138 of FBtr0084467. If not specified, Hrd3 RNAi refers to sequence #1; PI3K, nucleotides 358–857 of Pi3K92E coding sequence (FBpp0083348); PERK, nucleotides 1045–1506 of FBpp0078417; Ire1, nucleotides 391–883 of FBpp0112161; and GFP, nucleotides 17–633 of EGFP2 were used as control. S2 cells were treated with 37 nM dsRNA. Cells were transfected using Effectene (Qiagen) with pMT-FOXO-GFP, pMT-Gal4, and pUASP-14-3-3ε-HA (provided by Pernille Rørth). CuSO$_4$ (0.7 mM) was used to induce FOXO 14-3-3ε and Ire1 expression.

Human cell culture and treatments

H1299, MCF7, and HepG2 cells were maintained at 37°C in DMEM supplemented with 10% FBS. Cells were transfected using FuGENE 6 or Lipofectamine 2000 with pcDNA-FOXO1 (Puig and Tjian 2005), pcDNA-FOXO3 (generated by Aurelio Teleman from Addgene1787), pCMV-PERK (SC117132 from OriGene), and pEGFP-N1. pEGFP-N1-FOXO3 was generated at XhoI and BamHI sites using pcDNA-FOXO3 as a template. PERK siRNA (Qiagen, SI02223718) was transfected using HiPerFect reagent. For SILAC mass spectrometry experiments, MDA-MB231cells were maintained in SILAC-DMEM (Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (Thermo Fisher Scientific) and penicillin/streptomycin for at least six cell divisions in order to achieve uniform incorporation. Light medium was supplemented with 0.8 mM L-lysine:HCl and 0.4 mM L-arginine:HCl (Sigma). Heavy medium was supplemented with 0.8 mM L-lysine:2HCl (U-13C6 and U-15N2) and 0.4 mM L-arginine:HCl (U-13C6 and U-15N4) (Cambridge Isotope). Cells were transfected with pcDNA-FOXO1 using TurboFect to express FOXO1-V5 for 2 d. Cells in light medium were left untreated, and cells in heavy medium were treated with tunicamycin for 4 h. FOXO1-V5 was purified using Protein-G Sepharose beads (GE) and anti-V5 (Invitrogen) in RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor (Roche).

Antibodies

Antibody to *Drosophila* FOXO was from Puig et al. (2003). Antibodies to phospho-S505-AKT (*Drosophila*), phospho-S473-AKT (human), AKT, and Myc were from Cell Signaling Technology. Anti-GFP was from Invitrogen (A6455). HA antibody was from Roche. Anti-kinesin was from Cytoskeleton. For immunoblotting, samples were homogenized in SDS sample buffer, boiled, and resolved by SDS-PAGE before transfer to nitrocellulose membranes for antibody labeling.

In vitro kinase assay

Purified PERK was purchased from Invitrogen (PV5106). Kinase assays were conducted according to the manufacturer's protocol in 1× kinase buffer (PV3189) for 1 h at 25°C. His-tagged FOXO1 was expressed in *Escherichia coli* and purified by nickel chelate chromatography.

Mass spectrometry

SDS-PAGE and in-gel digestion   Immunoprecipitated samples were run on an SDS-PAGE using a NuPAGE 4%–12% Bis-Tris gel (Invitrogen). The FOXO bands were excised followed by in-gel digestion (Shevchenko et al. 2006), with minor modifications. Gel pieces were washed with 50 μL of 50 mM ammonium bicarbonate and destained with 50 μL of 50% acetonitrile/25 mM ammonium bicarbonate for 10 min. Reduction was carried out by covering the gel pieces with 10 mM DTT for 30 min at 56°C; alkylation was carried out with 55 mM iodoacetamide for 15 min in the dark at room temperature. Fifty microliters of 50 mM ammonium bicarbonate was used for washing, and 50 μL of 100% acetonitrile was used for shrinking twice for 10 min. Thirty microliters of 13 ng/μL sequencing-grade trypsin (Promega) was added to each well for 30 min at 4°C before 25 mM ammonium bicarbonate was added to cover the gel pieces. Samples were incubated for 3 h at 37°C. Supernatants containing peptides were cleared by centrifugation. Twenty microliters of 5% formic acid was added to each well followed by 20 μL of 100% acetonitrile for peptide extraction. Both steps were repeated.

Liquid chromatography/mass spectrometry analysis   Vacuum-dried sample was reconstituted in 0.1% formic acid and analyzed using nanoHPLC coupled to an LTQ Orbitrap classic, LTQ Orbitrap XL, Q-Exactive, or LTQ Velos (Thermo Fisher Scientific). Peptides were trapped onto a C18 precolumn and separated on an analytical column using a 2- to 4-h gradient ranging from 2% to 40% acetonitrile/0.1% formic acid, followed by a 5-min gradient ranging from 40% to 80% acetonitrile/0.1% formic acid. For Q-Exactive, survey full-scan mass spectrometry spectra (m/z 310–2000) were acquired with a resolution of $r = 70,000$, an AGC target of 1×10^6, and a maximum injection time of 2 msec. The 10 most intense peptide ions in each survey scan with an intensity threshold of 40,000, underfill ratio of 1%, and charge state ≥2 were isolated sequentially with an isolation window of 2 Th to a target value of 20,000 and a maximum injection time of 50 msec and fragmented in the high-energy collision cell by high-energy collision dissociation using a normalized collision energy of 27%. The tandem mass spectrometry was acquired with a resolution of 17,500 and a starting mass of m/z 100. A dynamic exclusion was applied using an exclusion duration of 10 sec. For LTQ Orbitrap, survey full-scan mass spectrometry spectra (m/z 310–1400) were acquired with a resolution of $r = 60,000$ at m/z 400, an AGC target of 1×10^6, and a maximum injection time of 1000 msec. The 10 most intense peptide ions in each survey scan with an ion intensity of >2000 counts and a charge state ≥2 were isolated sequentially to a target value of 5000 and fragmented in the linear ion trap by collision-induced dissociation (CID) using a normalized collision energy of 35%. A dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count, repeat duration of 45 sec, and exclusion duration of 30 sec. For LTQ Velos, we used mass spectrometry scans ranging from 310 to 1400 m/z, AGC target 3×10^4, and maximum injection time of 10 msec. The 20 most intense ions with an ion intensity >1000 and a charge state excluding 1 were sequentially isolated to a maximum AGC target value of 1×10^4 for a maximum of 100 msec and fragmented by either CID using a normalized collision energy of 30% or electron transfer dissociation (ETD). For ETD, the following parameters were used: reagent ion source temp of 160°C, reagent ion source emission current of 50 μA, reagent ion source electron energy of −70.00 V, reagent ion source CI pressure of 20 psi, reagent vial 1 ion time of 50, reagent vial 1 AGC target of 1×10^5, reagent vial 2 ion time of 50, reagent vial 2 AGC target of 1×10^5, and supplemental activation energy of 15. For both ETD and CID, a dynamic exclusion list was applied using an exclusion list size of 500, one repeat count, repeat duration of 45 sec, and exclusion duration of 30 sec, as well as a mass width of 1.0 low and 1.5 high. Expiration count was disabled.

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Data processing and database search

Raw file processing for unlabeled samples was carried out using Mascot Daemon (version 2.3.2, Matrix Science). A data import filter for precursor masses from 700 to 4000 Da with a minimum scans per group of 1 and a minimum peak count of 10 was used. Mascot search was performed using the UniProt *Drosophila* database (released on_2010_09, 34043 entries) or IPI Human database (ipi.HUMAN.v3.68.decoy.fasta or ipi.HUMAN.v3.86. decoy.fasta). For the SILAC-labeled samples, the raw files were processed using MaxQuant version 1.2.0.18 using the default parameters. An Andromeda search was carried out using the IPI Human database (ipi.HUMAN.v3.86.decoy.fasta). In both the Mascot and Andromeda searches, trypsin was specified as the enzyme, and there were two allowed missed cleavages. Carbamidomethyl (C) was set as a static modification, while the dynamic modifications were acetyl (protein N-term), oxidation (M), and phosphorylation (S/T/Y). The tolerance for the precursor masses was 7 parts per million (ppm), and the tolerance for fragments was 0.5 Da for samples analyzed on LTQ Orbitrap. Tolerance for the precursor masses was 2 Da, and the tolerance for fragments was 0.8 Da for samples analyzed on LTQ Velos. The tolerance for fragments was 20 ppm for samples analyzed on Q-Exactive.

Statistical analysis

Statistical analysis used the Fisher's exact test (for categorical data) and Student's t-test (for normally distributed data). All data are presented as average $\pm$ SD. $P < 0.05$ was considered significant.

Acknowledgments

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ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation

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The Hippo pathway acts via p53 and microRNAs to control proliferation and proapoptotic gene expression during tissue growth

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Summary

The Hippo pathway has a central role in coordinating tissue growth and apoptosis. Mutations that compromise Hippo pathway activity cause tissue overgrowth and have been causally linked to cancer. In *Drosophila*, the transcriptional coactivator Yorkie mediates Hippo pathway activity to control the expression of cyclin E and Myc to promote cell proliferation, as well as the expression of *bantam* miRNA and DIAP1 to inhibit cell death. Here we present evidence that the Hippo pathway acts via Yorkie and p53 to control the expression of the proapoptotic gene *reaper*. Yorkie further mediates *reaper* levels post-transcriptionally through regulation of members of the miR-2 microRNA family to prevent apoptosis. These findings provide evidence that the Hippo pathway acts via several distinct routes to limit proliferation-induced apoptosis.

Introduction

Cell proliferation is intimately linked with cell death. Cues that drive cell growth and division also induce apoptosis (Pelengaris et al., 2002). In an abnormal cell proliferation scenario, such as cancer, cells adopt a variety of strategies to overcome cell death (Hanahan and Weinberg, 2011). Many signaling pathways that drive tissue growth have been found to coordinate cell proliferation and apoptosis during animal development. Defects in these pathways quite often cause tissue overgrowth or cancer.

The Hippo pathway is one such signaling pathway, acting as a negative growth regulator. Mutations in several members of the pathway lead to tumorigenesis, implicating them as tumor suppressors (Cai et al., 2010; Pan, 2010). The core pathway comprises a kinase cascade including the Hippo and Warts kinases with their adaptors Salvador (Sav) and Mats (Moberg et al., 2001; Tapon et al., 2002; Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003). Several proteins have been implicated as upstream regulators of this kinase cascade by genetic studies, including Merlin/NF2, Expanded and the atypical cadherin, Fat (Hamaratoglu et al., 2006; Silva et al., 2006; Yu et al., 2010). Downstream, the Warts kinase directly phosphorylates and inactivates transcriptional coactivators including YAP, TAZ, and in *Drosophila*, Yorkie (Yki) (Huang et al., 2005; Zhao et al., 2007; Zhao et al., 2008; Zhao et al., 2010). YAP/TAZ and Yki function to promote cell proliferation and inhibit apoptosis. These proteins possess no DNA binding activity and therefore bind to transcription factors including Scalloped/TEAD to activate their targets.

Genetic studies have identified functional targets of Yki with positive roles in cell proliferation, including cycE and Myc, as well as negative regulators of apoptosis, including the *Drosophila* Inhibitor of Apoptosis Protein (DIAP1) and the antiapoptotic microRNA *bantam* (Moberg et al., 2001; Moberg et al., 2004; Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006; Neto-Silva et al., 2010). These studies have suggested that the Hippo pathway can balance proliferative drive with limitation of proliferation-induced apoptosis. This combination of roles may also explain the potency of mammalian YAP in control of tissue growth and its ability to induce cancer when overexpressed (Dong et al., 2007; Pan, 2010).

The tumor suppressor p53 is another key regulator coordinating cell division and cell death. Activation of p53 by the DNA damage checkpoint or other cell cycle abnormalities, leads to growth arrest, and initiates apoptosis. Activated p53 binds DNA and directs expression of downstream genes including p21, which inhibits the activity of cyclin–CDK complexes and activates cell cycle checkpoints to halt cell division (Gartel and Radhakrishnan, 2005). In addition, p53 promotes transcription of the proapoptotic genes Bax, PUMA and Apaf-1 to induce cell death. Recent studies in human cells have identified the ASPP1 protein (apoptosis-stimulating protein of p53-1) as a key mediator of p53-induced apoptosis (Aylon et al., 2010; Vigneron et al., 2010). The Hippo pathway kinase Lats,

the mammalian homolog of Warts, phosphorylates ASPP1 and forms a complex with ASPP1 and p53 to activate the proapoptotic transcription program. Phosphorylation of ASPP1, however, can be antagonized by another Lats substrate YAP.

The upstream control of the apoptosis program is conserved in *Drosophila*, with p53 serving as a mediator of the DNA damage checkpoint. However, the effector program involves a set of insect-specific proapoptotic genes: *reaper, head involution defective (hid), grim and sickle (skl)* (Steller, 2008). The proapoptotic activity of these four proteins results from their ability to bind and inactivate DIAP, which in turn inhibits caspases. In mammals the corresponding functions are provided by Apaf-1 to cleave and activate caspases instead of derepression of caspases (Pop et al., 2006). Previous studies in *Drosophila* have shown that the *bantam* microRNA acts to repress *hid* to limit proliferation induced apoptosis (Brennecke et al., 2003). *bantam* mediates interaction between the EGFR and Hippo growth control pathways (Herranz et al., 2012). microRNAs of the miR-2 seed family have also been shown to regulate the expression of the proapoptotic genes *reaper*, *grim* and *skl* (Stark et al., 2003; Brennecke et al., 2005; Leaman et al., 2005; Thermann and Hentze, 2007) and to limit apoptosis in the developing nervous system (Ge et al., 2012).

In view of the importance of the Hippo pathway in regulating proliferation-induced apoptosis, we have examined other modes of action for Yki. Here we provide evidence for additional parallel pathways involving Yki, p53 and the miR-2 family of microRNAs in controlling the expression of *reaper* another key proapoptotic gene. Yki acts via regulation of p53 on *reaper* transcription. In some tissues, Yki acts independently via members of the miR-2 family to regulate expression of *reaper* post-transcriptionally. Our findings place Yki at the center of a network of regulatory relationships balancing cell proliferation, p53-dependent checkpoints, proapoptotic genes and miRNAs in control of tissue growth.

Results

Hippo pathway controls apoptosis by limiting reaper expression

The transcription coactivator Yorkie mediates Hippo pathway activity to control gene expression in *Drosophila*. We used RNAi to deplete *yorkie* (*yki*) mRNA from S2 cells, to assess the contribution of the Hippo pathway to expression of genes involved in regulation of apoptosis. Depletion of *yki* mRNA was effective, and resulted in increased expression of *reaper* mRNA and a smaller increase in *hid* mRNA (Fig. 1A, $**P<0.01$). To test this relationship in a growing tissue *yki* was overexpressed in the wing imaginal disc under control of *nubbin-Gal4*. *yki* overexpression decreased the level of *reaper* mRNA (Fig. 1B, $P<0.01$; control for *yki* mRNA level in supplementary material Fig. S1A). Thus Yki appears to negatively regulate expression of *reaper*.

Does regulation of *reaper* contribute to the growth regulatory activity of the Hippo pathway *in vivo*? We made use of *patched-Gal4* (*ptc-Gal4*) to direct depletion of *yki* in a defined region of the wing (shaded in Fig. 1C). Expression of a *UAS-yki^RNAi* transgene under *ptc-Gal4* control reduced the area of the relevant region of the wing (Fig. 1C). This effect was quantified by measuring the ratio of the width of the vein 3–4 region to that of the vein 4–5 region (indicated by solid and dashed red lines, upper left panel of Fig. 1C). Depletion of *yki* reduced the relative size of the region where the *Gal4* driver was expressed (Fig. 1D).

This effect was partially offset by concurrently limiting *reaper* expression using a chromosomal deletion, *Df(3L)XR38*, which removes *reaper* and *skl*, but not the adjacent *grim* and *hid* genes (Peterson et al., 2002). *Df(3L)XR38* on its own showed no effect on growth, but limited the undergrowth caused by *yki* depletion (Fig. 1C,D; $P<0.001$). These observations suggest that increased expression of *reaper* contributes to the effects of *yki* depletion *in vivo*.

Yki acts via induction of p53 activity

Previous reports have shown that p53 can directly regulate *reaper* expression in *Drosophila* (Brodsky et al., 2000; Peterson et al., 2002; Zhou and Steller, 2003). This raised the possibility that Yki might act via p53 to control *reaper* during tissue growth *in vivo*. To test this we used the *ptc-Gal4 UAS-yki^RNAi* undergrowth assay. Coexpression of a dominant negative form of p53 (p53DN) partially suppressed the tissue undergrowth caused by depletion of *yki* (Fig. 2A; $P<0.001$). Expression of p53DN on its own had no effect on growth. Similarly, reducing p53 activity by introducing a null allele of the *p53* gene also partially suppressed the effects of *engrailed-Gal4 UAS-yki^RNAi* on tissue growth (Fig. 2B; $P<0.05$). The *p53* mutant had no effect on its own.

p53 can also be activated through the caspase Dronc (Nedd2-like caspase, Nc (Wells et al., 2006; Shlevkov and Morata, 2012)). This raised the possibility that depletion of Yki by RNAi could lead to reduced DIAP1 expression and thereby trigger Dronc-mediated activation of p53. To address this possibility, we depleted both Yki and Dronc from S2 cells and found that the increase in *reaper* mRNA levels was not reduced compared to cells depleted of Yki only, as might have been expected if the effects of Yki depletion were mediated through this feedback loop (Fig. 2C). Furthermore, *reaper* mRNA levels were higher in wing discs coexpressing *UAS-DIAP1* and *UAS-Yki^RNAi* compared to *UAS-Yki^RNAi* alone (Fig. 2D, $P<0.05$; DIAP1 overexpression quantified in supplementary material Fig. S1B). The increase in *reaper* levels may reflect improved survival of Yki-depleted cells when expressing DIAP1. Caspase activation due to low DIAP1 levels also seems unlikely to explain the effects of Yki depletion on *reaper* mRNA levels.

In mammalian cells expressing the oncogenic form of H-Ras, Lats, a component of the Hippo pathway, has been shown to phosphorylate ASPP1 and form a complex with ASPP1 and p53 to direct expression of pro-apoptotic genes (Aylon et al., 2010; Vigneron et al., 2010). To ask whether *Drosophila* ASPP (CG18375) might also be involved in the context of Yki regulation of p53 activity in normal tissue growth, we assessed the effects of removing one copy of the *ASPP* gene on *reaper* mRNA levels in wing discs. Quantitative RT-PCR showed that *reaper* mRNA was reduced by ~25%, when ASPP mRNA was reduced to ~50% in these discs (Fig. 2E; $**P<0.01$). Next, we assessed the effects of depleting ASPP by RNAi and the effects of removing one copy of the *ASPP* gene in the *ptc-Gal4 UAS-yki^RNAi* undergrowth assay. In both scenarios reduced ASPP activity partially restored growth of the *yki*-depleted tissue (Fig. 2F,G; $***P<0.001$).

Taken together, these observations suggest that the Hippo pathway acts through Yki and p53 to control *reaper* expression. The involvement of ASPP, suggests that this regulation is likely to be mediated through Yki binding to Lats/Wts and competing for ASPP1 phosphorylation, as described in mammalian cell culture models (Aylon et al., 2010; Vigneron et al., 2010). Here
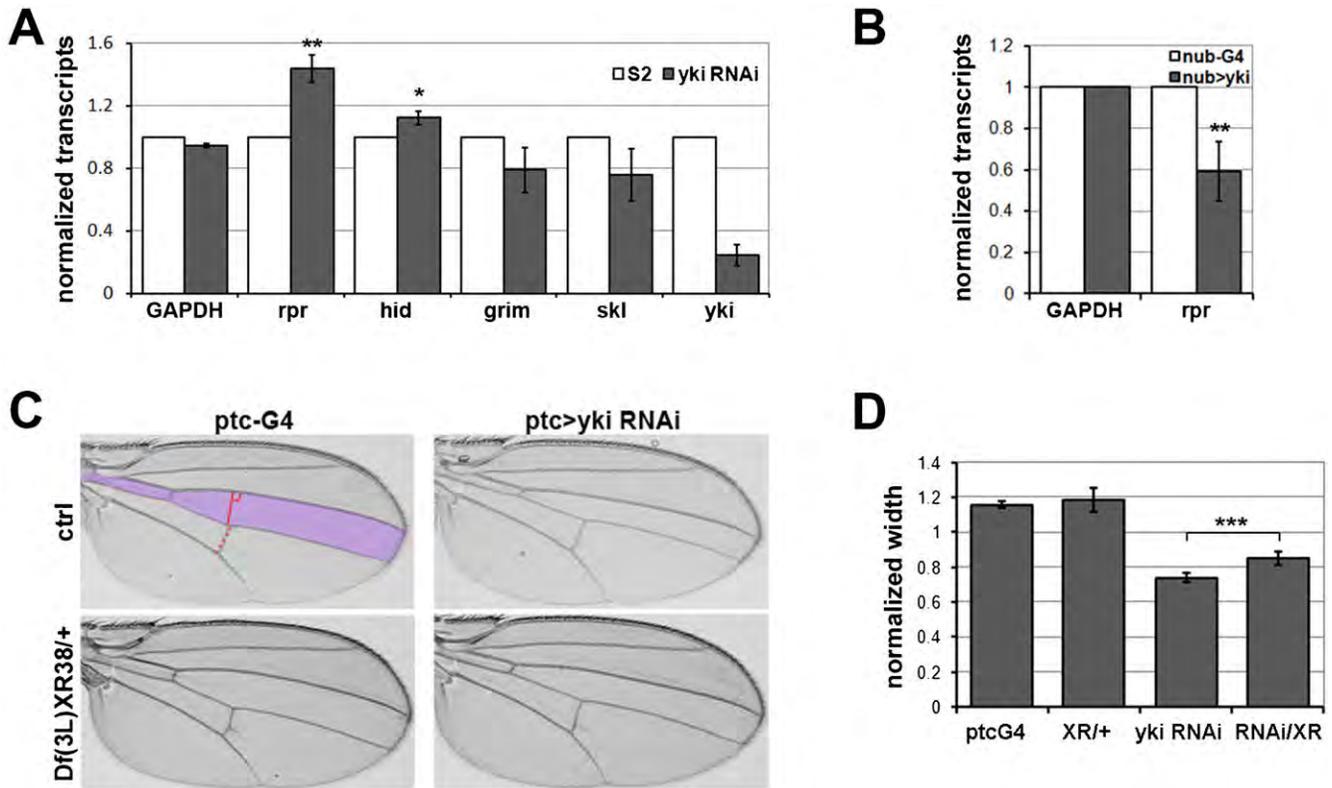
Fig. 1. Yorkie regulates *reaper* activity in tissue growth control. (**A**) Histogram showing the levels of *reaper* (*rpr*), *hid*, *grim*, *skl*, *GAPDH* and *yki* mRNAs measured by quantitative RT-PCR. S2 cells were treated with dsRNA to deplete *yki* (grey bars) or GFP as control (white bars). The *yki* RNA measurement shows that the RNAi treatment was effective. *GAPDH* serves as a control. Total RNA was extracted and normalized for cDNA synthesis. RNA levels were normalized to *kinesin* mRNA. Error bars represent standard deviation from 3 independent experiments. (*) Student's t-test for *hid* vs *GAPDH*: $P<0.05$; (**) Student's t-test for *rpr* vs *GAPDH*: $P<0.01$. (**B**) Histogram showing the levels of *rpr* and *GAPDH* mRNAs measured by quantitative RT-PCR. RNA was extracted from wing imaginal discs expressing *nub-Gal4* alone (white bars) or *nub-Gal4* with a *UAS-Yki* transgene. Error bars represent standard deviation from 3 independent experiments. (**) Student's t-test for *rpr* vs *GAPDH*: $P<0.01$. (**C**) Photomicrographs of adult wings of the indicated genotype. The domain of *ptc-Gal4* expression is shaded in the upper left panel. Left panels: *ptc-Gal4* control flies. Right panels *ptc-Gal4* driving expression of a *UAS-yki^{RNAi}* transgene to reduce *yki* mRNA levels in the *ptc-Gal4* expression domain. Upper panels: control flies with 2 copies of the proapoptotic genes. Lower panels: flies carrying one copy of *Df(3L)XR38*. (**D**) Quantification of the effects of the treatments in panel C on the size of the *ptc-Gal4* expression domain. Data are represented as the ratio of the width of the region between veins 3–4 (solid red line) to the region between veins 4–5 (dashed red line, measured along the posterior crossvein). In normal flies this ratio is ~1.2:1. Note that there was no effect of *ptc-Gal4* driven Yki RNAi on the size of the region between veins 4–5 (not shown), so the ratio reflects reduction of the L3–4 region. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when one copy of the *rpr* and *skl* genes were removed ($P<0.001$).

we present evidence that limiting *reaper* levels by manipulating p53-ASPP1 activity contributes to suppressing the tissue growth effects of the Hippo pathway. This observation is consistent with a model in which the Hippo pathway regulates p53 activity to control proliferation-induced apoptosis.

Yki regulates miRNA expression to control *reaper* level

Previous reports have shown that microRNAs of the miR-2 seed family (Fig. 3A) can regulate *reaper, grim* and *skl* (Stark et al., 2003; Leaman et al., 2005; Brennecke et al., 2005; Thermann and Hentze, 2007). This prompted us to ask whether there might be a miRNA-based mechanism by which the Hippo pathway controls *reaper* expression. As a first step we asked which of the miR-2 family miRNAs is subject to regulation by the Hippo pathway in S2 cells. Depletion of *yki* in S2 cells by RNAi led to a significant reduction in the levels of expression of *miR-2a* and *b* ($P<0.05$, Fig. 3B; *miR-13a/b* were on average lower, but the effect was variable and so not statistically significant). *miR-11* was not significantly changed. *miR-6* is expressed at very low levels in S2 cells.

As a first step to address how Yki might regulate *miR-2* expression, we sought to identify cis-regulatory control elements that direct expression of *miR-2* loci in S2 cells. *miR-2a-1*, *miR-2a-2* and *miR-2b-2* are expressed as a cluster of 3 miRNAs located in an intron of the *spitz* gene (Fig. 3C). A 1.9 Kb DNA fragment covering the intronic sequences upstream of the miRNA cluster and spanning the next upstream exon proved sufficient to direct expression of a luciferase reporter gene in S2 cells (Fig. 3C; supplementary material Fig. S2A). We then used this luciferase reporter to assess the effects of depleting *yki* by RNAi. Expression of the *miR-2a* cluster reporter decreased significantly in *yki*-depleted cells (Fig. 3D), suggesting that Yorkie regulates transcription of the *miR-2a* cluster.

To further assess this regulation *in vivo*, we first asked whether overexpressing members of the miR-2 family could rescue the *ptc-Gal4 UAS-yki^{RNAi}* undergrowth assay. Coexpression of a *miR-2a/2b* cluster transgene or a *miR-11* transgene suppressed the undergrowth of yki-depleted tissue caused by elevated *reaper* mRNA (Fig. 4A,B; $P<0.001$). Expression of *miR-2a/2b* or *miR-11* on their own had no effect on growth. Next we introduced a
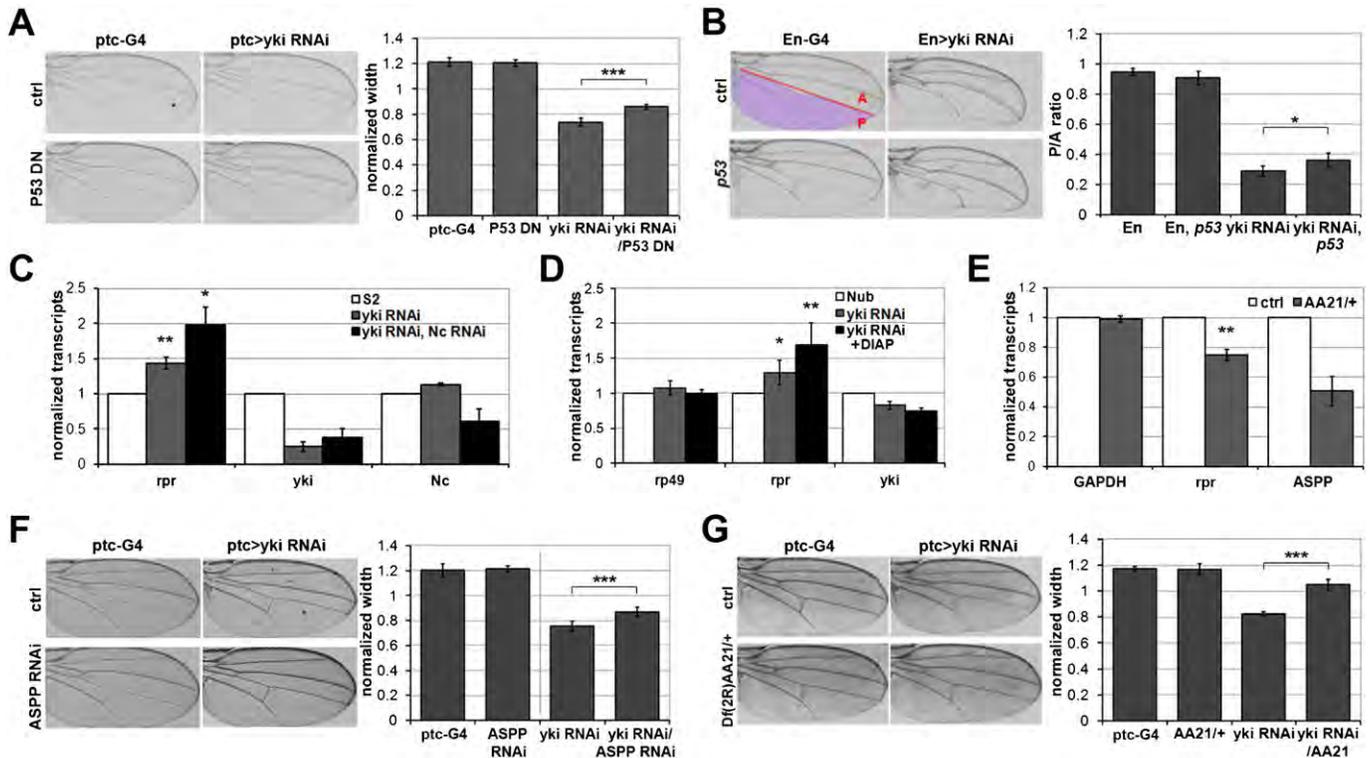
Fig. 2. p53 mediates the effects of Yorkie. (**A**) Photomicrographs of adult wings of the indicated genotype. Left panels: *ptc-Gal4* control flies. Right panels *ptc-Gal4* driving expression of a *UAS-yki^RNAi* transgene to reduce *yki* mRNA levels in the *ptc-Gal4* expression domain. Upper panels: control flies without expressing any other transgene. Lower panels: flies expressing a dominant negative form of p53, UAS-p53DN transgene. Histogram at right shows quantification of the effects on growth of the *ptc-Gal4* expression domain. Error bars represent standard deviation from measurement of at least 8 wings for each genotype. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when p53 activity was reduced ($P<0.001$). (**B**) Photomicrographs of adult wings of the indicated genotype, as in panel A, except that *en-Gal4* was used to drive transgene expression in the posterior compartment (shaded), and the ratio of anterior (A) to posterior (P) was measured. Lower panels: flies carrying two copies of a null allele of p53^5A-1-4. Histogram shows quantification of the effects on growth of the P compartment. Error bars represent standard deviation from at least 4 wings for each genotype. * indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when p53 activity was reduced ($P<0.05$). (**C**) Histogram showing the levels of *rpr*, *yki* and *Dronc (Nc)* mRNAs. S2 cells were treated with dsRNA to deplete *yki* (grey bars) or both yki and Nc (black bars) or GFP as a control (white bars). Total RNA was extracted and normalized for cDNA synthesis. RNA levels were normalized to *kinesin* mRNA. Error bars represent standard deviation from 3 independent experiments. (*) Student's t-test for *rpr* vs *GAPDH*: $P<0.05$; (**) Student's t-test for *rpr* vs *GAPDH*: $P<0.01$. (**D**) Histogram showing the levels of *rpr*, *rp49* and *yki* mRNAs measured by quantitative RT-PCR. RNA was extracted from wing imaginal discs expressing *nub-Gal4* alone (white bars) or *nub-Gal4* with *UAS-Yki RNAi* and *UAS-DIAP1* transgenes. RNA levels were normalized to *kinesin* mRNA. Error bars represent standard deviation from 6 independent experiments. (*) Student's t-test for *rpr* vs *rp49*: $P<0.05$; (**) Student's t-test for *rpr* vs *rp49*: $P<0.01$. (**E**) Histogram showing the levels of *rpr*, *GAPDH* and *ASPP* mRNAs. RNA was extracted from wing imaginal discs of 3rd instar control larvae (+/+) or *Df(2R)AA21/+* larvae (gray bars). RNA levels were normalized to *rp49* mRNA. Error bars represent standard deviation from 3 independent experiments. (**) Student's t-test for *rpr* vs *GAPDH*: $P<0.01$. (**F**) Photomicrographs of adult wings of the indicated genotype, as in panel A. Lower panels: flies expressing a *UAS-ASPP^RNAi* transgene to reduce *ASPP* mRNA levels in the *ptc-Gal4* domain. Histogram shows quantification of the effects of the *UAS-ASPP^RNAi* transgene alone (left) and together with *UAS-yki^RNAi*. Left and right pairs were from separate experiments. The ratio of the L3–4 to L4–5 width is constant at ~1.2:1 in all experiments. Error bars represent standard deviation from at least 7 wings for each genotype. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when ASPP activity was reduced ($P<0.001$). (**G**) Photomicrographs of adult wings of the indicated genotype, as in panel A. Lower panels: flies carrying one copy of *Df(2R)AA21*, which removes the *ASPP* gene. Error bars indicate standard deviation from at least 5 wings for each genotype. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when one copy of ASPP was removed ($P<0.001$).

miR-2a sensor into the *ptc-Gal4 UAS-yki^RNAi* assay to report *miR-2a* activity *in vivo*. The sensor transgene expresses GFP under control of the ubiquitously-expressed tubulin promoter and carries two *miR-2a* sites in its 3′ UTR (as described (Brennecke et al., 2005)). However, depletion of *yki* had no effect on the expression of the *miR-2a* reporter in wing imaginal discs (supplementary material Fig. S3). Although ectopically expressing members of *miR-2* family could suppress undergrowth of yki RNAi tissue, the negative result with the *miR-2a* reporter suggests that the effects of Yki on *reaper* are not mediated by regulation of *miR-2a* expression in the wing discs. To ask whether this regulation occurred in other tissues *in vivo*, in addition to S2 cells, we expressed UAS-*yki^RNAi* ubiquitously

under tubulin-Gal4 control and found a significant reduction of *miR-2a* and *b* in the whole 3rd instar larvae (Fig. 4C; $P<0.05$). These findings suggest that the Hippo pathway contributes to control of apoptosis through regulation of *miR-2* expression in some but not all tissues.

Discussion

Studies conducted in mammals and *Drosophila* have suggested that the downstream effectors of the Hippo pathway, YAP/TAZ and Yki direct expression of multiple targets linking cell division and cell death. Identified targets include the cell cycle regulator cycE and the cellular growth effector Myc (Huang et al., 2005; Neto-Silva et al., 2010). When the level of Hippo pathway
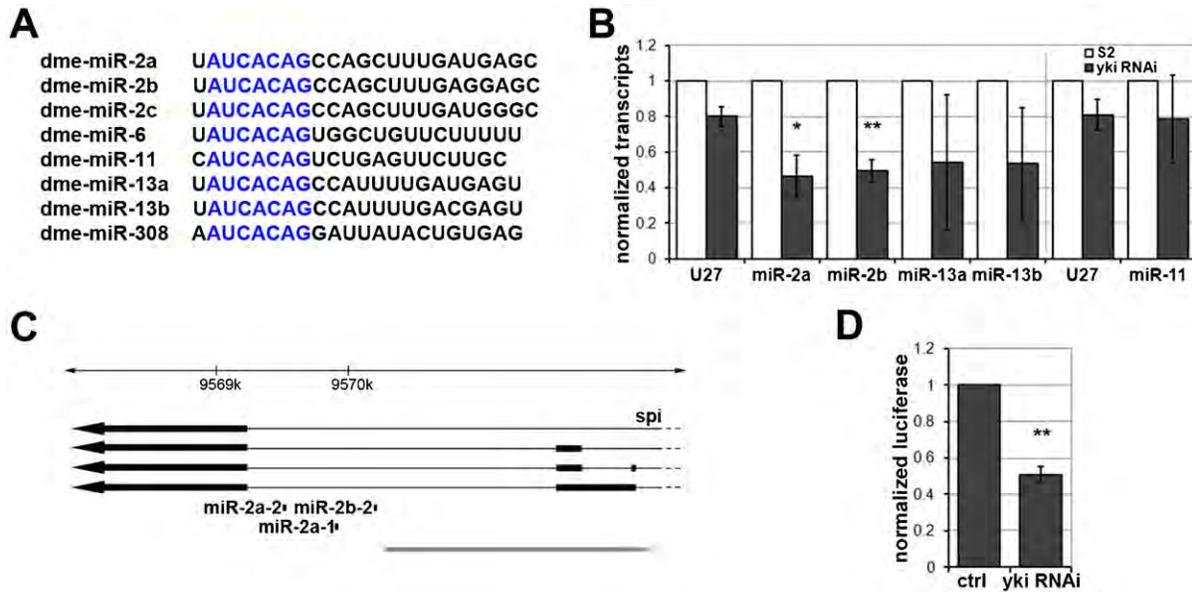
Fig. 3. Yorkie regulates miR-2a cluster expression in S2 cells. (**A**) Sequence alignment of *Drosophila* miR-2 family miRNAs. The seed region is shown in blue to recognize *reaper* mRNA. (**B**) Histogram showing the levels of *miR-2*, *miR-13*, *miR-11* and *U27* measured by quantitative RT-PCR. S2 cells were treated with dsRNA to deplete *yki* (grey bars) or left untreated (white bars). Small RNA *U27* serves as a control. Total RNA was extracted and normalized for cDNA synthesis. RNA levels were normalized to small RNA *U14*. Gray line indicates separate experiments. The ratio of *U27* between untreated and yki RNAi sample is constant at 1:~0.8 in all experiments. Error bars represent standard deviation from at least 3 independent experiments. Student's t-test for *miR-2* vs *U27*: *$P<0.05$, **$P<0.01$. (**C**) Schematic representation of the miR-2a cluster locus. Arrow lines represent transcripts of *spi* gene: thick parts indicate exons; thin parts indicate introns. microRNAs of miR-2 family were represented as black dots. The 1.9 kB cis-regulatory fragment directing luciferase reporter is shown as thick gray line below. (**D**) Luciferase assays showing activation of reporter directed by a 1.9 Kb DNA fragment of *miR-2a* cluster cis-regulatory element (C). S2 cells were treated with dsRNA to deplete *yki* (right bar) or GFP as a control (left bar). Error bars represent standard deviation from 3 independent experiments. (**) Student's t-test <0.01.

activity is sufficient, more cycE binds to CDK2 to promote the transition from G1 to S phase promoting cell division (Hinds et al., 1992). Meanwhile, elevated Myc activates numerous target genes for ribosome assembly and cellular growth (Grewal et al., 2005). Myc activation is sufficient to induce apoptosis (Pelengaris et al., 2002), and YAP/TAZ/Yki act in parallel to limit apoptosis to ensure balance in the coordinated drive for cells to grow and divide.

Yki acts at multiple levels to control apoptosis. Yki directs expression of the *Drosophila* Inhibitor of Apoptosis Protein, DIAP1 (Huang et al., 2005). We have provided evidence that Yki acts via regulation of p53 activity to regulate *reaper* transcription. Yki acts in parallel in some tissues via regulation of miR-2 family miRNAs to regulate *reaper* activity. *miR-2* has been shown to regulate translation of *repaer* mRNA (Thermann and Hentze, 2007). In addition, Yki mediated regulation of *bantam* miRNA expression (Nolo et al., 2006; Thompson and Cohen, 2006) controls *hid* transcript levels (Brennecke et al., 2003). Thus, Yki is at the center of a network of regulatory relationships involving p53-dependent checkpoints, proapoptotic genes and expression of multiple miRNAs in control of proliferation induced apoptosis (illustrated in Fig. 4D).

Why use a variety of parallel effector mechanisms? Our findings suggest that there may be tissue-specific differences in pathway use. As well, use of multiple pathways allows for the possibility that their activity may be regulated in a manner that depends on physiological context. This may be advantageous in adapting control of growth and apoptosis to the needs of different tissue types during development and in the adult for homeostasis and tissue repair. Diverse modes of regulation may also reflect the importance

of having adequate checkpoints to limit proliferation. Bypassing apoptosis and negative growth regulatory signals are important steps along the path to cancer (Hanahan and Weinberg, 2011).

## Materials and Methods

Fly strains

Df(3L)XR38, which removes *rpr* and *skl*, but not *hid* and *grim*, is described by Peterson et al. (Peterson et al., 2002) and was provided by Kristin White. UAS-p53DN is described by Brodsky et al. (Brodsky et al., 2000). *GUS-p53DN* (p53.Ct), p53[5A-1-4], *Df(2R)AA21* flies were obtained from the Bloomington Stock Center. *UAS-RNAi-yki* (transformant ID: 40497 and 104523) and *UAS-RNAi-ASPP* lines were from the Vienna Drosophila RNAi center. *UAS-miR-2a/2b* and *miR-2a* GFP sensor flies were described by Stark et al. (Stark et al., 2003). *UAS-miR-11* transgene was described by Szuplewski et al. (Szuplewski et al., 2012).

Cell culture and treatments

S2 cells were grown at 25℃ in SFM (Gibco) supplemented with L-glutamine. dsRNA was prepared using MegascriptT7 (Ambion) with the following templates: *yki*, nucleotides 331–875 of yki 215AA isoform coding sequence; Dronc, nt649–1122 of the ORF; GFP, nt 17–633 of EGFP2. S2 cells were treated with 37 nM dsRNA. The primers used to clone the 1.9 Kb DNA fragment before miR-2a cluster into pGL3-Basic by SLIC at XhoI site were: forward, 5′-GCGTGCTAGCCCGGGCTCGAGAAACTTTTGTTTGGTTTTGGAATATA-CATATATGTATGTGTG-3′; reverse, 5′-AAGCTTACTTAGATCGCAGATC-TGTTTCGATTCGATGAGAGCCGAGGTG-3′. The primers used to clone the 1.5 Kb DNA fragment before miR-2b-1 using the same method were: forward, 5′-GCGTGCTAGCCCGGGCTCGAGTTTAAATGTGCTTTTTTAAATAGCGA-GCCACTG-3′; reverse, 5′-AAGCTTACTTAGATCGCAGATTGAATATTGT-TGACAACATGTCCACTGCCAC-3′.

Quantitative RT-PCR

Total RNA was extracted from S2 cells or wing imaginal discs and treated with DNase-1 to eliminate genomic DNA contamination. Reverse transcription to synthesize the first strand used oligo-dT primers and Superscript RT-III (Invitrogen). PCR was performed and analyzed on Applied Biosystems 7500 fast real-time PCR system. The following primers were used: yki-f,
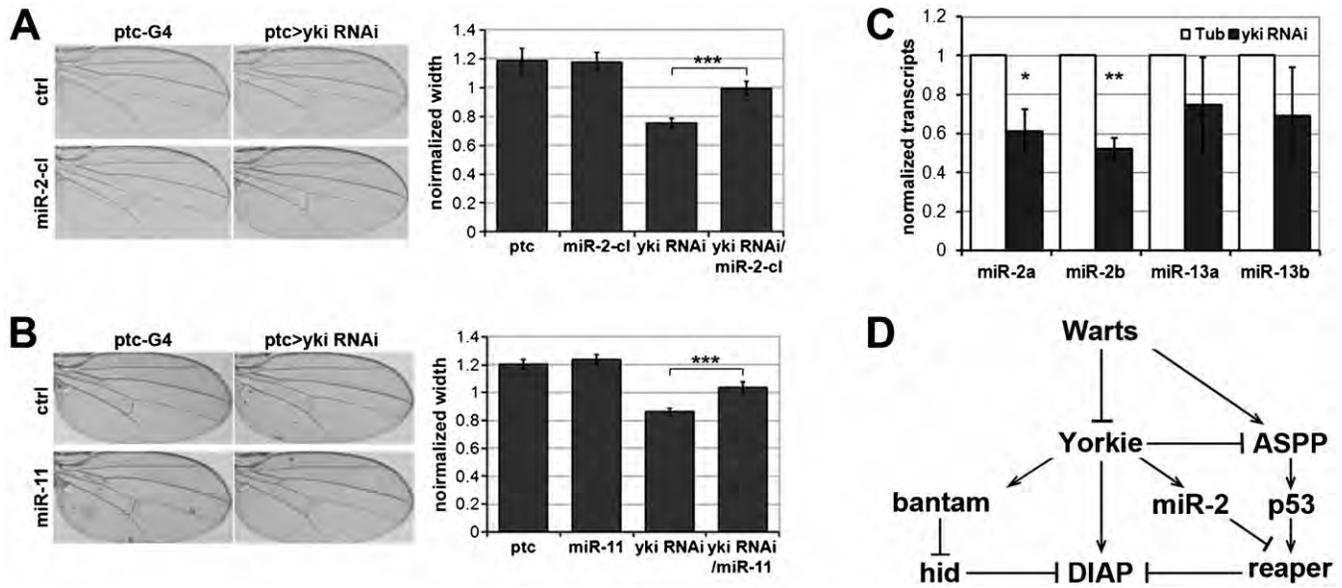
Fig. 4. Expression of miR-2 family mediates tissue growth of Hippo pathway. (**A**) Photomicrographs of adult wings of the indicated genotype. Left panels: *ptc-Gal4* control flies. Right panels *ptc-Gal4* driving expression of a *UAS-yki^RNAi* transgene to reduce *yki* mRNA levels in the *ptc-Gal4* expression domain. Upper panels: control flies without expressing any other transgene. Lower panels: flies expressing *UAS-miR-2a/2b* cluster transgene to increase miR-2a and 2b levels. Histogram at right shows quantification of the effects on growth of the *ptc-Gal4* expression domain. Error bars represent standard deviation from measurement of at least 8 wings for each genotype. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when miR-2 cluster level was increased (*P*<0.001). (**B**) Photomicrographs of adult wings of the indicated genotype, as in panel A. Lower panels: flies expressing a *UAS-miR-11* transgene to increase miR-11 level in the *ptc-Gal4* domain. Histogram shows quantification of the effects of the *UAS-miR-11* transgene alone and together with *UAS-yki^RNAi*. Error bars represent standard deviation from at least 6 wings for each genotype. *** indicates statistically significant increase in the width of the *ptc-Gal4* expression domain when *miR-11* level was increased (*P*<0.001). As a member of the *miR-2* seed family, *miR-11* is expected to regulate the same targets as *miR-2*. Regulation of *reaper* by *miR-11* has been confirmed in the embryo by Leaman et al. and Ge et al. (Leaman et al., 2005; Ge et al., 2012). (**C**) Histogram showing the levels of *miR-2* and *miR-13* measured by quantitative RT-PCR. Wandering 3rd instar larvae expressed UAS-*yki^RNAi* under ubiquitous *tubulin-Gal4* control. Control (white bars) expressed *tubulin-Gal4* without the UAS-RNAi transgene. Total RNA from whole larvae was extracted and normalized for cDNA synthesis. RNA levels were normalized to small RNA *U14*. Error bars represent standard deviation from 3 independent experiments. Student's t-test for *miR-2* vs *U14*: *P<0.05, **P<0.01. (**D**) Yki acts at multiple levels to control apoptosis. Arrows indicate activation, whereas bar-ended lines indicate inhibitory interactions.

5′-GAGCAGGCAGTTACCGAGTC-3′; yki-r, 5′-TCCATGAAGTCGTTCGA-TCA-3′; rpr-f, 5′-TTGCGGGAGTCACAGTGGA-3′; rpr-r, 5′-TGCGATGGC-TTGCGATATTT-3′; hid-f, 5′-CCTCTACGAGTGGGTCAGGA-3′; hid-r, 5′-CGTGCGGAAAGAACACATC-3′; grim-f, 5′-TGGGAAAGGCAGGC-TCAATCAAAG-3′; grim-r, 5′-ACTCGTTCCTCCTCATGTGTCC-3′; skl-f, 5′-ACCAACTTAAGCACCAACTAAGGC-3′; skl-r, 5′-TGCGCTAGTTCT-CACCAACG-3′; DIAP1-f, 5′-TTGTGCAAGATCTGCTACGG-3′; DIAP1-r, 5′-CACAGCGGACACTTTGTCAC-3′; Dronc-f, 5′-GAAGTCGGCCGATAT-TGTGGAC-3′; Dronc-r, 5′-GCTCATTCCGGAGCTTGCTAAC-3′; ASPP-f, 5′-GACCGACGATGTCCTGTGAATATC-3′; ASPP-r, 5′-GCGACAACTGA-TTGCGGTACATC-3′. Kinesin, rp49 and GAPDH, which were mentioned by Zhang et al., were used as house-keeping genes (Zhang et al., 2011). Data were normalized at least to the two having similar behaviors.

For microRNA quantification, reverse transcription and PCR were performed using TaqMan® MicroRNA Assays from Applied Biosystems.

Immunostaining and microscopy

Wandering 3rd instar larvae were collected and dissected. Tissues were fixed in PBS with 4% paraformaldehyde at room temperature for 20 min, then rinsed and washed in PBST (PBS+0.05% Triton X-100) before blocked in PBST+5% BSA. Anti-GFP and Anti-Gal4 were incubated at 4℃ overnight. Secondary antibodies were incubated at room temperature for 2 hrs with DAPI. Wing imaginal discs were mounted and imaged using a Zeiss LSM700 confocal microscope.

Competing Interests

The authors have no competing interests to declare.

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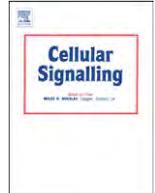
Feedback regulation on PTEN/AKT pathway by the ER stress kinase PERK mediated by interaction with the Vault complex

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ABSTRACT

The high proliferation rate of cancer cells, together with environmental factors such as hypoxia and nutrient deprivation can cause Endoplasmic Reticulum (ER) stress. The protein kinase PERK is an essential mediator in one of the three ER stress response pathways. Genetic and pharmacological inhibition of PERK has been reported to limit tumor growth in xenograft models. Here we provide evidence that inactive PERK interacts with the nuclear pore-associated Vault complex protein and that this compromises Vault-mediated nuclear transport of PTEN. Pharmacological inhibition of PERK under ER stress results is abnormal sequestration of the Vault complex, leading to increased cytoplasmic PTEN activity and lower AKT activation. As the PI3K/PTEN/AKT pathway is crucial for many aspects of cell growth and survival, this unexpected effect of PERK inhibitors on AKT activity may have implications for their potential use as therapeutic agents.

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1. Introduction

The phosphatidylinositol-3-kinase (PI3K)/PTEN/AKT pathway is crucial for many aspects of cell growth and survival. PI3K and AKT are overactive in many cancers because of PTEN inactivation [1]. Activation of PI3K via the insulin-signaling pathway leads to phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2) to produce phosphatidylinositol-triphosphate (PIP3) at the plasma membrane. Elevated PIP3 level promotes phosphorylation and activation of AKT by PDK1. Thus kinase activity of PI3K on membrane lipids is a key mediator of growth factor signaling. The output of PI3K activity is counteracted by the phosphoinositide phosphatase PTEN, which converts PIP3 to PIP2, thereby reducing the increase in AKT activity in response to growth factors. AKT transduces growth factor signals by phosphorylation of transcription factors including members of the FoxO Forkhead transcription factor family. Elevated growth factor signaling leads to increased AKT-mediated phosphorylation of FoxO proteins, which promotes their retention in the cytoplasm. Reciprocally, low AKT activity allows higher nuclear activity of FoxO proteins.

In normal cells, PTEN is detected in both cytoplasmic and nuclear compartments. It is clear that cytoplasmic PTEN acts as a negative regulator of PI3K/AKT, but the function of nuclear PTEN remains unknown [2]. PTEN is imported into the nucleus by different mechanisms,

including the nuclear pore associated Vault complex [3], a conserved ribonucleoprotein particle found in higher eukaryotes. The Vault complex has a barrel-shaped structure. Constituents include the major vault protein (MVP, which constitutes >70% of total complex mass), poly (ADP-ribose) polymerase 4 (PARP4), telomerase-associated protein (TEP1) and a small RNA (vRNA) [4]. Expression of MVP has been reported to correlate with the degree of malignancy in certain cancer types, implying a direct involvement of Vaults in tumor development and progression [5]. It has also been demonstrated that the Vaults are upregulated in multidrug-resistant (MDR) human cancer cells and downregulated in MDR-revertant cell lines. However, the function of this complex is still largely unclear.

Endoplasmic reticulum (ER) stress response is an adaptive mechanism that responds to protein misfolding in the secretory pathway. ER stress signaling is mediated by three parallel pathways, involving the protein kinases Ire1 and PERK and the ATF6 transcription factor [6]. ER stress has been linked to the PTEN/AKT pathway via Ire1-mediated activation of JNK signaling. JNK phosphorylates the insulin receptor substrate (IRS) proteins and limits activation of PI3K/AKT [7]. In addition, PERK inhibits protein translation by phosphorylating eIF2α.

ER stress plays an important role in cancer cell survival and resistance to anti-cancer treatment [8–14]. The high proliferation rate of cancer cells challenges the cells capacity to fold, assemble and transport proteins through the secretory pathway, a condition that can induce ER stress. Moreover, as tumors grow, cells experience nutrient deprivation and hypoxia, which can activate the ER stress pathway

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[9–11]. Indeed, phosphorylation of eIF2α by eIF2α Kinase3 (also known as PRKR-like endoplasmic reticulum kinase, PERK) has been shown to confer a survival advantage for tumor cells under hypoxic stress [15]. A recent report has presented evidence that a small molecule inhibitor of PERK can inhibit growth of human tumor samples in xenograft models [16].

Previously we reported that ER stress potentiates insulin resistance through PERK-mediated FoxO phosphorylation [17]. PERK-mediated phosphorylation was shown to potentiate FoxO transcription factor activity. PERK opposes the activity of the insulin/AKT pathway, mediated via regulation of FoxO activity. We hypothesized that PERK might be a promising pharmacological target for ameliorating insulin resistance. PERK inhibition was predicted to reduce FoxO activity. Here we report that pharmacological inhibition of PERK had a paradoxical effect, leading to increased FoxO activity. We provide evidence that inactive PERK protein interacts with Vault complex proteins leading to increased cytoplasmic PTEN activity. This lowers PI3K/AKT signaling, resulting in an increase in nuclear FoxO activity. The unexpected effects of PERK inhibitors on activity of the PI3K/AKT pathway have implications for the potential use of PERK inhibitors as therapeutic agents in cancer and metabolic disease.

2. Material and methods

2.1. Human cell culture and treatments

HEK293T, HeLa and MDA-MB-231H1299 and HepG2 cells were purchased from ATCC (Manassas, VA). Cell lines were cultured with high glucose DMEM media supplemented with 10% FBS at 37 °C and 5% CO_2. Cells were transfected using FuGENE 6 with pcDNA-FoxO1, pcDNA-FoxO3, pCMV-PERK and pEGFP-N1. pCMV-MVP was from OriGene (SC114118). pcDNA-GFP-PTEN was from Addgene (10759). K622M mutation was introduced into pCMV-PERK using QuickChange II XL kit with primers 5′-ACTGCAATTATGCTATCATGAGGATCCGTCTC CCC-3′ and 5′-GGGGAGACGGATCCTCATGATAGCATAATTGCAGT-3′.

For SILAC mass spectrometry experiments, H1299 cells were maintained in SILAC-DMEM (Thermo Fisher Scientific) and penicillin/ streptomycin for at least six cell divisions. Light medium was supplemented with 0.8 mM L-lysine: HCl and 0.4 mM L-arginine: HCl (Sigma). Heavy medium was supplemented with 0.8 mM L-lysine: 2HCl (U-13C6 and U-15 N2) and 0.4 mM L-arginine: HCl (U-13C6 and U-15 N4) (Cambridge Isotope). Light-labeled cells were transfected to express PERK-Flag and heavy-labeled cells were transfected to express PERK^{K622M}-Flag.

2.2. Kinase assay

PERK kinase was purchased from Carna and Invitrogen. 65nM PERK and 1 μl of compound at 26X assay concentrations in 15 μl of reconstitution buffer were added to a 384-well plate and incubated at room temperature for 15 min. FL-PP20 and ATP in substrate buffer were added and the plate was incubated at room temperature for 3 hours. The reaction was terminated with 45 μl of termination buffer. The reactions in the absence of ATP or compounds were used as controls. Data were collected on a LabChip EZ Reader II (Caliper).

2.3. Cell proliferation assay

Cell proliferation was measured using Cell Titer-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions.
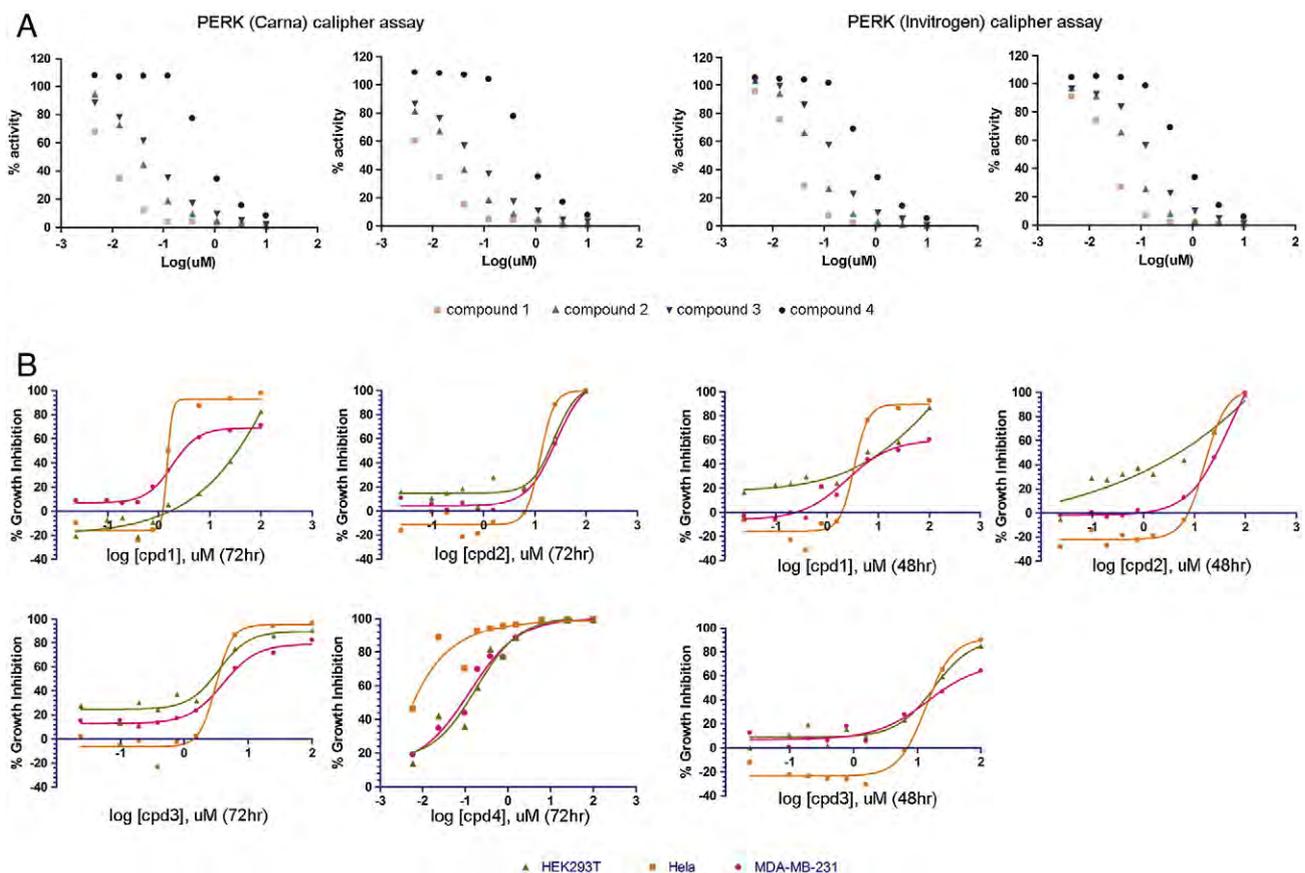
Fig. 1. Characterization of PERK inhibitors. (A) In vitro kinase assays using purified PERK protein (purchased from Carna and Invitrogen). The dose response of the 4 compounds was tested at the indicated concentrations. Compounds 1–3 are described in [20]. Compound 4 is described in [21]. (B) Cytotoxicity of the compounds was determined for HEK293T, HeLa and MDA-MB-231 cells. Cells were incubated with compounds for 48 h or 72 h at the indicated concentrations.

Table 1
Summary of compound activity.

	1	2	3	4
Structure				
Compound	1	2	3	4
IUPAC Name	1-(5-(4-amino-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-5-yl)indolin-1-yl)-2-(2,5-difluorophenyl)ethan-1-one	1-(5-(4-amino-7-azetidin-3-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl)indolin-1-yl)-2-(2,5-difluorophenyl)ethan-1-one	1-(5-(4-aminothieno[3,2-c]pyridin-3-yl)indolin-1-yl)-2-(2,5-difluorophenyl)ethan-1-one	5-bromo-N4-(pyridin-2-yl)-N2-(3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indol-5-yl)pyrimidine-2,4-diamine
MW (Dalton)	419.44	460.48	421.46	462.35
PERK IC$_{50}$ (µM) Carna protein	0.008	0.031	0.061	0.835
PERK IC$_{50}$ (µM) Invitrogen prot	0.025	0.064	0.152	0.729
PERK IC$_{50}$ (µM) [CSK assay][a]	0.0004		0.0025	
Toxicity IC$_{50}$ (µM) HEK293T, 72 h	>50	24.09	3.470	0.182
Toxicity IC$_{50}$ (µM) Hela, 72 h	1.486	12.82	3.136	<0.006
Toxicity IC$_{50}$ (µM) MDA-MB-231, 72 h	1.860	26.10	4.045	0.127
Toxicity IC$_{50}$ (µM) HEK293T, 48 h	>10	>3	18.18	
Toxicity IC$_{50}$ (µM) Hela, 48 h	3.38	16.17	13.50	
Toxicity IC$_{50}$ (µM) MDA-MB-231, 48 h	2.43	51.06	14.54	
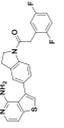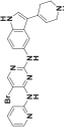
[a] J. Med. Chem., 2012, **55**, pp 7193–7207.

Cells were seeded at 2000/well the day before the experiment. Serial dilutions of the compounds were added to each well and cultured at 37 °C in 5% CO$_2$. After 48 h or 72 h, an equal volume of Cell Titer Glo reagent was added to each well. Plates were rocked on rotator for 2 h. 100 µl solution from each well was transferred to a 96 well opaque plate, and total light emitted was measured on the Tecan Safire II.

2.4. Quantitative RT-PCR

Total RNA was extracted using QIAGEN RNeasy Mini Kit and treated with On-Column DNase (QIAGEN RNase-free DNase Set) to eliminate genomic DNA contamination. Reverse transcription to synthesize the first strand was done using oligo-dT primers and Superscript RT-III (Invitrogen). PCR was performed using power sybr green Master Mix (Applied Biosystems) and analyzed on ABS 7500 fast real-time PCR system.

Results were normalized to KIF, Rp132 and Actin. The following primers were used: KIF, 5′-GCTCAACAGATGGCGTAATGGG-3′ and 5′-GAAAGCTTCCAAGTTGGCTTTCTC-3′; Rp132, 5′-ATCTCCTTCTCGCTGGCG ATTG-3′ and 5′-AGGTGAGGAAGAATCCTGGAAGG-3′; Actin, 5′-GATGCG TAGCATTTGCTGCATGG-3′ and 5′-TGAGGCTAGCATGAGGTGTGTG-3′; Firefly, 5′-CCGCCGTTGTTGTTTTG-3′ and 5′-CTCCGCGCAACTTTTTC-3′; Renilla, 5′-CGGACCCAGGATTCTTTT-3′ and 5′-TTGCGAAAAATGAAGA CCT-3′; PERK, 5′-TACAGCTGGCCTCTATACATTCCC-3′ and 5′-AAGACATT GTAGAAGCTG CCAGAG-3.

2.5. Immunoaffinity purification of protein complexes

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Proteins complexes were purified with anti-FlagM2 agarose beads (Sigma) or GFP-trap beads (Chromotek) and eluted using 3X Flag peptide or SDS sample buffer. The affinity-purified protein complexes were subjected to liquid chromatography-mass spectrometry analysis as described below or were used for western blot analysis.

2.6. Mass spectrometry and data analysis

Eluted protein complexes were separated by one-dimensional 4–12% NuPage Novex Bis–Tris Gel (Invitrogen), stained using the Colloidal Blue Staining Kit (Invitrogen) and digested with trypsin using published procedures [18]. Samples were analyzed on an Orbitrap XL (Thermo Fisher). Survey full scan MS spectra (m/z 300–1400) were acquired with a resolution of r = 60,000 at m/z 400, an AGC target of 1e6, and a maximum injection time of 500 ms. The ten most intense peptide ions in each survey scan with an ion intensity of >2000 counts and a charge state ≥2 were isolated sequentially to a target value of 1e4 and fragmented in the linear ion trap by collisionally-induced dissociation using a normalized collision energy of 35%. A dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count, repeat, and exclusion duration of 30 s. SILAC peptide and protein quantification was performed with MaxQuant [19] version 1.3.0.5 using default settings. Database searches of MS data were performed using Uniprot human fasta (2013 July release, 88354 proteins) with tryptic specificity allowing maximum two missed cleavages, two labeled amino acids, and an initial mass tolerance of 6 ppm for precursor ions and 0.5 Da for fragment ions. Cysteine carbamidomethylation was searched as a fixed modification, and N-acetylation and oxidized methionine were searched as variable modifications. Labeled arginine and lysine were specified as fixed or variable modifications, depending on the prior knowledge about the parent ion. Maximum false discovery rates were set to 0.01 for both protein and peptide. Proteins were considered identified when supported by at least one unique peptide with a minimum length of seven amino acids.

2.7. Immunoblotting

Cells were homogenized in SDS sample buffer, boiled and resolved by SDS-PAGE before transfer to nitrocellulose membranes for antibody labeling. Odyssey Infrared System or Image J was used for protein quantification. Antibodies to PERK, AKT p308, AKT p473 and AKT were from Cell Signaling. Anti-GFP was from Molecular Probes. MVP antibody was from BD Signal Transduction.

MVP siRNA (Qiagen, SI00118391 and SI03057516), PARP4 siRNA (Qiagen, SI03063172) and PTEN siRNA (Qiagen, SI03116092) was transfected using DharmaFECT and HiPerFect reagents.

3. Results

3.1. PERK regulates FoxO independent of its kinase activity

Four previously described small molecule inhibitors of PERK kinase [20,21] were synthesized and screened by *in vitro* PERK kinase assay (Fig. 1A, Table 1), cell proliferation assays (Fig. 1B, Table 1) and using the KINOMEscan® platform (Tables S1–S2). The compounds were further tested for interaction with three other eIF2α kinases (Tables S3–S4). Compounds 2 and 3 (cpd2, cpd3) showed better selectivity for PERK while cpd2 was the least cytotoxic. cpd2 showed the best overall profile (Table 1). Next, we tested the inhibitory efficacy of cpd2 *in vivo*. HepG2 cells were treated with Tunicamycin to induce ER stress and endogenous PERK protein was visualized by immunoblotting (Fig. 2A, Figure S1). Activation of PERK by ER stress leads to auto-phosphorylation and a shift in electrophoretic mobility during SDS-PAGE. cpd2 showed inhibition of PERK in HEPG2 cells at concentrations greater than 500 nM (Fig. 2A, Figure S1).

We have previously reported that PERK overexpression increased FoxO transcription factor activity in luciferase reporter assays, and reciprocally that depletion of PERK by siRNA treatment reduced FoxO activity [17]. To assess the effects of the PERK inhibitors on FoxO activity we performed luciferase assays in H1299 cells expressing FoxO3 and a luciferase reporter containing four synthetic FoxO3 sites with and without PERK overexpression (4FRE; Fig. 2B). cpd2 and cpd3 had little effect on FoxO3 activity in control cells. In cells overexpressing PERK, we observed a modest decrease in FoxO3 activity at concentrations up to ~150 nM, and a sharp increase at higher concentrations (Fig. 2B).

Activation of FoxO at higher concentrations of PERK inhibitor was unexpected, as depletion of PERK protein by siRNA treatment lowers
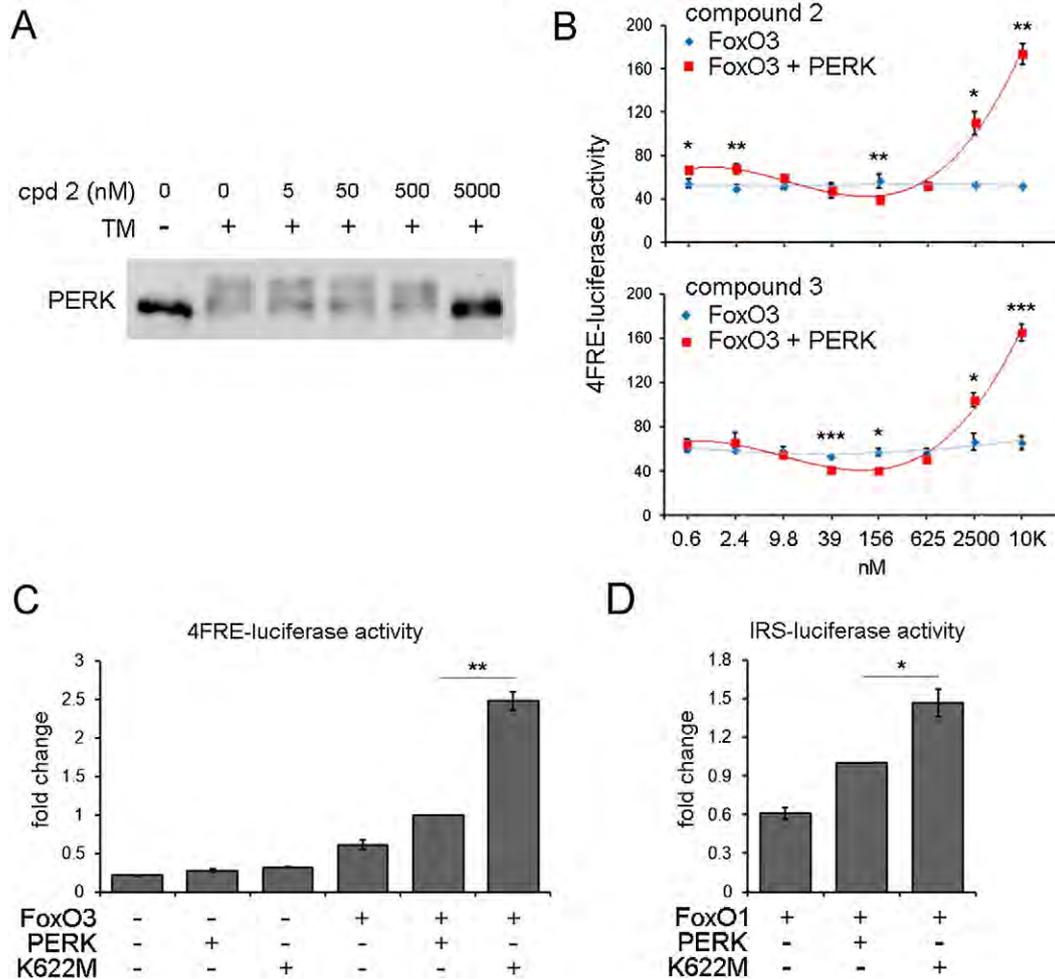
Fig. 2. PERK regulates FoxO independent of its kinase activity. (A) Immunoblot to visualize the level of PERK phosphorylation in HepG2 cells treated with Tunicamycin (TM) and series of dilutions of compound 2 (cpd2). The slower migrating forms correspond to phosphorylated PERK. (B) Luciferase reporter assays for human FoxO3 activity. H1299 cells were transfected to express a luciferase reporter containing four synthetic FoxO3 sites. Cells were cotransfected to express human FoxO3 and PERK with series dilutions of compound 2 or 3 as indicated. Data represent the average of 3 independent experiments. Error bars: ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t*-test. (C) FoxO3 luciferase assays as in B, except cells were cotransfected to express PERK or PERK[K622M] and FoxO3 as indicated. Data were normalized to the average of the FoxO3 + PERK samples expressing, and represent the average of 3 independent experiments. Error bars: ± SD. ** $p < 0.01$, Student's *t*-test. (D) Luciferase reporter assays for human FoxO1 activity. H1299 cells were transfected to express a FoxO1-responsive luciferase reporter derived from the IRS-2 gene. Cells were cotransfected to express intact PERK or PERK[K622M] and FoxO1 as indicated. Data were normalized to the average of the FoxO1 + PERK samples expressing, and represent the average of 3 independent experiments. Error bars: ± SD. * $p < 0.05$, Student's *t*-test.

FoxO activity [17]. We therefore considered the possibility that the presence of an inhibited PERK protein might have indirect effects on FoxO activity. To test this, we produced a catalytically inactive mutant, PERK^{K622M}. Expression of PERK^{K622M} or native PERK had little effect on FoxO3 reporter activity in H1299 cells; however when co-expressed with FoxO3, PERK^{K622M} increased reporter activity considerably more than native PERK (Fig. 2C, ** $p < 0.01$, Figure S2). Similar results were obtained in cells co-transfected to express FoxO1 and a luciferase reporter containing the human IRS-2 promoter as readout of FoxO1 (Fig. 2D, * $p < 0.05$, Figure S3). We conclude that an inactive PERK protein, whether catalytically inactive or chemically inhibited, can increase FoxO activity. This poses an apparent paradox: depletion of PERK protein produced an opposite effect than inhibiting PERK activity.

3.2. Inactive PERK interacts with Vault complex

To test the possibility that active and inactive forms of PERK might interact differently with other proteins, we performed SILAC mass spectrometry on proteins that immunopurified together with Flag tagged PERK and PERK^{K622M} (Fig. 3A). The MVP, PARP4 and TEP1 proteins were among the top 10 proteins enriched for binding to PERK^{K622M} compared to native PERK ($p < 0.01$, Table S5, Fig. 3B). eIF2α, another PERK target, was not enriched (eIF2S1, Fig. 3B). The occurrence of these 3 proteins among the top 10 was striking because all 3 are components of the Vault complex (Fig. 3C). The Vault complex is located in the cytoplasm and on the outer surface of the nuclear envelope [22]. Many different roles have been proposed for the Vault complex, including nucleo-cytoplasmic transport [23,24].
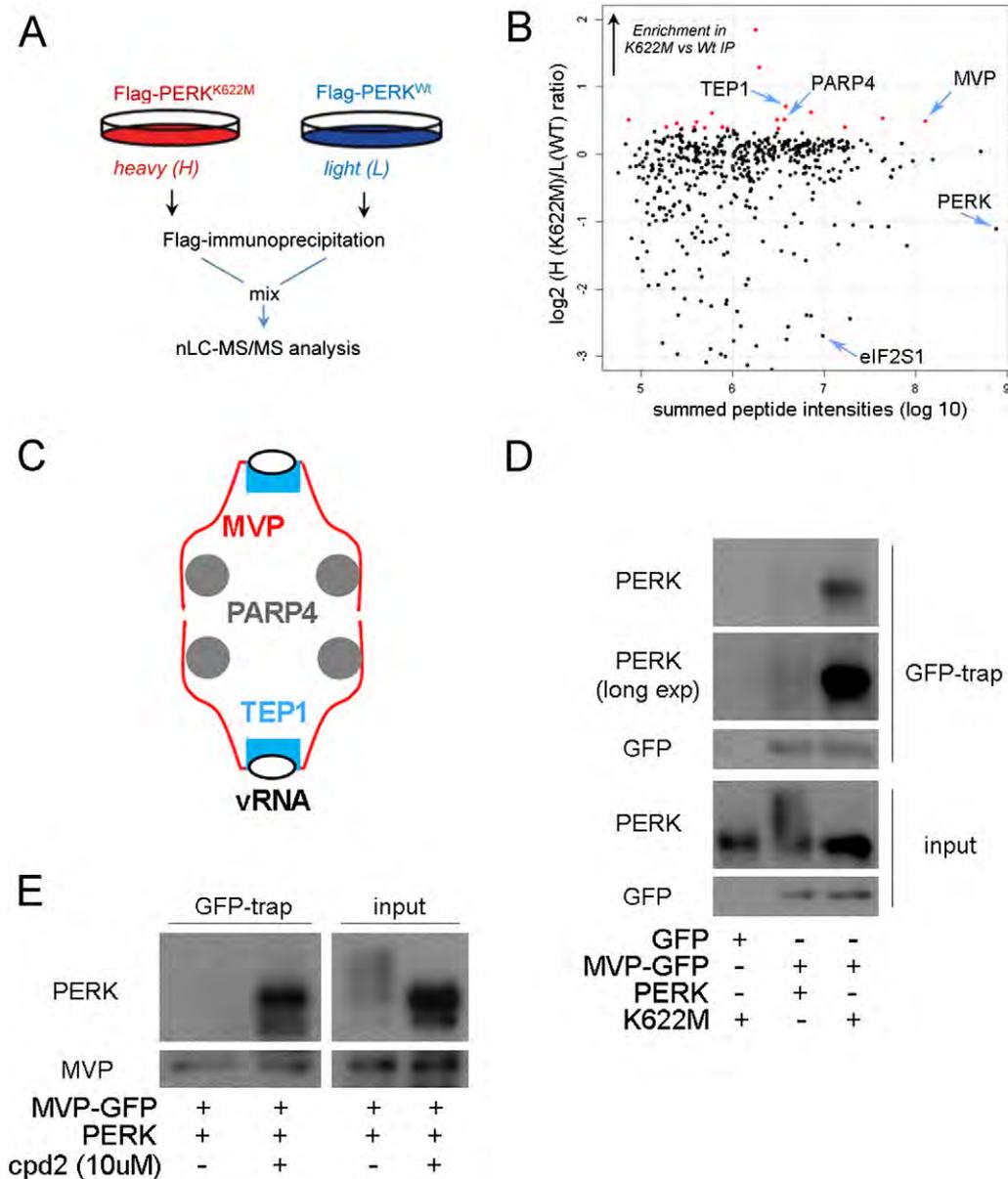
Fig. 3. Inactive PERK interacts with Vault complex. (A) SILAC pull-down workflow. H1299 cells were transfected to express PERK^{K622M}-Flag or intact PERK-Flag and grown in Heavy vs Light SILAC media. Flag immunoprecipitation was used to recover PERK and bound proteins for mass spectometric analysis. (B) Ratio–intensity plot. The red data points indicate proteins that preferentially interacted with PERK^{K622M} vs native PERK with a p value (Ratio Significance) < 0.05. (C) Diagram of the Vault complex modified from [4]. The capsid structure consists of MVP molecules (red). Gray circles indicate PARP4. vRNA (empty circles) at the end of cap region co-localizes with TEP1 (blue). (D) Immunoblots to monitor the interaction between MVP-GFP and PERK or PERK^{K622M}. H1299 cells were transfected to express the indicated proteins. GFP-tagged MVP was recovered using GFP-trap beads, and blots were probed with anti-GFP and anti-PERK. Input shows 1% of the lysate. IP and input samples were run on the same gel. Intervening lanes have been removed. (E) Immunoblots to monitor the interaction between MVP-GFP and PERK as in D. H1299 cells were treated with cpd2, as indicated. Input shows 1% of the lysate. IP and input samples were run on the same gel. Intervening lanes have been removed.

To further explore the interaction between PERK and Vault complex components, we transfected cells to express a GFP-tagged MVP. Purification of GFP-MVP on GFP trap-beads showed much stronger binding to PERK^{K622M}, compared to native catalytically-active PERK (Fig. 3D, Figure S4A). Interestingly, a longer exposure of the immunoblot showed that the native PERK protein that was recovered bound to MVP was mostly the faster-migrated unphosphorylated form of the protein, which co-migrated with the catalytically-inactive PERK^{K622M} protein (Fig. 3D). However, native catalytically-active PERK was effectively recovered by binding to MVP when its catalytic activity was inhibited by treatment with compounds 2, similar to PERK^{K622M} (Fig. 3E, Figure S4B and S4C). This suggested that the (auto)-phosphorylation status of PERK influences its interaction with the vault complex.

3.3. PERK regulates PI3K/AKT pathway via Vault-mediated PTEN localization

MVP was previously identified as a PTEN-binding protein in a yeast two-hybrid screen [25]. MVP binding was shown to be critical for nuclear transport of PTEN in human cells [3]. In light of this, we asked whether the difference in binding of the two PERK forms to MVP would affect PTEN localization. Expression of the PERK^{K622M} mutant, which binds MVP more effectively, led to an increase in cytoplasmic PTEN compared to native PERK (Fig. 4A). This suggested that interaction with the PERK^{K622M} mutant protein compromised Vault-complex activity.

PTEN is a tumor suppressor that downregulates AKT signaling by reducing the output of PI3kinase at the cell membrane. To address the contribution of the Vault complex to PTEN activity, we made use of siRNAs to deplete the Vault proteins MVP or PARP4, and monitored insulin-induced activation of AKT. Depletion of MVP or PARP4 suppressed insulin-dependent phosphorylation of AKT (Fig. 4B, C). Similarly, expression of PERK^{K622M}, which compromises Vaults activity and consequently elevates cytoplasmic PTEN, also resulted in a reduction of AKT phosphorylation level compared to native PERK (Fig. 4D).

Taken together these findings are consistent with a role for MVP in nuclear transport and an increase in cytoplasmic PTEN activity [3].

To further assess the effect of inhibiting PERK on AKT activation, cells were treated with insulin to activate the AKT pathway and with Tunicamycin to induce ER stress. Tunicamycin treatment, due to activation of Ire-1 [7], caused a mild reduction in AKT phosphorylation, which was further reduced by treatment with the PERK inhibitor compound 2 (Fig. 5A, B). PERK inhibitors had little or no effect in cells that had not been treated to induce ER stress (Fig. 5C, Figure S5). In cells depleted of MVP (Fig. 5A) or PTEN (Fig. 5B) by siRNA treatment, the PERK inhibitor no longer had any discernible effect on AKT phosphorylation level. This implicates MVP/PTEN as an essential part in the mechanism by which inhibition of PERK influences the AKT pathway.

4. Discussion

Our findings provide evidence that the effect of PERK inhibition on the PI3K/AKT pathway depends on the presence of MVP. The data are consistent with a model in which binding to inhibited PERK lowers Vault complex activity, resulting in increased cytoplasmic PTEN activity. We noted that ER stress increased the level of PERK mRNA (Fig. 5D), suggesting an adaptation mechanism to increase PERK levels under chronic stress conditions. Under normal conditions, when ER-stress has not been induced, PERK inhibitors appear to have little or no measurable effect on the PI3K/AKT pathway.

4.1. Implications for use of PERK inhibitors

The observations reported here have implications for the prospective use of PERK inhibitors as cancer therapeutics [16,20]. Tumors are subject to hypoxia and nutrient deprivation, features that can be exploited therapeutically [8,9]. Hypoxia triggers ER stress and ER stress responses have been observed in a variety of cancers (e.g. [10,11,26]. Manipulations to block ER stress pathway activity have been shown to
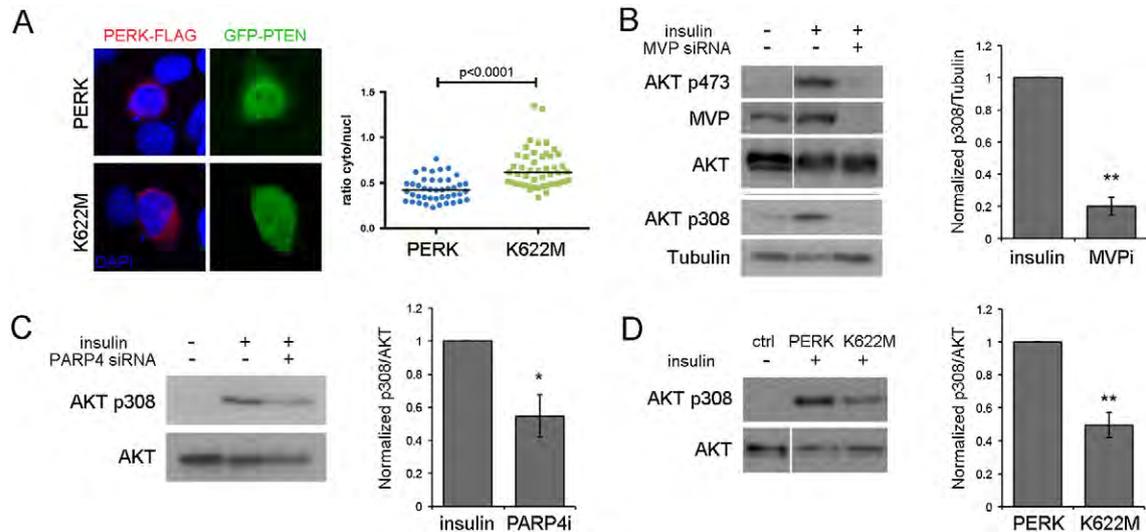
Fig. 4. PERK regulates AKT via Vaults-mediated PTEN. (A) Left panels: GFP-PTEN (green) in cells expressing PERK-Flag or PERK^{K622M}-Flag. Anti-Flag is shown in red. Nuclei were labeled with DAPI (blue). Images were selected to present cells showing the averages of quantifications on the right panel. Right panel: scatter plot showing the ratio of cytoplasmic to nuclear PTEN-GFP was quantified using DAPI to define the nucleus. p-value: Mann-Whitney test. (B) Left panels: AKT phosphorylation status in HepG2 cells treated with insulin and/or siRNA to deplete MVP. Antibody specific to pS473 and pT308 were used to monitor AKT phosphorylation. Anti-AKT and anti-Tubulin were used as loading controls. Upper panel: samples were run on the same gel, with intervening lanes removed (vertical gap). Right panel: Histogram showing the normalized ratio of pT308/Tubulin indicated for representative lanes in left panels. Data represent the average of 3 independent experiments. Error bars: ± SD. ** p < 0.01, Student's t-test. (C) Left panels: Immunoblot to visualize the level of AKT T308 phosphorylation and total AKT in cells treated with siRNA to deplete PARP4 and insulin, as indicated. Right panel: Histogram showing the normalized ratio of pT308/AKT indicated for representative lanes in left panels. Data represent the average of 3 independent experiments. Error bars: ± SD. * p < 0.05, Student's t-test. (D) Left panels: Immunoblot to visualize AKT and pT308 AKT in cells treated with insulin. Cells were transfected to express PERK or PERK^{K622M} as indicated. Samples were run on the same gel, with intervening lanes removed. Right panel: Histogram showing the normalized ratio of pT308/AKT indicated for representative lanes in left panels. Data represent the average of 3 independent experiments. Error bars: ± SD. ** p < 0.01, Student's t-test.
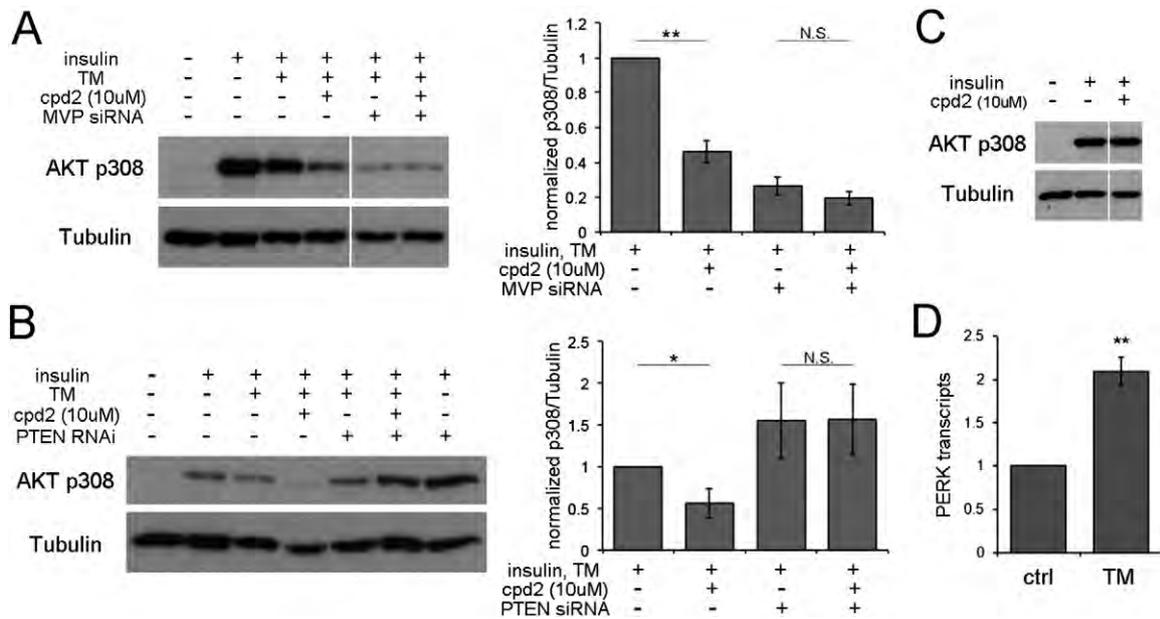
Fig. 5. Inhibiting PERK under ER stress regulates AKT via MVP/PTEN. (A) Left panels: Immunoblot to visualize Tubulin and pT308 AKT in cells treated with insulin, Tunicamycin, compound 2 and/or siRNA to deplete MVP, as indicated. Samples were run on the same gel, with intervening lanes removed. Right panel: Histogram showing the normalized ratio of pT308/Tubulin indicated for representative lanes in left panels. Data represent the average of 3 independent experiments. Error bars: ± SD. (N.S.) not significant; ** $p < 0.01$, Student's t-test. (B) Immunoblot to visualize Tubulin and pT308 AKT in cells treated with insulin, Tunicamycin, compound 2 and/or siRNA to deplete PTEN, as indicated. (C) Left panels: Immunoblots to visualize the level of AKT T308 phosphorylation. Cells were treated with insulin and compound 2 as indicated. Tubulin was used as loading control. Samples were run on the same gel, but intervening lanes have been removed as indicated. Right panel: Histogram showing the normalized ratio of pT308/Tubulin indicated for representative lanes in left panels. Data represent the average of 3 independent experiments. Error bars: ± SD. (N.S.) not significant; * $p < 0.05$, Student's t-test. (D) Histogram showing the levels of PERK mRNA measured by quantitative RT-PCR. HepG2 cells were treated with Tunicamycin for 24 h to induce ER stress or DMSO as control. Data represent the average of 3 independent experiments. Error bars: ± SD. ** $p < 0.01$, Student's t-test.

limit growth of cancer cells in mouse xenograft models [10,11,15]. Small molecule inhibitors of PERK have also been reported to slow the growth of human cancers in xenograft models [16,20].

Our findings have highlighted the molecular interaction between pharmacologically inhibited PERK and the PI3K/AKT pathway. Inactivated PERK interacts with Vault complex proteins and limits their ability to promote nuclear localization of PTEN. The resulting shift in PTEN to the cytoplasm leads to reduced activation of AKT by growth-factor PI3K signaling pathways. This interaction has little functional consequence in normal cells. But, in ER-stressed cells, treatment with PERK inhibitors leads to reduced AKT activation. Given the prevalence of ER stress in tumors, this interaction may have functional significance. In this context, it is interesting that elevated expression of MVP is frequently observed in multidrug resistant cancers [27]. Given that inhibited PERK binds MVP and lowers the ratio of nuclear PTEN, our model raises the possibility that cancers with low MVP and high PERK expression might be more sensitive to the effects of PERK inhibition on the AKT pathway, whereas those with elevated MVP might be relatively resistant.

5. Conclusions

ER stress elevates the expression and activity of PERK kinase. Pharmacological inhibition of PERK under ER stress then enhances the sequestration of nuclear pore associated Vault-complex by inactive PERK, and consequently compromises the nuclear transport of PTEN, creating an unexpected effect on the AKT pathway. This drug-induced link may have implications for therapeutic use of PERK inhibitors.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.12.010.

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标题：Feedback regulation on PTEN/AKT pathway by the ER stress kinase PERK mediated by interaction with the Vault complex

作者：Zhang, W (Zhang, Wei); Neo, SP (Neo, Suat Peng); Gunaratne, J (Gunaratne, Jayantha); Poulsen, A (Poulsen, Anders); Liu, BP (Liu Boping); Ong, EH (Ong, Esther Hongqian); Sangthongpitag, K (Sangthongpitag, Kanda); Pendharkar, V (Pendharkar, Vishal); Hill, J (Hill, Jeffrey); Cohen, SM (Cohen, Stephen M.)

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标题：The Hippo pathway acts via p53 and microRNAs to control proliferation and proapoptotic gene expression during tissue growth

作者：Zhang, W (Zhang, Wei); Cohen, SM (Cohen, Stephen M.)

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标题：ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation

作者：Zhang, W (Zhang, Wei); Hietakangas, V (Hietakangas, Ville); Wee, S (Wee, Sheena); Lim, SC (Lim, Siew Choo); Gunaratne, J (Gunaratne, Jayantha); Cohen, SM (Cohen, Stephen M.)

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标题：MAPK/ERK Signaling Regulates Insulin Sensitivity to Control Glucose Metabolism in Drosophila

作者：Zhang, W (Zhang, Wei); Thompson, BJ (Thompson, Barry J.); Hietakangas, V (Hietakangas, Ville); Cohen, SM (Cohen, Stephen M.)

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(54) **COMPOUNDS AND METHODS FOR TREATING INSULIN RESISTANCE SYNDROME**

(76) Inventors: **Stephen Michael Cohen**, Singapore (SG); **Wei Zhang**, Singapore (SG)

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(57) **ABSTRACT**

The present invention relates to a method of treating or pre-venting insulin resistance syndrome in an animal body by administering an inhibitor of protein kinase RNA-like endo-plasmic reticulum kinase (PERK) gene, or a functional vari-ant thereof, or an inhibitor of PERK protein or a functional variant thereof or a method of reducing activity of transcrip-tion factors of the FOXO family (Foxo 1, 3a, 4 and 6) by administering an inhibitor of protein kinase RNA-like endo-plasmic reticulum kinase (PERK) gene, or a functional vari-ant thereof, or an inhibitor of PERK protein or a functional variant thereof. The present invention also relates to different compounds and methods for using PERK gene or PERK protein.
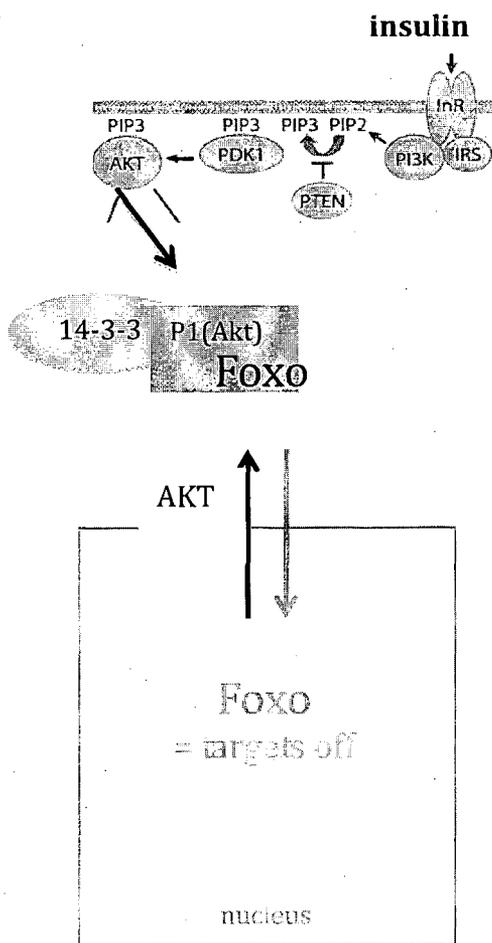
FIG. 1(a)
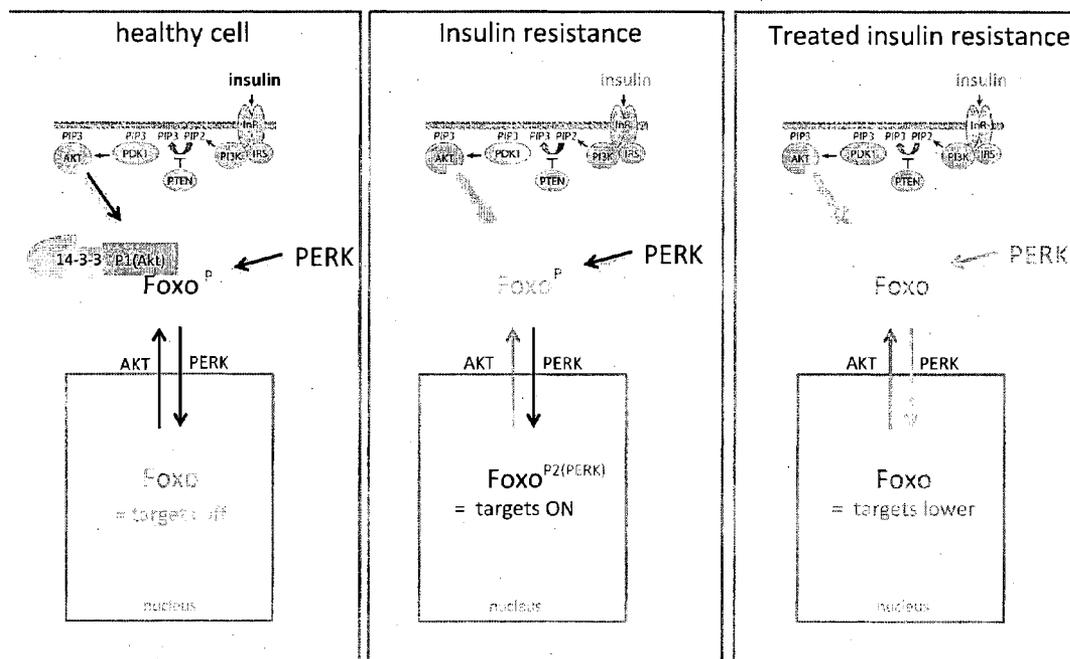
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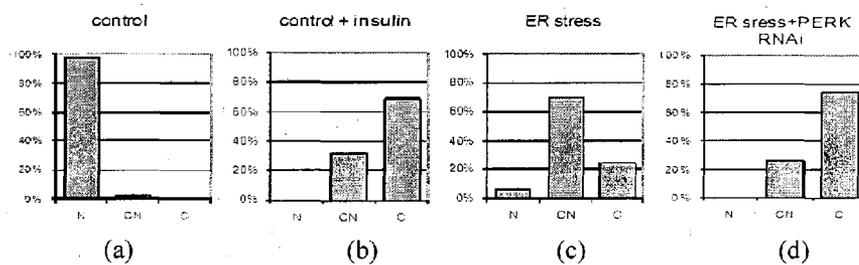
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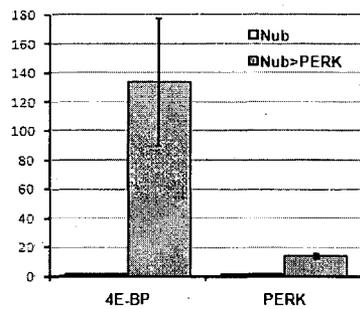
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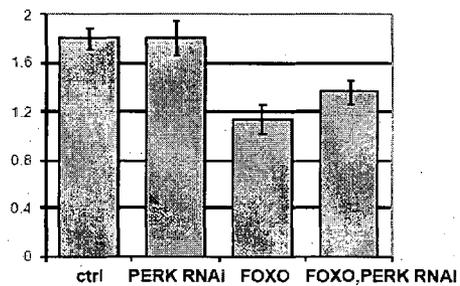
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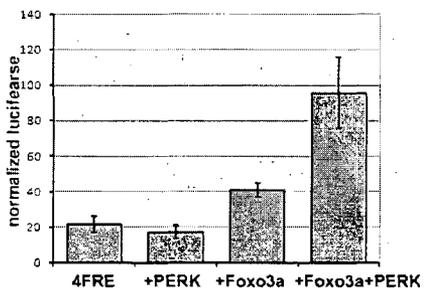
FIG. 4A
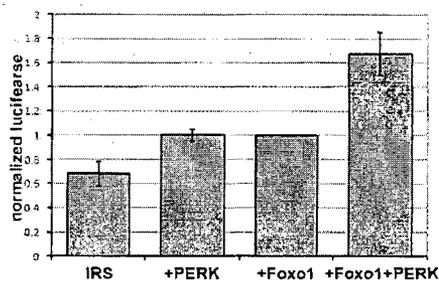
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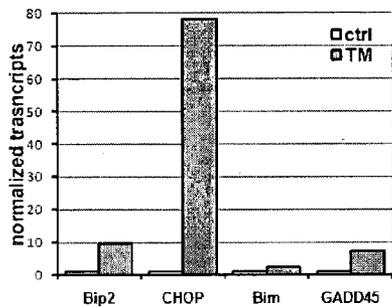
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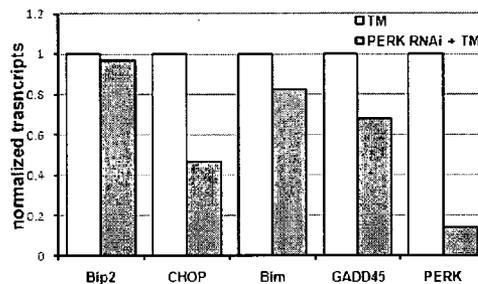
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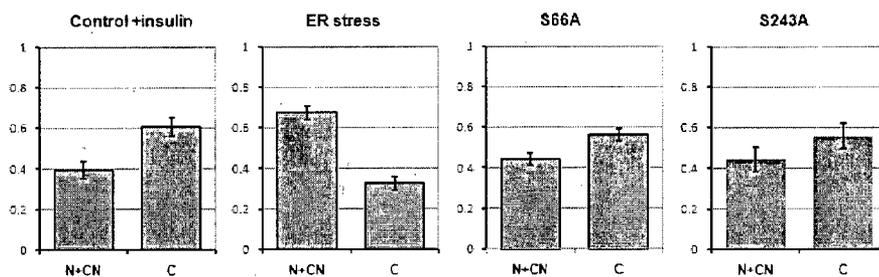
FIG. 6
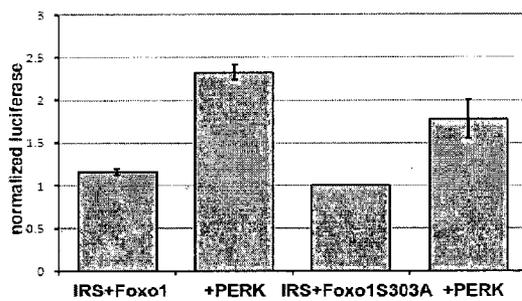
FIG. 7

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FOXO1    --------MAEAPQVVEIDPDFEPLPRPRSCTWPLPRPEFSQSNSATSSPAPSGSAAANP
              .:  ::..::.:::  :::::::::: ::::.. :.  :.. . . :
FOXO3    MAEAPASPAPLSPLEVELDPEFEPQSRPRSCWPLQRPELQASPAKPSGETAADSMIPEE


FOXO1    -----DAAAGLPSASAAAVSADFMSNL---SLLEESEDFPQAPGSVAAAVAAAAAAATG
             :  .:  ..::  :...  :.   .:: :.   :::.   . . :.:: :
FOXO3    EDDEDDEDGGGRAGSAMAIGGGGGSGTLGSGLLLEDSARVLAPGGQDPGSGPATAA---G


FOXO1    GLCGDFQGPEAGCLHPAPPQPPPPGPLSQHPPVPPAAAGPLAGQPRKSSSSRRNAWGNLS
         :: : :.   :.: : :::     :.::: ..::::: :: ::::::::::
FOXO3    GLSGGTQA----LLQPQQPLPPPQ----------PGAAGG-SGQPRKCSS-RRNAWGNLS


FOXO1    YADLITKAIESSAEKRLTLSQIYEWMVKSVPYFKDKGDSNSSAGWKNSIRHNLSLHSKFI
         :::::::.::::: .::::::::::::::. :::::::::::::::::::::::::::::.:.
FOXO3    YADLITRAIESSPDKRLTLSQIYEWMVRCVPYFKDKGDSNSSAGWKNSIRHNLSLHSRFM


FOXO1    RVQNEGTGKSSWWMLNPEGGKSGKSPRRRAASMDNNSKFAKSRSRAAKKKA.LQGGQEGA
         :::::::::::::::..::.:::::::.:::::.::::..:..::.:::::::.::.. :.:
FOXO3    RVQNEGTGKSSWWIINPDGGKSGKAPRRRAVSMDNSNKYTKSRGRAAKKKAALQTAPESA


FOXO1    GDSPGSQFSKWPASPGSHSNDDFDNWSTFRPRTSSNASTISGRLSPIM--TEQDDLGEGD
         ::: ::.::::.:::.|::.::.:.: :. :: ::.::::::.:::::: :: :... :
FOXO3    DDSPSSQLSKWPGSPSSRSSDELDAWTDFRSRTNSNASTVSGRLSPIMASTELDEVQDDD


FOXO1    --VHSMVYPPSAAKMAST-------LPSLSEISNPENMEN-LLDNL-NLLSSPTSLTVST
          . :.: ::. :. ::  :.....  :... : .:: . : .  .: :
FOXO3    APLSPMLYSSSASLSPSVSKPCTVELPRLTDMAGTMNLNDGLTENLMDDLLDNITLPPSQ


FOXO1    QSSPGTMMQQTPCYSFAPPNTSLNSPSPNYQKYTYGQSSMSPLPQMPIQTLQDNK-SSYG
         :  :  .::..  . ..    ...:.::  .... ..: ::.. : : :.::.:.:: ....
FOXO3    PSPTGGLMQRSSSFPYTTKGSGLGSPTCSFNSTVFGPSSLNSLRQSPMQTIQENKPATFS


FOXO1    GMSQYNCAPGLLKELLTSDSPPHNDIMSTPVDPGVAQPNSRVLGQNV---MMGPNSVMST
         .::.:.  :.:..:::::: :.:.:  : .:.: .. .. .:::   .: :.  .
FOXO3    SMSHYGNQ--TLQDLLTSDSLSHSDVMMTQSDPLMSQASTAVSAQNSRRNVMLRNDPMMS


FOXO1    YGSQASHNKMMNPSSHTHPGHAQQTSAVNGRPLPHTVSTMPHTSGMNRLTQVKTPVQVPL
         ...: .......: ..  :  : :  ..: : ..::.:  :  .: ..:  : :.
FOXO3    FAAQPNQGSLVN-QNLLHHQHQTQGALGGSRALSNSVSNMG-LSESSSLGSAKHQQQSPV


FOXO1    PHPMQMSALGGYSSVSSCNGYGRMGLLHQEKLPSDLD-GMFIERLDCDMESIIRNDLMDG
         . ::  .   . :. :     .   .. ..::.::::: ::   :.:::::::::..:::.
FOXO3    SQSMQ-TLSDSLSGSSLYSTSANLPVMGHEKFPSDLDLDMFNGSLECDMESIIRSELMDA


FOXO1    DTLDFNFDNVLPNQS--------FPHSVKTTTHSWVSG
         : :::::::... ..:.        : . .....::: :
FOXO3    DGLDFNFDSLISTQNVVGLNVGNFTGAKQASSQSWVPG
```

FIG. 8
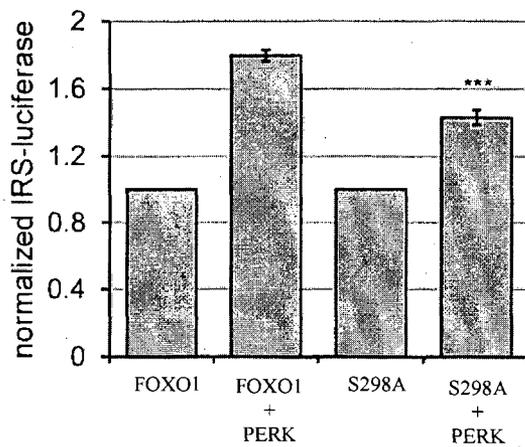
FIG. 9
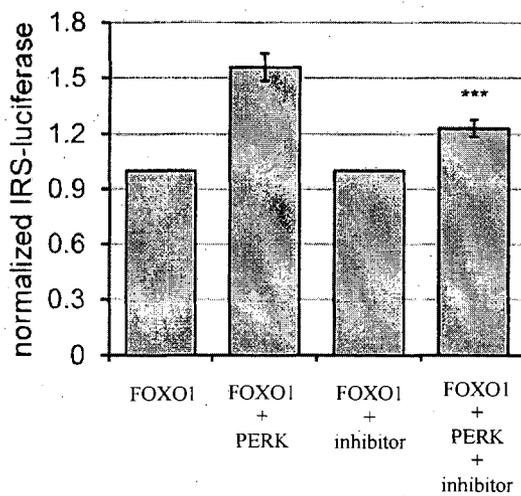
FIG. 10
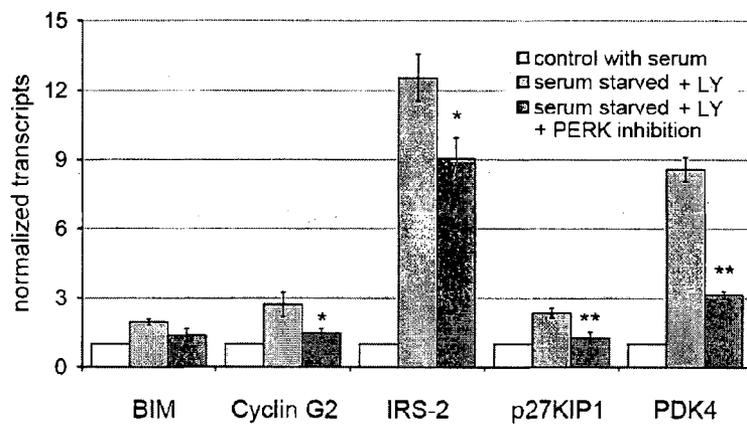
FIG. 11A
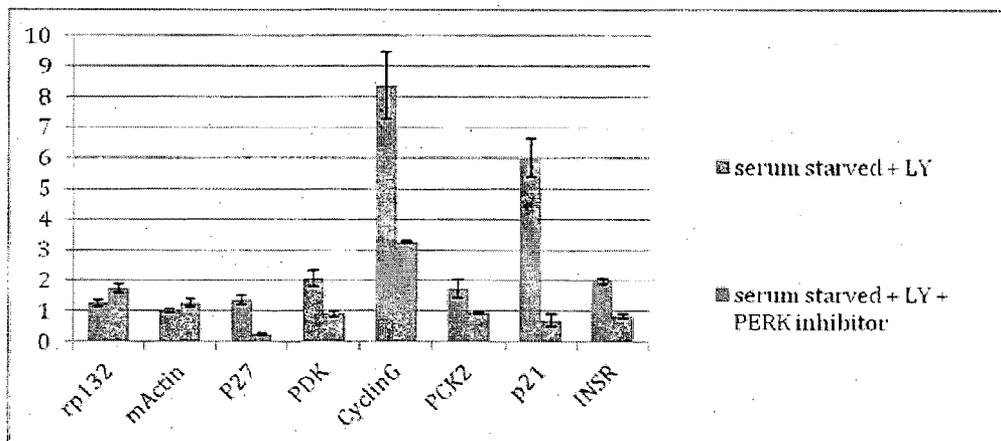

FIG. 11B
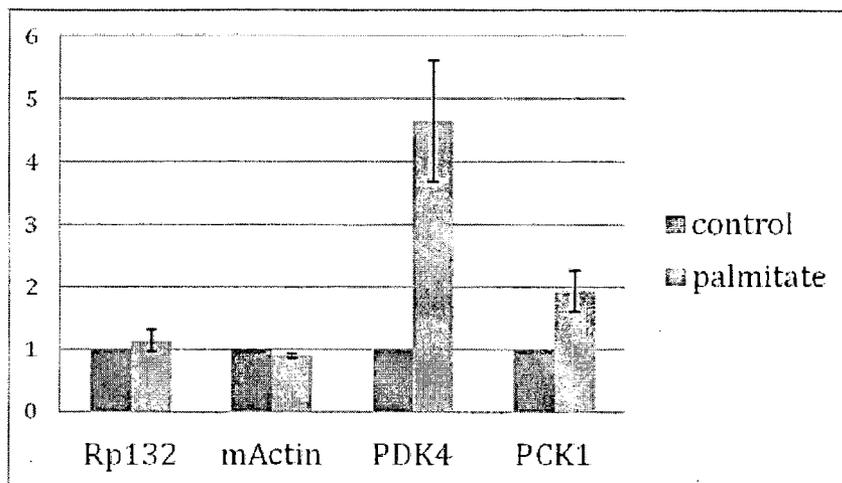
FIG. 12A
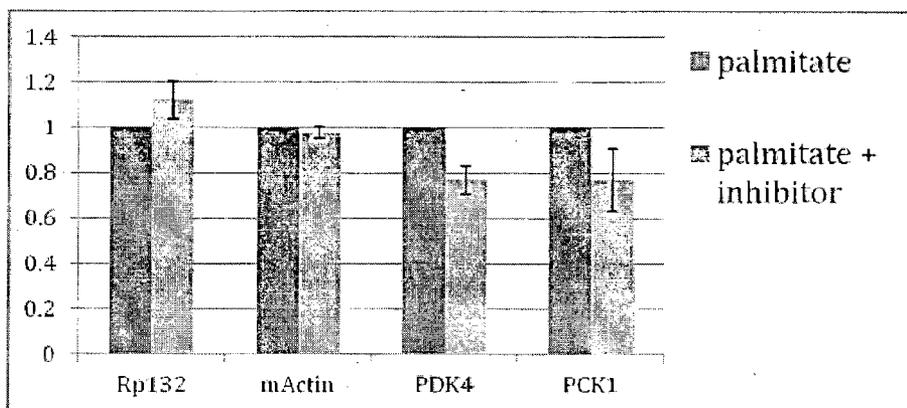
FIG. 12B

COMPOUNDS AND METHODS FOR TREATING INSULIN RESISTANCE SYNDROME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore patent application no. 201103478-2, filed May 13, 2011, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to biochemistry and medical applications of biochemical molecules.

BACKGROUND OF THE INVENTION

[0003] Insulin resistance is when the cells of the body become resistant to the effects of insulin. Accordingly, the body provides a smaller than expected biological response to a given dose of insulin and thus, a higher amount of insulin is required in order to be effective. Insulin resistance may lead to certain diseases. For example, Type 2 diabetes is associated with resistance to insulin leading to elevated blood glucose and elevated insulin levels. Typically, insulin resistance precedes the development of Type 2 diabetes. Obesity-related insulin resistance underlies metabolic syndrome, which is characterized by excess abdominal fat, and can also lead to the development of diabetes. Insulin resistance is thus an important medical problem.

[0004] The insulin signal transduction pathway has been extensively studied. In brief, binding of insulin to the extracellular portion of the transmembrane insulin receptor triggers a cascade of molecular events within the cell, leading to activation of PI3 Kinase (PI3K). Activated PI3K increases the level of the phosphatidylinositol (3,4,5)-triphosphate (PIP(3, 4,5)P$_3$) protein at the intracellular portion of the cell membrane, in turn leading to activation of the protein kinase known as AKT (or protein kinase B (PKB)). Activation of AKT triggers a chain of sequences, leading to the stimulation of glucose uptake into the cell and the promotion of glucose storage in the cell as glycogen.

[0005] However in an insulin resistant subject, AKT activity will be lowered. Hence, increasing AKT activity could be used to counter insulin resistance by stimulating glucose uptake. One way to increase AKT activity would be to inhibit the activity of the lipid phosphatase and tensin homolog (PTEN), which opposes PI3K activity. However, PTEN has been identified as a tumor suppressor gene, so it is likely that a drug reducing PTEN activity might pose an increased cancer risk. Furthermore, hyperactivation of AKT is also widely found in cancer. Accordingly, manipulation of the insulin-PI3K-AKT pathway would pose excessive risks to be used as a therapy for a chronic disease.

[0006] There is therefore a need to provide an alternative therapy for insulin resistance that overcomes, or at least ameliorates, one or more of the disadvantages described above. There is a need to provide further drugs that can be used to treat insulin resistance or related diseases.

SUMMARY OF THE INVENTION

[0007] According to a first aspect, there is provided a method of treating or preventing insulin resistance syndrome in an animal body by administering an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0008] According to a second aspect, there is provided a method of reducing activity of transcription factors of the FOXO family (Foxo1, 3a, 4 and 6) by administering an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0009] According to a third aspect, there is provided siRNA directed against the nucleic acid transcribed from the PERK gene.

[0010] According to a fourth aspect, there is provided an antibody, or a functional variant thereof, or a fragment of the antibody capable of binding to PERK protein.

[0011] According to a fifth aspect, there is provided a pharmaceutical composition comprising an inhibitor of PERK gene or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0012] According to a sixth aspect, there is provided a method of identifying a compound that modulates expression of PERK gene in a cell, the method comprising:

[0013] a. exposing cells expressing the PERK gene with a test compound;

[0014] b. determining the expression level of the PERK gene in the cells which were exposed to the test compound under (a);

[0015] c. comparing the level of expression of the PERK gene determined under (b) with the expression of the PERK gene in control cells which were not exposed to the test compound; wherein a difference in the expression level between the cells under (b) compared to the control cells identifies the compound that modulates expression of the PERK gene in a cell.

[0016] According to a seventh aspect, there is provided a method of identifying a compound that modulates the amount or activity of PERK protein comprised in a cell, the method comprising:

[0017] a. exposing cells expressing PERK protein with a test compound;

[0018] b. determining the amount or activity of PERK protein in the cells which were exposed to the test compound under (a);

[0019] c. comparing the amount or activity of PERK protein determined under (b) with the activity of PERK protein in control cells not exposed to the test compound; wherein a difference in the amount or activity of PERK protein between the cells under (b) compared to the control cells identifies the compound that modulates the amount of PERK protein in the cells.

[0020] According to an eighth aspect, there is provided a method of identifying a compound that modulates the amount of PERK protein comprised in a cell, the method comprising:

[0021] a. exposing cells expressing PERK protein with a test compound;

[0022] b. determining the amount of PERK protein in the cells which were exposed to the test compound under (a);

[0023] c. comparing the amount of PERK protein determined under (b) with the amount of PERK in control cells not exposed to the test compound; wherein a difference in the amount of PERK protein between the cells

under (b) compared to the control cells identifies the compound that modulates the amount of PERK protein in the cells.

[0024] According to a ninth aspect, there is provided a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises identifying and determining the PERK activity in the patient, wherein an increased PERK activity indicates that the person may be receptive for a treatment with a PERK inhibitor.

[0025] According to a tenth aspect, there is provided a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises measuring activity of a protein kinase AKT and/or PI3Kinase activity in a subject, wherein in comparison to a control a lowered AKT activity and/or lowered PI3Kinase activity indicates that the patient may be receptive for a treatment with a PERK inhibitor.

[0026] According to an eleventh aspect, there is provided a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises measuring the relative levels of phosphorylation on the AKT site and on the PERK site(s), wherein a lower ratio of AKT site phosphorylation to PERK site phosphorylation indicates that the patient is receptive for a treatment with a PERK inhibitor.

[0027] According to a twelfth aspect, there is provided a kit for use in treating or preventing insulin resistance syndrome in a patient, said kit comprises one of the following selected from the group consisting of a siRNA as disclosed herein, an antibody as disclosed herein, an organic molecule as disclosed herein, and a pharmaceutical composition as disclosed herein.

[0028] According to a thirteenth aspect, there is provided a kit for determining whether a patient suffering from insulin resistance syndrome is receptive for a treatment with a PERK inhibitor, wherein the kit comprises:

[0029] a. antibodies specific to one or more of the AKT phosphorylation site(s) on one or more of the human FOXO proteins; and

[0030] b. antibodies specific for one or more of the PERK phosphorylation site(s) on one or more of the human FOXO proteins or for one or more of the PERK phosphorylation site(s) on PERK protein or on eIF2α.

[0031] According to a fourteenth aspect, there is provided the use of an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of the activity of PERK protein, or a functional variant thereof, in the manufacture of a medicament for the treatment or prevention of insulin resistance syndrome.

[0032] According to a fifteenth aspect, there is provided the use of an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of the activity of PERK protein or a functional variant thereof, in the manufacture of a medicament to reduce activity of transcription factors of the FOXO family (Foxo1, 3a, 4 and 6) for the treatment or prevention of insulin resistance syndrome.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0034] FIG. 1(a) shows a schematic diagram of the connection between insulin signaling and FOXO in a cell. The binding of insulin to the transmembrane insulin receptor triggers a chain of events which increases AKT activity. AKT phosphorylates the FOXO transcription factors in the cell nucleus and brings the phosphorylated FOXO out of the nucleus into the cytoplasm, thereby preventing the expression of the target genes of FOXO, such as Bim, GADD45 and CHOP. FIG. 1(b) shows a schematic diagram of the connection between FOXO and PERK in a healthy cell, an insulin resistant cell and a cell treated for insulin resistance. AKT is less active in an insulin resistant cell, thereby reducing AKT-mediated phosphorylation of FOXO. This results in an increase in nuclear FOXO localization and up-regulation of FOXO targets by PERK-mediated phosphorylation of FOXO which promotes nuclear localization. Consequently, a cell can be treated to reduce PERK activity, thereby down-regulating the expression of FOXO targets and offsetting the effects of insulin resistance.

[0035] FIGS. 2(a) to 2(d) show the nuclear and/or cytoplasmic localization of FOXO-GFP in samples (a) to (d) referred to in Example 1. Sample (a) refers to the control cells, sample (b) refers to insulin treated cells, sample (c) refers to endoplasmic reticulum (ER) stress induced cells and sample (d) refers to ER stress induced cells that have PERK depleted by RNA interference (RNAi). From FIG. 2(a), it can be seen that the FOXO-GFP protein was predominantly nuclear, but insulin treatment shifts the location of FOXO towards the cytoplasm as seen in FIG. 2(b). From FIG. 2(c), it can be seen that ER stress counteracts the effects of insulin by increasing the proportion of nuclear FOXO, but depletion of PERK by RNAi increases cytoplasmic FOXO as seen in FIG. 2(d).

[0036] FIG. 3A shows the levels of expression of the FOXO target, 4E-BP (a regulator of overall translation levels in cells), and PERK in Drosophila wing disc tissue expressing the nub-Gal4 transgene alone ("Nub") or together with an upstream activation sequence (UAS)-PERK transgene ("Nub>PERK") referred to in Example 2A. The data is presented as fold change relative to the level in the "Nub" control sample. It can be seen that overexpression of PERK mRNA increased the levels of 4E-BP in "Nub>PERK" as compared to "Nub".

[0037] FIG. 3B shows the relative eye size in Drosophila expressing the GMR-Gal4 transgene ("ctrl") referred to in Example 2B. The total eye areas were measured in pixels from digital microscopic images using standardized magnification and the pixels were plotted in arbitrary units for each sample. PERK was then depleted by RNA interference (RNAi) and this sample is denoted as "PERK RNAi". Overexpression of an upstream activation sequence (UAS)— FOXO transgene was done to the control fly ("FOXO"). PERK was further depleted by RNAi from the "FOXO" fly and this sample is denoted as "FOXO,PERK RNAi". As seen in FIG. 3B, depletion of PERK had no effect on the control fly, when comparing "ctrl" with "PERK RNAi". However, when comparing "FOXO" with "FOXO,PERK RNAi", it can be seen that depletion of PERK counteracted the effects of FOXO overexpression, as evidenced by an increase in relative eye size.

[0038] FIG. 4A shows normalized luciferase levels of the FOXO luciferase reporter referred to in Example 3A in human MCF-7 cells. Control cells expressed a human Foxo3a luciferase reporter alone ("4FRE"). Cells were further co-transfected to express Foxo3a ("+Foxo3a"), PERK ("+PERK"), or both Foxo3a and PERK together ("+Foxo3a+PERK"). As seen in FIG. 4A, a higher level of luciferase in "+Foxo3a" indicates that expression of Foxo3a increased reporter gene expression when compared with "4FRE". PERK expression alone had little effect on the control, when comparing "4FRE" with "PERK". However, co-expression of PERK potentiated the effects of Foxo3a in the sample "+Foxo3a+PERK".

[0039] FIG. 4B shows normalized luciferase levels of the FOXO luciferase reporter referred to in Example 3B in human MCF-7 cells. Control cells expressed a human Foxo1 luciferase reporter alone ("IRS"). Cells were further co-transfected to express Foxo1 ("+Foxo1"), PERK ("+PERK"), or both Foxo1 and PERK together ("+Foxo1+PERK"). As seen in FIG. 4B, a higher level of luciferase in "+PERK" as compared to "IRS" presumably indicates that overexpression of PERK was acting on endogenous Foxo1. A higher level of luciferase in "+Foxo1" indicates that expression of Foxo1 increased reporter gene expression when compared with "IRS". Co-expression of PERK potentiated the effects of Foxo1 in the sample "+Foxo1+PERK".

[0040] FIG. 5A shows normalized levels of mRNA transcripts of FOXO targets referred to in Example 4A: CCAAT/enhancer binding protein epsilon (CHOP), Bim (anti- or pro-apoptotic regulators), Growth Arrest and DNA Damage gene (GADD45) and PERK, in human AGS cells. Bip2, which is not a FOXO target, was also measured as a control. Non-treated human AGS cells ("ctrl") were treated with antibiotic tunicamycin to induce ER stress ("TM"). In FIG. 5A, it can be seen that "TM" cells had significantly higher levels of Bip2, CHOP, Bim and GADD45 as compared to "ctrl" cells. Thus, it can be concluded that ER stress up-regulates FOXO activity. FIG. 5B shows normalized levels of mRNA transcripts of the FOXO targets referred to in Example 4B in human AGS cells. "TM" cells were further treated with siRNA treatment to deplete PERK ("PERK RNAi+TM"). In FIG. 5B, it can be seen from "PERK RNAi+TM" that PERK depletion down-regulates FOXO activity, thereby reducing the degree of induction of the FOXO targets. The levels of Bip2 transcripts were not affected since Bip2 is not a FOXO target.

[0041] FIG. 6 shows the nuclear and/or cytoplasmic localization of PERK in insulin treated *Drosophila* cells ("Control+insulin") and ER stress induced cells ("ER stress") referred to in Example 6. The Serine residues of *Drosophila* FOXO phosphorylation sites 1 and 4 were mutated to Alanine and denoted as "S66A" and "S243A" and treated with tunicamycin to induce ER stress. In FIG. 6, it can be seen that ER stress counteracted the effects of insulin when comparing "Control+insulin" and "ER stress" as nuclear localization was increased in "ER stress". However, ER stress did not up-regulate the activity of mutated FOXO evidenced by higher cytoplasmic localization of PERK in "S66A" and "S243A". It can thus be concluded that *Drosophila* FOXO phosphorylation sites 1 and 4, i.e. S66 and S243, are required to induce movement of *Drosophila* FOXO into the nucleus.

[0042] FIG. 7 shows normalized luciferase levels of the human Foxo1 luciferase reporter referred to in Example 7. The S303 residue of human Foxo1 phosphorylation site was mutated to Alanine (corresponding to phosphorylation site 4,

S243, in *Drosophila* FOXO), denoted as "S303A". Control cells were co-transfected to express the luciferase reporter and Foxo1 ("IRS+Foxo1") and PERK ("+PERK"). The mutated "S303A" was also co-transfected to express the luciferase reporter and Foxo1 ("IRS+Foxo1 S303A") and PERK ("+PERK"). As seen in FIG. 7, "IRS+Foxo1S303A" was slightly less effective than "IRS+Foxo1". When Foxo1 ("IRS+Foxo1") is co-expressed with PERK ("+PERK"), the activity of human Foxo1 was enhanced by about 2 fold. However, the effect of PERK was attenuated when co-expressed with Foxo1 S303A, evidenced by the lower levels of normalized luciferase in the "+PERK" samples. This indicates that S303 in human Foxo1 plays an important role in mediating the effects of PERK on Foxo1 activity.

[0043] FIG. 8 shows the DNA sequences of Foxo1 and Foxo3 and the corresponding phosphorylated sites and phospho-peptides in ER-stress induced human H1299 cells referred to in Example 8.

[0044] FIG. 9 shows normalized luciferase levels of the human Foxo1 luciferase reporter referred to in Example 9. The S298 residue of human Foxo1 was mutated to Alanine (corresponding to phosphorylation site 4, S243, in *Drosophila* FOXO), denoted as "S298A", and tested for responsiveness to PERK. H1299 cells were transfected to express natural Foxo1 ("FOXO1") or the S298A mutant version of Foxo1 ("S298A") and each were co-transfected to overexpress PERK ("FOXO1+PERK" and "S298A+PERK"). As seen in FIG. 9, the activity of "S298A" was comparable to that of "FOXO1" in the reporter assay without added PERK. However, "S298A" shows a lower response to PERK overexpression (S298A+PERK") as compared to "FOXO1+PERK". It can thus be concluded that S298 is one of the sites on human Foxo1 that contributes to mediating the effects of PERK on Foxo1 activity.

[0045] FIG. 10 shows normalized luciferase levels of the human Foxo1 luciferase reporter referred to in Example 10. Human H1299 cells were transfected to express human Foxo1 ("FOXO1"), co-transfected to express PERK ("FOXO1+PERK") and both were treated with PERK kinase inhibitor ("FOXO1+inhibitor" and "FOXO1+PERK+inhibitor"). In FIG. 10, it can be seen from "FOXO1" and "FOXO1+PERK" that PERK potentiates the effect of Foxo1 alone. However, partial inhibition of PERK activity reduced the level of Foxo1 activity when comparing "FOXO1+PERK" and "FOXO1+PERK+inhibitor". It can thus be concluded that PERK kinase inhibitor partially inhibits PERK activity, thereby down-regulating Foxo1 activity.

[0046] FIG. 11A shows the normalized expression levels of endogenous Foxo1 mRNA targets in MCF-7 cells that were serum starved to remove insulin ("control with serum") and treated with a PI3K inhibitor to further reduce AKT activity ("serum starved+LY") to simulate extreme insulin resistance, as described in Example 11A. The cells were further treated with PERK kinase inhibitor ("serum starved+LY+PERK inhibition"). The endogenous FOXO targets are BIM, Cyclin G2, IRS-2, p27KIP1 and PDK4. In FIG. 11A, it can be seen that reduction of AKT activity increases Foxo1 activity, evidenced by the substantial increase of target gene expression of "serum starved+LY" as compared to "control with serum". Further, PERK inhibition counteracted the effects of AKT reduction, thereby reducing the target gene expression of "serum starved+LY+PERK inhibition" as compared to "serum starved+LY". FIG. 11B shows the normalized expression levels of FOXO targets in HEPG2 cells instead, referred

to in Example 11B. FIG. **11**B confirms that PERK inhibition counteracted the effects of AKT reduction and reduced target genes expression. The FOXO targets are p27, PDK, Cyclin G, PCK2, p21 and INSR. However, PERK inhibition did not reduce the expression of rp132 and mActin, which are not FOXO targets.

[0047] FIG. **12**A shows the normalized expression levels of Foxo1 target genes in HEPG2 liver cells treated with palmitate to induce insulin resistance. The data was normalized to Kinesin mRNA levels and to the level of the rp132 control mRNAs. The Foxo1 target genes measured were PDK4 and PCK1. In FIG. **12**A, it can be seen that the palmitate treated cells had substantially higher levels of PDK4 and PCK1 expression. However, the levels of rp123 and mActin, which are not Foxo1 targets, were not substantially altered. FIG. **12**B shows the normalized expression levels of Foxo1 targets, PDK4 and PCK1, in palmitate treated cells and palmitate treated cells further treated with PERK kinase inhibitor. The data was normalized to Kinesin mRNA levels and to the level of the rp132 control mRNAs. In FIG. **12**B, it can be seen that PERK inhibition decreased the expression of PDK4 and PCK1. However, the levels of rp123 and mActin, which are not Foxo1 targets, were not substantially altered. It can thus be concluded that a reduction of Foxo1 activity is a consequence of PERK inhibition in cells induced with insulin resistance.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0048] It is known that an increase in the activity of the protein kinase known as AKT (or protein kinase B (PKB)) could be used to treat insulin resistance. However, hyperactivation of AKT is also widely found in cancer.

[0049] The present inventors have now surprisingly found alternative methods and compounds for treating insulin resistance and related diseases. Specifically, the present inventors have surprisingly found a way to downregulate the activity of the transcription factors of the Forkhead box class 0 (FOXO) family to provide a means to treat insulin resistance and related diseases.

[0050] One of the targets of AKT is the FOXO transcription factors (Foxo1, 3a, 4 and 6) whose nuclear localization is regulated by AKT-mediated phosphorylation. The connection between insulin signaling and FOXO in a cell is schematically shown in FIG. **1**(*a*). Referring to FIG. **1**(*a*), the binding of insulin to the transmembrane insulin receptor (InR) triggers a cascade of molecular events leading to the activation of PI3 Kinase (PI3K). Activated PI3K increases the level of the phosphatidylinositol (3,4,5)-triphosphate (PIP(3, 4,5)P$_3$) protein at the cell membrane, in turn leading to activation of AKT. Phosphorylation of Foxo1 by AKT is known to create a binding site for the 14-3-3 proteins, which brings FOXO out of the nucleus and into the cytoplasm, thereby leading to the retention of the phosphorylated FOXO in the cytoplasm. The effects of AKT are comparable on all FOXO family members.

[0051] For the avoidance of doubt, it is to be noted that the use of the term "FOXO" herein refers to all members of the FOXO transcription factor family, while references to a specific family member will be defined by number e.g. Foxo1.

[0052] From FIG. **1**(*a*), it is clear that an outcome of the insulin signal is the increase in AKT activity, which increases AKT-mediated FOXO phosphorylation and increases retention of FOXO in the cytoplasm, where it cannot act as a

transcription factor to induce the expression of its target genes in the nucleus. Thus, insulin signaling can be described as a reduction of FOXO activity and the consequential reduction of the expression of genes whose expression is regulated by FOXO. Insulin resistance can therefore be described as increased nuclear FOXO activity, leading to increased expression of FOXO regulated (targeted) genes.

[0053] However, FOXO are transcription factors and as such are not conventional druggable targets. The present inventors have now identified that protein kinase RNA-like endoplasmic reticulum kinase (PERK), also known as eukaryotic translation initiation factor 2, α-subunit, kinase-3 (eIF2α K$_3$), regulates FOXO. Specifically, as seen in FIG. **1**(*b*) it was found by the inventors that PERK phosphorylates FOXO, which promotes the nuclear localization of FOXO. PERK activity therefore acts in opposition to AKT because AKT increases retention of FOXO in the cytoplasm, while PERK increases retention of FOXO in the nucleus. As AKT activity stimulates glucose uptake and thus counters insulin resistance, PERK activity can therefore be seen to be representative of insulin resistance. In an insulin resistant cell, AKT is less active, thereby reducing AKT-mediated phosphorylation of FOXO. This results in an increase in nuclear FOXO localization and up-regulation of FOXO targets by PERK-mediated phosphorylation of FOXO which promotes nuclear localization. Accordingly, inhibition of PERK provides a means to treat or prevent insulin resistance and related diseases by reducing nuclear FOXO activity.

[0054] As a result, in one embodiment, there is provided a method of treating or preventing insulin resistance syndrome in an animal body by administering an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0055] In another embodiment, there is provided a method of reducing activity of transcription factors of the FOXO family by administering an inhibitor of PERK gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0056] Members of the FOXO family in mammals include Foxo1, Foxo2, Foxo3, Foxo4 and Foxo6, while Foxo5 is the fish ortholog of Foxo3. Foxo3 is known as Foxo3a, while Foxo3b is a pseudogene. In one embodiment, the FOXO family members are Foxo1, 3a, 4 and 6.

[0057] As used in the context of the specification, the phrases "inhibiting the PERK gene" or "inhibitor of the PERK gene", or variants thereof, mean that the expression of the PERK gene is decreased or absent. Further, in the context of the specification, the phrases "inhibiting the PERK protein" or "inhibitor of the PERK protein", or variants thereof, mean that the activity of the PERK protein is decreased or absent. Absent means that there is completely no expression of the PERK gene or activity of the PERK protein. Functional variants of an inhibitor of the PERK gene within the context of the specification refers to genes which possess a biological activity (either functional or structural) that is substantially similar to the PERK gene disclosed herein. Functional variants of an inhibitor of PERK protein may be construed similarly to refer to a protein that is altered by one or more amino acids. The term "functional variant" also includes a fragment, a variant based on the degenerative nucleic acid code or a chemical derivative. A functional variant may have conservative changes, wherein a substituted amino acid or nucleic acid has similar structural or chemical properties to the replaced

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amino acid/nucleic acid, e.g. replacement of leucine with isoleucine. A functional variant may also have non-conservative changes, e.g. replacement of a glycine with a tryptophan, or a deletion and/or insertion of one or more amino acids, or a deletion and/or insertion of one or more nucleic acids. It is understood that the functional variant at least partially retains its biological activity, e.g. function, of the PERK gene or PERK protein, or even exhibits improved biological activity.

[0058] It is understood that the inhibition of the PERK gene decreases the expression of the PERK protein. Accordingly, as PERK phosphorylates FOXO and promotes the nuclear localization of FOXO, the inhibition of the PERK gene or inhibition of the activity of the PERK protein down-regulates the downstream phosphorylation of FOXO. Consequently, nuclear FOXO activity is down-regulated.

[0059] Insulin resistance syndrome makes up a broad clinical spectrum and is defined as any abnormalities associated with insulin resistance. Abnormalities such as the resistance to insulin, diabetes, hypertension, dyslipidemia and cardiovascular disease constitute the insulin resistance syndrome.

[0060] The insulin resistance syndrome may be diet-induced insulin resistance and/or obesity-induced insulin resistance. Diet-induced insulin resistance means that the resistance to insulin is induced by a diet high in saturated fat and carbohydrates. Obesity-induced insulin resistance means that the resistance to insulin is induced by a genetic predisposition to obesity or obesity which is due to dietary habits.

[0061] The disclosed methods may be used for treating any one of the following conditions which are caused by insulin resistance syndrome: insulin resistance, hypertension, dyslipidemia, Type 2 diabetes or coronary artery disease.

[0062] Hypertension refers to the sustained elevation of resting systolic blood pressure, diastolic blood pressure or both. Dyslipidemia refers to elevated plasma cholesterol and/or triglyceride concentration or a low high-density-lipoprotein level that contributes to the development of artherosclerosis. Coronary artery disease involves the impairment of blood flow through the coronary arteries, most commonly by atheromas.

[0063] Insulin resistance refers to a decreased ability to respond to the action of insulin, which is compensated by a downstream increase in the endogenous secretion of insulin. Over time, a person suffering from insulin resistance can develop high plasma sugar levels as the increased secretion of insulin can no longer compensate for elevated plasma sugar levels.

[0064] Insulin resistance typically precedes the development of Type 2 diabetes. In certain individuals who subsequently develop Type 2 diabetes, insulin resistance may be present for many years before the actual onset of diabetes. In Type 2 diabetes, insulin secretion is inadequate. Accordingly, Type 2 diabetes relates to relative insulin deficiency. On the other hand, Type 1 diabetes is an absolute insulin deficiency due to the destruction of insulin-producing cells in the pancreas. Accordingly, treatment of Type 1 diabetes may be linked to the restoration of insulin production. In contrast, treatment of Type 2 diabetes is linked to the restoration of sensitivity to insulin.

[0065] In general, the term "inhibition" as used in the context of the specification means a decrease in the expression of PERK gene or the activity of PERK protein and a corresponding downstream decrease in FOXO activity.

[0066] The inhibitor of any of the genes referred to herein may comprise at least one oligonucleotide or at least one antibody or at least one inorganic molecule or at least one organic molecule.

[0067] The oligonucleotide may be an interfering ribonucleic acid (iRNA), or protein nucleic acid (PNA) or locked nucleic acid (LNA).

[0068] The term "oligonucleotide" generally refers to a single-stranded nucleotide polymer made of more than 2 nucleotide subunits covalently joined together. Preferably between 10 and 100 nucleotide units are present, most preferably between 12 and 50 nucleotides units are joined together. The sugar groups of the nucleotide subunits may be ribose, deoxyribose or modified derivatives thereof such as 2'-O-methyl ribose. The nucleotide subunits of an oligonucleotide may be joined by phosphodiester linkages, phosphorothioate linkages, methyl phosphonate linkages or by other rare or non-naturally-occurring linkages that do not prevent hybridization of the oligonucleotide. Furthermore, an oligonucleotide may have uncommon nucleotides or normucleotide moieties. An oligonucleotide, as defined herein, is a nucleic acid, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), or have a combination of ribo- and deoxyribonucleotides covalently linked

[0069] The term "oligonucleotide" may also refer, in the context of the specification, to a nucleic acid analogue of those known in the art, for example Locked Nucleic Acid (LNA), or a mixture thereof. The term "oligonucleotide" includes oligonucleotides composed of naturally occurring nucleobases, sugars and internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly or with specific improved functions. A fully or partly modified or substituted oligonucleotide is often preferred over native forms because of several desirable properties of such oligonucleotides such as for instance, the ability to penetrate a cell membrane, good resistance to extra- and intracellular nucleases, high affinity and specificity for the nucleic acid target. Methods of modifying oligonucleotides in this manner are known in the art.

[0070] In some oligonucleotides, sometimes called oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a protein nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

[0071] A further modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom, wherein n is 1 or 2. The term "LNA" generally refers to a nucleotide containing one bicyclic nucleoside analogue, also referred to as a LNA monomer, or an oligonucleotide containing one or more bicyclic nucleoside analogues.

[0072] Examples of modified oligonucleotides include, but are not limited to oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones,

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and in particular-CH_2—NH—O—CH_2—, —CH_2—N
(CH_3)—O—CH_2— [known as a methylene (methylimino) or
MMI backbone], —CH_2—O—N(CH_3)—CH_2—, —CH_2—
N(CH_3)—N(CH_3)—CH_2— and —O—N(CH_3)—CH_2—
CH_2— [wherein the native phosphodiester backbone is rep-
resented as —O—P—O—CH_2—]. Also usable are
oligonucleotides having morpholino backbone structures.

[0073] The interfering ribonucleic acid may be a small
interfering ribonucleic acid (siRNA) or small hairpin ribo-
nucleic acid (shRNA) or micro ribonucleic acid (miRNA).

[0074] Modified oligonucleotides used as interfering ribo-
nucleic acids may also contain one or more substituted sugar
moieties. Preferred oligonucleotides comprise one of the fol-
lowing at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or
N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein
the alkyl, alkenyl and alkynyl may be substituted or unsub-
stituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl.
Particular examples include, but are not limited to O[(CH_2)
$_nO]_mCH_3$, O(CH_2)$_nOCH_3$, O(CH_2)$_nNH_2$, O(CH_2)$_nCH_3$,
O(CH_2)$_nONH_2$, and O(CH_2)$_nON[($CH_2$)$_nCH_3$)]$_2$, where n
and m are from 1 to about 10. Other exemplary oligonucle-
otides comprise one of the following at the 2' position: C_1 to
C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl,
alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl,
Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2,
heterocycloalkyl, heterocycloalkaryl, aminoalkylamino,
polyalkylamino, substituted silyl, an RNA cleaving group, a
reporter group, an intercalator, a group for improving the
pharmacokinetic properties of an oligonucleotide, or a group
for improving the pharmacodynamic properties of an oligo-
nucleotide, and other substituents having similar properties.
One exemplary modification includes 2'-methoxyethoxy(2^1-
O—$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or
2'-MOE), i.e., an alkoxyalkoxy group.

[0075] As used herein, the term "siRNA" refers to a ribo-
nucleic acid (RNA) or RNA analog comprising between
about 10 to 50 nucleotides (or nucleotide analogs) capable of
directing or mediating the RNA interference pathway. These
molecules can vary in length and can contain varying degrees
of complementarity to their target messenger RNA (mRNA)
in the antisense strand. The term "siRNA" includes duplexes
of two separate strands, i.e. double stranded RNA, as well as
single strands that can form hairpin structures comprising of
a duplex region. The siRNA may have a length of between
about 10 to 50 nucleotides, or between about 15 to 50 nucle-
otides, or between about 20 to 50 nucleotides, or between
about 25 to 50 nucleotides, or between about 30 to 50 nucle-
otides, or between about 35 to 50 nucleotides, or between
about 40 to 50 nucleotides, or between about 10 to 45 nucle-
otides, or between about 10 to 40 nucleotides, or between
about 10 to 35 nucleotides, or between about 10 to 30 nucle-
otides, or between about 10 to 25 nucleotides, or between
about 10 to 20 nucleotides, or between about 15 to 50 nucle-
otides, or between about 15 to 35 nucleotides, or between
about 15 to 30 nucleotides, or between about 15 to 25 nucle-
otides. In one embodiment, the siRNA has a length of
between 15 to 30 nucleotides.

[0076] The application of siRNA to down-regulate the
activity of its target mRNA is known in the art. In some
embodiments, mRNA degradation occurs when the anti-
sense strand, or guide strand, of the siRNA directs the RNA-
induced silencing complex (RISC) that contains the RNA
endonuclease Ago2 to cleave its target mRNA bearing a
complementary sequence. Accordingly, the siRNA may be

complementary to any portion of varying lengths on the
PERK gene. The siRNA may also be complementary to the
sense strand and/or the anti-sense strand of the PERK gene.
Accordingly, siRNA treatment may be used to silence the
PERK gene, thereby depleting the PERK protein down-
stream.

[0077] Hence, in one embodiment, there is provided siRNA
directed against the nucleic acid transcribed from the PERK
gene.

[0078] The siRNA may be directed against fragments of the
nucleic acid transcribed from the PERK gene. Accordingly,
the siRNA may comprise a sequence that is complementary to
any fragment of the PERK gene. In one embodiment, the
siRNA comprises the following sequence: 5'-CAAACU-
GUAUAACGGUUUAUT-3' (SEQ ID NO: 1), or functional
variants thereof. Such functional variants thereof may com-
prise at least one modified or substituted nucleotide. Func-
tional modifications and/or substitutions of the siRNA may be
performed by methods known in the art.

[0079] The term "shRNA", as used herein, refers to a uni-
molecular RNA that is capable of performing RNAi and that
has a passenger strand, a loop and a guide strand. The pas-
senger and guide strand may be substantially complementary
to each other. The term "shRNA" may also include nucleic
acids that contain moieties other than ribonucleotide moi-
eties, including, but not limited to, modified nucleotides,
modified internucleotide linkages, non-nucleotides, deoxy-
nucleotides, and analogs of the nucleotides mentioned
thereof.

[0080] miRNAs down-regulate their target mRNAs. The
term "miRNA" generally refers to a single stranded molecule,
but in specific embodiments, may also encompass a region or
an additional strand that is partially (between 10% and 50%
complementary across length of strand), substantially
(greater than 50% but less than 100% complementary across
length of strand) or fully complementary to another region of
the same single-stranded molecule or to another nucleic acid.
Thus, nucleic acids may encompass a molecule that com-
prises one or more complementary or self-complementary
strand(s) or "complements" of a particular sequence compris-
ing a molecule. For example, precursor miRNA may have a
self-complementary region, which is up to 100% comple-
mentary. miRNA probes or nucleic acids of the invention can
include, can be or can be at least 60%, 65%, 70%, 75%, 80%,
85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% complemen-
tary to their target.

[0081] The inhibitor may comprise of at least one antibody.
The term "antibody" refers to an antigen-binding protein
having a basic four-polypeptide chain structure consisting of
two heavy and two light chains, said chains being stabilized,
for example, by interchain disulfide bonds, which has the
ability to specifically bind antigen. The term "antibody"
includes monoclonal antibodies (including full-length mono-
clonal antibodies), polyclonal antibodies, multispecific anti-
bodies (for example, bispecific antibodies), chimeric antibod-
ies, CDR-grafted antibodies, humanized antibodies, human
antibodies and single chain antibodies (scFvs). The term
"antibody" also includes anticalins, which are artificial pro-
teins that are able to bind to antigens. Anticalins are not
structurally related to antibodies and thus are a type of anti-
body mimetic.

[0082] PERK is a transmembrane receptor protein. Hence,
fragments of PERK may be from the luminal domain or the
cytosolic domain. Antibodies may be used to inhibit PERK

protein such that substrate binding to PERK, or an isolated PERK fragment thereof, may partially be inhibited. The degree of inhibition of the substrate binding may differ. Accordingly, in one embodiment, there is provided an antibody, or functional variant thereof, or a fragment of the antibody capable of binding to PERK protein. In this embodiment, the antibody, or the functional variant thereof, or the fragment of the antibody may be a monoclonal or polyclonal antibody. Antibodies may be generated by methods known in the art using a PERK protein or a fragment thereof.

[0083] The term "isolated", when used in the context of an isolated protein, or fragment of protein, refers to a protein that has been removed from its natural environment, for example, as part of an organ, tissue or organism. The term does not imply that the protein is the only protein present, but does indicate that the protein is sufficiently separated from other proteins with which it would naturally be associated with, so that it is the predominant protein present (at least 10-20% more than any other proteins). "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity of the protein and that may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into, for example, immunogenic preparations or pharmaceutical acceptable preparations.

[0084] The term "fragment", when used in the context of a protein, refers to a part or portion of a protein comprising fewer polypeptide chains than an intact or complete protein. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete protein.

[0085] The inhibitor may comprise of at least one organic molecule or at least one inorganic molecule. The term "organic molecule" refers, for example, to any molecule that is made up predominantly of carbon and hydrogen, such as, alkanes and arylamines. The term "inorganic molecule" refers, for example, to molecules that are not organic molecules.

[0086] In one embodiment, the organic molecule is selected from the group consisting of:

[0087] 5-bromo-N_4-2-pyridinyl-N_2-[3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine;

[0088] 1-[5-(4-Amino-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-2,3-dihydro-1H-indol-1-yl]-2-[3-fluoro-5-(trifluoromethyl)phenyl]ethanone;

[0089] 1-methyl-3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0090] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0091] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0092] 7-methyl-5-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0093] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

[0094] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

[0095] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(3-pyridinyl)thieno[3,2-c]pyridin-4-amine;

[0096] 1-methyl-4-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-indazol-3-amine;

[0097] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(4-pyridinyl)thieno[3,2-c]pyridin-4-amine;

[0098] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-pyridinyl)thieno[3,2-c]pyridin-4-amine;

[0099] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-3-yl)thieno[3,2-c]pyridin-4-amine;

[0100] 4-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-indazol-3-amine;

[0101] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(1H-pyrazol-4-yl)thieno[3,2-c]pyridin-4-amine;

[0102] 7-(1-methyl-1H-pyrazol-4-yl)-3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

[0103] 3-{1-[(2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0104] 3-{1-[(3-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0105] 1-methyl-3-{1-[(2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0106] 1-methyl-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0107] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(1,2,3,6-tetrahydro-4-pyridinyl)thieno[3,2-c]pyridin-4-amine;

[0108] 3-(1-[3-(trifluoromethyl)phenyl]acetyl-2,3-dihydro-1H-indol-5-yl)thieno[3,2-c]pyridin-4-amine;

[0109] 3-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

[0110] 3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

[0111] 3-(1-[3-(methyloxy)phenyl]acetyl-2,3-dihydro-1H-indol-5-yl)thieno[3,2-c]pyridin-4-amine;

[0112] 3-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)thieno[3,2-c]pyridin-4-amine;

[0113] 3-[1-(2-naphthalenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

[0114] 3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(4-piperidinyl)thieno[3,2-c]pyridin-4-amine;

[0115] 7-{3-[(dimethylamino)methyl]phenyl}-3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

[0116] 3-{1-[(2,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0117] 3-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0118] 3-{1-[(3,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0119] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl} thieno[2,3-d]pyrimidin-4-amine;

[0120] 3-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0121] 7-methyl-5-{1-[(2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0122] 5-{1-[(2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0123] 5-{1-[(3-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0124] 3-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

[0125] 7-methyl-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0126] 3-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

[0127] 3-{2-[5-(4-aminothieno[3,2-c]pyridin-3-yl)-2,3-dihydro-1H-indol-1-yl]-2-oxoethyl}benzonitrile;

[0128] 3-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0129] 3-{1-[(2,3-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0130] 3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0131] 1-methyl-3-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0132] 7-methyl-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0133] 5-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0134] 5-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0135] 5-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0136] 7-methyl-5-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0137] 1-methyl-3-(1-{[3-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0138] 7-methyl-5-(1-{[3-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0139] 3-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0140] 1-methyl-3-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0141] 5-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0142] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0143] 1-methyl-3-{1-[(2,3,5-trifluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0144] 5-{1-[(2,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0145] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-4-yl)furo[3,2-c]pyridin-4-amine;

[0146] 3-{1-[(3,5-dichlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0147] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0148] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-4-yl)thieno[3,2-c]pyridin-4-amine;

[0149] 3-{1-[(3,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0150] 5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0151] 5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methyl-4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0152] 5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[2,3-d]pyrimidin-4-amine;

[0153] 3-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0154] 3-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0155] 3-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0156] 1-methyl-3-{1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0157] 3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0158] 5-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0159] 5-{1-[(2-fluoro-3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0160] 5-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0161] 5-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0162] 3-{1-[(2-fluoro-3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0163] 3-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0164] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methyl-4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0165] 5-{1-[(3-chloro-4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0166] 5-{1-[(3-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0167] 3-{1-[(3-chloro-4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0168] 3-{1-[(3-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0169] 5-{1-[(2,3-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0170] 1-(1-methylethyl)-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0171] 2-(4-amino-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)ethanol;

[0172] 5-{1-[(3,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0173] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0174] 1-ethyl-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0175] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methylfuro[3,2-c]pyridin-4-amine;

[0176] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-(1-methylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0177] 5-{1-[(3,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0178] 7-methyl-5-{1-[(2,3,5-trifluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0179] 5-{1-[(3,5-dichlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0180] 7-(3-azetidinyl)-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0181] 5-{1-[(4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0182] 7-methyl-5-{1-[(4-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0183] 5-{1-[(3-chloro-2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0184] 5-(1-{[3-fluoro-5-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0185] 7-[(methyloxy)methyl]-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0186] 7-methyl-5-{1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0187] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methylethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0188] 5-{1-[(5-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0189] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(4-morpholinyl)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0190] 5-{1-[(2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0191] 5-{1-[(3,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0192] phenylmethyl[2-(4-amino-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-7-yl)ethyl]carbamate;

[0193] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-methylbutyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0194] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(dimethylamino)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0195] 5-{1-[(6-chloro-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0196] 3-{1-[(3-chloro-2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0197] 7-(2-aminoethyl)-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0198] 4-amino-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridine-7-carbonitrile;

[0199] 5-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0200] 5-[4-fluoro-1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0201] 5-{4-fluoro-1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0202] 5-{1-[(2,5-difluorophenyl)acetyl]-4-fluoro-2,3-dihydro-1,4-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0203] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

[0204] 5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)furo[2,3-d]pyrimidin-4-amine;

[0205] 5-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

[0206] 5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

[0207] 5-(1-{[3-fluoro-5-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)furo[2,3-d]pyrimidin-4-amine;

[0208] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(4-piperidinyl)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0209] 7-methyl-5-{1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0210] 5-(1-{[4-fluoro-3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0211] 5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-oxetanyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

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[0212] 3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(dimethylamino)ethyl]furo[3,2-c]pyridin-4-amine;

[0213] 7-methyl-5-(1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0214] 7-(3-oxetanyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0215] 7-[2-(4-morpholinyl)ethyl]-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0216] 7-(1-methylethyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0217] 7-(3-methylbutyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0218] 4-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-c]pyridin-3-amine;

[0219] 7-chloro-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0220] 7-(3-azetidinyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0221] 7-(1-methyl-3-azetidinyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0222] 7-[2-(dimethylamino)ethyl]-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0223] 5-(4-fluoro-1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0224] 5-{4-fluoro-1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0225] 5-(4-fluoro-1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0226] 5-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-4-fluoro-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0227] 5-(4-fluoro-1-{[4-fluoro-3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0228] 3-{1-[(2,5-difluorophenyl)acetyl]-4-fluoro-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0229] 5-{4-fluoro-1-[(4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0230] 4-(1-[3-(trifluoromethyl)phenyl)acetyl]-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-c]pyridin-3-amine;

[0231] 1-methyl-4-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-c]pyridin-3-amine;

[0232] 7-(3-azetidinyl)-5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0233] 7-[2-(4-piperidinyl)ethyl]-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0234] 7-(2-aminoethyl)-3-{1-[(2,5-difluorophenyl)acetyl]-4-fluoro-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

[0235] 3-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

[0236] 5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrrolo[2,3-d]pyrimidin-4-amine;

[0237] 5-{4-chloro-1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine; and

[0238] 5-(4-chloro-1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

and salts thereof, including pharmaceutically acceptable salts thereof.

[0239] In one embodiment, the inhibitor is the organic molecule: 5-bromo-N_4-2-pyridinyl-N_2-[3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine. This organic molecule is a small molecule inhibitor of PERK protein. While PERK inhibitors are known in the art, known PERK inhibitors have not been used in the FOXO pathway. The present inventors have now surprisingly found that this organic molecule blocks the potentiating effects of PERK on FOXO. One normal function of PERK protein is to protect secretory cells from endoplasmic reticulum (ER) stress. It has been shown that an absence of PERK during embryonic development causes Type 1 diabetes due to loss of pancreatic islet cells. Further, loss of ER homeostasis and an accumulation of misfolded proteins can contribute to cardiovascular and degenerative diseases. PERK also responds to unfolded proteins in a cell. ER stress due to unfolded proteins is elevated in cancers and inhibition of the ER stress response can limit tumor growth. However, there has been no link until now to show that inhibition of PERK can treat or prevent insulin resistance syndrome by targeting the FOXO pathway.

[0240] The disclosed methods may further comprise administration of a peroxisome proliferator-activated receptor (PPAR) agonist and/or an anti-diabetic agent. The PPAR agonist may include, but is not limited to, PPAR-alpha agonists, PPAR-beta agonists, PPAR-gamma agonists, and PPAR-delta agonists. In one embodiment, the PPAR agonist is selected from the group consisting of mono-PPAR-alpha agonists, mono-PPAR-beta agonists, mono-PPAR-gamma agonists, mono-PPAR-delta agonists, dual PPAR-alpha and gamma agonists, N-[(4-methoxyphenoxy)carbonyl]-N-{4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy]benzyl}glycine (Muraglitazar), 2-[(4-methoxyphenoxy)carbonyl-[(1S)-1-[4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy]phenyl]ethyl]amino]acetic acid (peliglitazar), N-(o-benzoylphenyl)-O-[2-(5-methyl-2-phenyl-4-oxazolypethyl]-L-tyrosine (Farglitazar), thiazolidinediones class of PPAR-agonists, (RS)-5-(4-[(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methoxy]benzyl)thiazolidine-2,4-dione (Troglitazone), (RS)-5-(4-[2-(5-ethylpyridin-2-yl)ethoxy]benzyl)thiazolidine-2,4-dione (Pioglitazone), (RS)-5-[4-(2-[methyl (pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-di one (Rosiglitazone), 5-[[6-[(2-fluorophenyl)methoxy]naphthalen-2-yl]methyl]-1,3-thiazolidine-2,4-dione (netoglitazone or MCC555), 5-[(2,4-Dioxo-1,3-thiazolidin-5-yl)methyl]-2-methoxy-N-4-(trifluoromethyl)benzyl)benzamide (KRP 297), 4-[[4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy]phenyl]methyl]-1,

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2-oxazolidine-3,5-dione (JTT-501), 12-(4-Chlorophenyl)-2, 2-dichlorododecanoic acid (BM 17.0744), L764486, 2-[2-methyl-4-([4-methyl-2-[4-(trifluoromethyl)phenyl)thiazol-5-yl)methylthio)phenoxy)acetic acid (GW501516), (25)-2-ethoxy-3-[4-(2-phenoxazin-10-ylethoxy)phenyl]propanoic acid (NN622), 2-(4-{2-[(4-chlorobenzoyl)amino] ethyl}phenoxy)-2-methylpropanoic acid (bezafibrate), 5-(2, 5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid (gemfibrozil), fibrate class of PPAR-agonists, (S)-3-[4-[2-(Phenoxazin-10-yl)ethoxy]phenyl]-2-ethoxypropanoicacid (DRF 2725), [[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]-acetic acid (WY 14,643), 3-{4-[2-(Benzooxazol-2-yl-methyl-amino)-ethoxy]-phenyl}-2-ethoxy-propionic acid (SB 213068), (2S)-2-Ethoxy-3-[4-[2-(4-methylsulfonyloxyphenyl)ethoxy]phenyl]propanoic acid (Tesaglitazar; AZ 242), (±)-5-[[4-[2-(methyl-2-pyridinylamino)ethoxy]phenyl]methyl]-2,4-thiazolidinedione, (Z)-2-butenedioate and 1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Avandaryl), 2-methoxy-3-[4-[3-[4-(phenoxy)phenoxy]propoxy]phenyl]propanoic acid (Naveglitazar), (2S)-3-[4-[2-(4a,10a-dihydrophenoxazin-10-yl)ethoxy]phenyl]-2-ethoxypropanoic acid (Ragaglitazar; NN622), PLX 204, PLX 134, PLX 203, 5-(4-((6-(4-amino-3,5-dimethylphenoxy)-1-methyl-1H-benzo[d] imidazol-2-yl)methoxy)benzypthiazolidine-2,4-dione (CS 7017), DRF 10945, AVE 0847, AVE 8134, 641597 (GSK), 590735 (GSK), MK 767, AA 10090, LY 674, LY 929, T 131, DRF 4158, CLX 0921, NS 220, LY 293111, DRF 4832, GW 7282, 501516 (GSK), LG 100754, GW 544, AR H049020, AK-109, E-3030 (Eisai), CS-7017 (Sankyo), DRF-10945, KRP-101, ONO-5129, TY-51501, GSK-677954, LSN-862, LY-518674, GW-590735, KT6-207, K-111 (Roche), Bay-54-9801 (GSK), R-483 (Roche), EMD-336340 (Merck KGaA), LR-90 (Merck KGaA), CLX-0940, CLX-0921, LG-100754, GW-409890, SB-219994, NIP-223, T-174 (Tanabe Seiyaku), (+/−)-5-(p-((3,4-Dihydro-3-methyl-4-oxo-2-quinazolinyl) methoxy)benzyl)-2,4-thiazolidinedione (balaglitazone; DRF-2593), VDO-52, GW-1929, NC-2100, 5-[[6-[(2-fluorophenyl)methoxy]naphthalen-2-yl]methyl]-1,3-thiazolidine-2,4-dione (netoglitazone), 5-{4-[(1-methylcyclohexyl) methoxy]benzyl}-1,3-thiazolidine-2,4-dione (ciglitazone), LGD 1268, LG 101506, LGD 1324, GW 9578, 5-[(2-benzyl-3,4-dihydro-2H-chromen-6-yl)methyl]-1,3-thiazolidine-2, 4-dione (Englitazone), and 5-[(4-[3-(5-methyl-2-phenyl-1,3-oxazol-4-yl)propanoyl]phenyl)methyl]-1,3-thiazolidine-2, 4-dione (Darglitazone).

[0241] The disclosed methods may further comprise administration of an agent used in the treatment of diabetes, obesity or insulin resistance syndrome.

[0242] As the insulin signaling pathway is conserved throughout the animal kingdom, the present inventors have now found that the endoplasmic reticulum (ER) stress pathway in *Drosophila* is an inducer of FOXO activity. Whereas, in mammals, ER stress effects three pathways: activating transcription factor 6 (ATF6), IRE1 and PERK (which in turn effects the FOXO pathway). ATF6 is an ER stress-regulated transmembrane transcription factor that activates the transcription of ER molecules. IRE1 is a ser/thr protein kinase that possesses endonuclease activity and is important in altering gene expression as a response to ER stress signals. Accordingly, as ER stress induces the nuclear localization

and activity of FOXO, modulators of ER stress would be useful in decreasing the effects of ER stress on the activity of FOXO.

[0243] The agent used in the treatment of diabetes, obesity or insulin resistance syndrome may include, but is not limited to, ER stress modulators such as PBA (4-phenyl butyrate), TUDCA (tauroursodeoxycholic acid), TMAO (trimethylamine N-oxide), and derivatives thereof; anti-diabetic agents, anti-obesity agents, anti-dyslipidemia agent or anti-atherosclerosis agent, aspirin, vitamins, minerals, and anti-hypertensive agents.

[0244] Anti-diabetic agent includes, but is not limited to, insulin or hypoglycemic agents. Hypoglycemic agents include, but are not limited to, oral hypoglycemic agents such as sulfonylureas, tolbutamide, metformin, chlorpropamide, acetohexamide, tolazamide, glyburide, etc. Anti-obesity agent includes, but is not limited to, appetite suppressants. Anti-dyslipidemia agent or anti-atherosclerosis agent includes, but is not limited to, cholesterol lowering agents. Cholesterol lowering agents include, but are not limited to, HMg-CoA and reductase inhibitors such as lovastatin, atorvastatin, simvastatin, pravastatin, fluvastatin, etc.

[0245] It has been shown that ER stress can also be induced by genetic models of obesity such as the ob/ob mouse as well as diet-induced obesity. Use of chemical chaperones to overcome ER stress has been reported to limit activation of the three pathways, to decrease insulin and glucose levels and to improve glucose tolerance and insulin tolerance test results in both insulin resistant ob/ob mice and in obese diabetic humans.

[0246] Accordingly, the anti-diabetic agent may be used in combination with a chemical chaperon. The chemical chaperon may include, but is not limited to, imidodicarbonimidic diamide (biguanides), sulfonylureas, insulin and analogs thereof, peroxisome proliferator-activated receptor-gamma agonists, dual PPAR agonists and PPAR pan agonists, combination therapies, meglitinides, alpha-glucosidase inhibitors, glucagon-like peptide-1 (GLP-1) analogues and agonists, dipeptidyl peptidase IV (DPP-IV) inhibitors, pancreatic lipase inhibitors, sodium glucose co-transporter (SGLT) inhibitors, and amylin analog.

[0247] An example of biguanides is metformin. Examples of sulfonylureas include glimepiride, glyburide, glibenclamide, glipizide and gliclazide. Analogs of insulin include insulin lispro, insulin glargine, exubera, AERx insulin diabetes management system, AIR inhaled insulin, oralin, insulin detemir and insulin glulisine. Peroxisome proliferator-activated receptor-gamma agonists include rosiglitazone, pioglitazone, isaglitazone, rivoglitazone, T-131, MBX-102 and R-483 CLX-0921. Dual PPAR agonists and PPAR pan agonists include BMS-398585, tesaglitazar, muraglitazar, naveglitazar, TAK-559, netoglitazone, GW-677594, AVE-0847, LY-929 and ONO-5129. Combination therapies include metformin/glyburide, metformin/rosiglitazone, metformin and glipizide. Meglitinides include repaglinide or nateglinide. Alpha-glucosidase inhibitors include acarbose, miglitol and voglibose. GLP-1 analogues and agonists include Exenatide, Exenatide LAR, Liraglutide, CJC-1131, AVE-0010, BIM-51077, NN-2501 and SUN-E7001. DPP-IV inhibitors include LAF-237, MK-431 (Merck and Co), dipeptidyl peptidase IV (PSN-9301; Probiodrug Prosidion), 815541 (GlaxoSmithKline-Tanabe), 823093 (GlaxoSmithKline), 825964 (GlaxoSmithKline) and (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo[3.1.

0]hexane-3-carbonitrile (BMS-477118). An example of pancreatic lipase inhibitors is orlistat. SGLT inhibitors include T-1095 (Tanabe-J&J), AVE-2268 and 2-(4-methoxybenzyl) phenyl 6-O-(ethoxycarbonyl)-β-D-glucopyranoside (869682; GlaxoSmithKline-Kissei). An example of amylin analog is pramlintide.

[0248] The disclosed chemical chaperone or the disclosed ER stress modulator may be used in combination with an anti-obesity agent. The anti-obesity agent may include, but is not limited to, pancreatic lipase inhibitors, serotonin and norepinephrine reuptake inhibitors, noradrenergic anorectic agents, peripherally acting agents, centrally acting agents, thermogenic agents, cannabinoid CB1 antagonists, cholecystokinin (CCK) agonists, lipid metabolism modulator, glucagon-like peptide 1 agonist, leptin agonist, beta-3 adrenergic agonists, peptide hormone, CNS modulator, neurotrophic factor, 5HT2C serotonin receptor agonist, methamphetamine HCl, 1426 (Sanofi-Aventis), 1954 (Sanofi-Aventis), c-2624 (Merck & Co), c-5093 (Merck & Co), and T71 (Tularik).

[0249] As previously mentioned, pancreatic lipase inhibitors may include orlistat. An example of serotonin and norepinephrine reuptake inhibitors is sibutramine. Noradrenergic anorectic agents include phentermine and mazindol. Peripherally acting agents include 2-(Hexadecyloxy)-6-methyl-4H-3,1-benzoxazin-4-one (ATL-962; Alizyme), HMR-1426 (Aventis) and G1-181771 (GlaxoSmithKline). Centrally acting agents include Recombinant human ciliary neurotrophic factor, 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide (Rimonabant (SR-141716; Sanofi-Synthelabo)), BVT-933 (GlaxoSmithKline/Biovitrum), (±)-2-(tert-Butylamino)-1-(3-chlorophenyl)propan-1-one (Bupropion SR; GlaxoSmith-Kline) and P-57 (Phytopharm). An example of thermogenic agents is TAK-677 (AJ-9677) (Dainippon/Takeda). Cannabinoid CB1 antagonists include acomplia and SLV319. An example of CCK agonists is GI 181771 (GSK). An example of lipid metabolism modulator is AOD$_{9604}$ (Monash University/Metabolic Pharmaceuticals). An example of glucagon-like peptide 1 agonist is AC137 (Amylin). An example of leptin agonist is second generation leptin (Amgen). Beta-3 adrenergic agonists include (7S)-5,6,7,8-Tetrahydro-7-[[(R)-2-hydroxy-2-(3-chlorophenyl)ethyl]amino]-2-[(ethoxycarbonyl)methoxy]naphthalene (SR58611; Sanofi-Aventis), CP 331684 (Pfizer), LY 377604 (Eli Lilly) and n5984 (Nisshin Kyorin Pharmaceutical). An example of peptide hormone is peptide YY [3-36] (Nastech). An example of CNS modulator is S2367 (Shionogi & Co. Ltd.). An example of neurotrophic factor is peg axokine. An example of 5HT2C serotonin receptor agonist is APD356.

[0250] The disclosed chemical chaperone or the disclosed ER stress modulator may be used in combination with an anti-dyslipidemia agent or anti-atherosclerosis agent. The anti-dyslipidemia agent or anti-atherosclerosis agent may include, but is not limited to, HMG-CoA reductase inhibitors, fibrates, bile acid sequestrants, niacin (immediate and extended release), anti-platelets, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor antagonists, acyl-CoA cholesterol acetyltransferase (ACAT) inhibitors, cholesterol absorption inhibitors, nicotinic acid derivatives, cholesterol ester transfer protein (CETP) inhibitors, microsomal triglyceride transfer protein (MTTP) inhibitors, other cholesterol modulators, bile acid modulators, peroxisome proliferation activated receptor (PPAR) agonists, gene-based therapies, composite vascular protectant, 4,6-Di-tert-

butyl-2,3-dihydro-2,2-dipentyl-5-benzofuranol (BO-653; Chugai), glycoprotein inhibitors, aspirin and analogs thereof, combination therapies, ileal bile acid transporter (IBAT) inhibitors, squalene synthase inhibitors, monocyte chemoattractant protein (MCP-1) inhibitors, liver X receptor agonists, and other new approaches.

[0251] HMG-CoA reductase inhibitors include atorvastatin, pravastatin, simvastatin, lovastatin, fluvastatin, cerivastatina, rosuvastatin and pitivastatin. Fibrates include ciprofibrate, bezafibrate, clofibrate, fenofibrate and gemfibrozil. Bile acid sequestrants include cholestyramine, colestipol and colesevelam. Anti-platelets include aspirin, clopidogrel and ticlopidine. ACE inhibitors include ramipril and enalapril. Angiotensin II receptor antagonists include losartan and potassium. ACAT inhibitors include avasimibe, eflucimibe, CS-505 (Sankyo and Kyoto), SMP-797 (Sumito). Cholesterol absorption inhibitors include ezetimibe and pamaqueside. An example of nicotinic acid derivatives is nicotinic acid. CETP inhibitors include CP-529414 (Pfizer), JTT-705 (Japan Tobacco), CETi-1 and torcetrapib. MTTP inhibitors include implitapide, R-103757 and CP-346086 (Pfizer). Other cholesterol modulators include NO-1886 (Otsuka/TAP Pharmaceutical), CI-1027 (Pfizer) and WAY-135433 (Wyeth-Ayerst). Bile acid modulators include GT102-279 (GelTex/Sankyo), HBS-107 (Hisamitsu/Banyu), BTG-511 (British Technology Group), BARI-1453 (Aventis), S-8921 (Shionogi), SD-5613 (Pfizer) and AZD-7806 (AstraZeneca). PPAR agonists include Tesaglitazar (AZ-242) (AstraZeneca), Netoglitazone (MCC-555) (Mitsubishi/Johnson & Johnson), (2S)-3-[4-[2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy] phenyl]-2-[(4-oxo-4-phenylbutan-2-yl)amino]propanoic acid (GW-409544; Ligand Pharmaceutical s/Glaxo Smith-Kline), 2-[2-methyl-4-([4-methyl-2-[4-(trifluoromethyl) phenyl]thiazol-5-yl]methylthio)phenoxy)acetic acid (GW-501516; Ligand Pharmaceuticals/GlaxoSmithKline), LY-929 (Ligand Pharmaceuticals and Eli Lilly), 2-methyl-2-[4-[2-[5-methyl-2-(4-phenylphenyl)-1,3-oxazol-4-yl]ethoxy]phenoxy]propanoic acid (LY-465608; Ligand Pharmaceuticals and Eli Lilly), 2-methyl-2-[4-[3-[1-[(4-methylphenyl)methyl]-5-oxo-2H-1,2,4-triazol-3-yl]propyl]phenoxy]propanoic acid (LY-518674; Ligand Pharmaceuticals and Eli Lilly) and MK-767 (Merck and Kyorin). Gene-based therapies include AdGVVEGF121.10 (GenVec), ApoA1 (UCB Pharma/Groupe Fournier), EG-004 (Trinam) (Ark Therapeutics) and ATP-binding cassette transporter-A1 (ABCA1) (CV Therapeutics/Incyte, Aventis, Xenon). An example of composite vascular protectant is AGI-1067 (Atherogenics). Glycoprotein IIb/IIIa inhibitors include Roxifiban (Bristol-Myers Squibb), Gantofiban (Yamanouchi) and Cromafiban (Millennium Pharmaceuticals). Aspirin and analogs thereof include asacard, slow-release aspirin and pamicogrel. Combination therapies include niacin/lovastatin, amlodipine/atorvastatin and simvastatin/ezetimibe. An example of IBAT inhibitors is S-89-21 (Shionogi). Squalene synthase inhibitors include BMS-188494 I (Bristol-Myers Squibb), CP-210172 (Pfizer), CP-295697 (Pfizer), CP-294838 (Pfizer) and TAK-475 (Takeda). MCP-1 inhibitors include RS-504393 (Roche Bioscience) and other MCP-1 inhibitors (GlaxoSmithKline, Teijin, and Bristol-Myers Squibb). Liver X receptor agonists include GW-3965 (GlaxoSmithKline) and TU-0901317 (Tularik). Other new approaches include MBX-102 (Metabolex), NO-1886 (Otsuka) and Gemcabene (Pfizer).

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[0252] The disclosed chemical chaperone or the disclosed ER stress modulator may be used in combination with an anti-hypertension agent. The anti-hypertension agent may include, but is not limited to, diuretics, alpha-blockers, beta-blockers, Ca^{2+} channel blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin II (AT-II) antagonists, vasopeptidase inhibitors, dual neutral endopeptidase and enotheline converting enzyme (NEP/ECE) inhibitors, angiotensin vaccines, ACE/NEP inhibitors, Na^+/K^+ ATPase modulators, vasodilators, naturetic peptides, angiotensin receptor blockers (ARBs), ACE crosslink breakers, endothelin receptor agonists, combination therapies, and MC4232 (University of Manitoba/Medicure).

[0253] Diuretics include chlorthalidone, metolazone, indapamide, bumetanide, ethacrynic acid, furosemide, torsemide, amiloride HCl, spironolactone and triamterene. Alpha-blockers include doxazosin mesylate, prazosin HCl and terazosin HCl. Beta-blockers include acebutolol, atenolol, betaxolol, bisoprolol fumarate, carteolol HCl, metoprolol tartrate, metoprolol succinate, nadolol, penbutolol sulfate, pindolol, propanolol HCl, timolol maleate and carvedilol. Ca^{2+} channel blockers include amlodipine besylate, felodipine, isradipine, nicardipine, nifedipine, nisoldipine, diltiazem HCl, verapamil HCl, azelnidipine, pranidipine, graded diltiazem formulation, (s)-amlodipine and clevidipine. ACE inhibitors include benazepril hydrochloride, captopril, enalapril maleate, fosinopril sodium, lisinopril, moexipril, perindopril, quinapril hydrochloride, ramipril and trandolapril. AT-II antagonists include losartan, valsartan, irbesartan, candesartan, telmisartan, eprosartan, olmesarta and YM-358 (Yamanouchi). Vasopeptidase inhibitors include omapatrilat, gemopatrilat, fasidotril, sampatrilat, AVE 7688 (Aventis), M100240 (Aventis), Z13752A (Zambon/GSK) and 796406 (Zambon/GSK). NEP/ECE inhibitors include SLV306 (Solvay), NEP inhibitors such as ecadotril, aldosterone antagonists such as eplerenone, renin inhibitors such as Aliskiren (Novartis), SPP 500 (Roche/Speedel), SPP600 (Speedel) and SPP 800 (Locus/Speedel). An example of angiotensin vaccines is PMD-3117 (Protherics). ACE/NEP inhibitors include AVE-7688 (Aventis) and GW-660511 (Zambon SpA). Na^+/K^+ ATPase modulators include PST-2238 (Prassis-Sigma-Tau) and endothelin antagonists such as PD-156707 (Pfizer). Vasodilators include NCX-4016 (NicOx) and LP-805 (Pola/Wyeth). An example of naturetic peptides is BDNP (Mayo Foundation). An example of ARBs is pratosartan. An example of ACE crosslink breakers is alagebrium chloride. Endothelin receptor agonists include tezosentan (Genentech), ambrisentan (Myogen), BMS 193884 (BMS), sitaxsentan (Encysive Pharmaceuticals), SPP301 (Roche/Speedel), Darusentan (Myogen/Abbott), J104132 (BanyulMerck & Co.), TBC3711 (Encysive Pharmaceuticals) and SB 234551 (GSK/Shionogi). Combination therapies include benazepril hydrochloride/hydrochlorothiazide, captopril/hydrochlorothiazide, enalapril maleate/hydrochlorothiazide, lisinopril/hydrochlorothiazide, losartan/hydrochlorothiazide, atenolol/chlorthalidone, bisoprolol fumarate/hydrochlorothiazide, metoprolol tartrate/hydrochlorothiazide, amlodipine besylatelbenazepril hydrochloride, felodipine/enalapril maleate, verapamil hydrochloride/trandolapril, lercanidipine and enalapril, olmesartan/hydrochlorothiazide, eprosartanlhydrochlorothiazide, amlodipine besylate/atorvastatin and nitrendipine/enalapril.

[0254] The disclosed chemical chaperone or the disclosed ER stress modulator may be used in combination with vitamin, mineral, and other nutritional supplements.

[0255] For the avoidance of doubt, the disclosed chemical chaperone may be used in any combination thereof as disclosed above. The disclosed ER stress modulator may also be used in any combination thereof as disclosed above.

[0256] In some embodiments, there is further provided the use of an inhibitor of PERK gene, or a functional variant thereof, or an inhibitor of the activity of PERK protein, or a functional variant thereof, in the manufacture of a medicament for the treatment or prevention of insulin resistance syndrome.

[0257] The term "preventing" refers to a method of barring the organism from acquiring the abnormal condition, while the term "treating" refers to a method of alleviating or abrogating the abnormal condition in the organism.

[0258] In other embodiments, there is also provided the use of an inhibitor of PERK gene, or a functional variant thereof, or an inhibitor of the activity of PERK protein or a functional variant thereof, in the manufacture of a medicament to reduce activity of transcription factors of the FOXO family (Foxo1, 3a, 4 and 6) for the treatment or prevention of insulin resistance syndrome.

[0259] The disclosed inhibitor of PERK gene or a functional variant thereof, or an inhibitor of PERK protein, or a functional variant thereof, may be used in a pharmaceutical composition. Accordingly, in one embodiment, there is provided a pharmaceutical composition comprising an inhibitor of PERK gene or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

[0260] The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier and/or diluent and/or excipient.

[0261] As used herein, the terms "administering" or "administration", or grammatical variants thereof, refer to the delivery of the disclosed inhibitor alone or in combination with the compounds disclosed herein to an organism for the purpose of prevention or treatment of insulin resistance syndrome.

[0262] As used herein, the term "pharmaceutically acceptable carrier" refers to media generally accepted in the art for the delivery of biologically active agents, i.e. the disclosed inhibitor alone or in combination with any of the disclosed compounds in the context of the specification, to mammals, e.g. humans. Such carriers are generally formulated according to a number of factors well within the purview of those of ordinary skill in the art to determine and account for. These include, without limitation, the type and nature of the active agent, being formulated; the subject to which the agent-containing composition is to be administered; the intended route of administration of the composition; and the therapeutic indication being targeted. Pharmaceutically acceptable carriers include both aqueous and non-aqueous liquid media, as well as a variety of solid and semi-solid dosage forms. Such carriers can include a number of different ingredients and additives in addition to the active agent, such additional ingredients being included in the formulation for a variety of reasons, e.g., stabilization of the active agent, well known to those of ordinary skill in the art. Non-limiting examples of a pharmaceutically acceptable carrier are hyaluronic acid and salts thereof, and microspheres (including, but not limited to,

poly(D,L)-lactide-co-glycolic acid copolymer (PLGA), poly (L-lactic acid) (PLA), poly(caprolactone) (PCL) and bovine serum albumin (BSA)).

[0263] The term "diluent" generally refers to a substance that usually makes up the major portion of the pharmaceutical composition. Suitable diluents include sugars such as lactose, sucrose, mannitol, and sorbitol; starches derived from wheat, corn rice, and potato; and celluloses such as microcrystalline cellulose. The amount of diluent in the composition can range from about 10% to about 90% by weight of the total composition, or from about 20% to about 90% by weight, or from about 30% to about 90% by weight, or from about 10% to about 80% by weight, or from about 20% to about 80% by weight, or from about 30% to about 80% by weight.

[0264] The term "excipient" refers to a pharmaceutically acceptable additive, other than the active ingredient, included in a formulation and having different purposes depending, for example, on the nature of the drug, and the mode of administration. Examples of excipients include, without limitation, carriers, co-solvents, stabilizing agents, solubilizing agents and surfactants, buffers, antioxidants, tonicity agents, bulking agents, lubricating agents, emulsifiers, suspending or viscosity agents, antibacterial agents, chelating agents, preservatives, sweeteners, perfuming agents, flavoring agents, administration aids, and combinations thereof. Some of the excipients or additives may have more than one possible function or use, depending on their properties and the nature of the formulation.

[0265] Suitable routes of administration may include, but is not limited to, oral, rectal, transmucosal or intestinal administration or intramuscular, subcutaneous, intramedullary, intrathecal, direct intraventricular, intravenous, intravitreal, intraperitoneal, intranasal, or intraocular injections.

[0266] The pharmaceutical composition may be in the forms normally employed, such as tablets, capsules, powders, syrups, solutions, suspensions and the like, may contain flavorants, sweeteners etc. in suitable solid or liquid carriers or diluents, or in suitable sterile media to form injectable solutions or suspensions. Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, drageemaking, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0267] Pharmaceutical compositions for use in accordance with the present invention may be formulated in any conventional manner using one or more physiologically acceptable carriers comprising excipients, diluents and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The active compound will be present in such pharmaceutical compositions in the amounts sufficient to provide a desired dosage. Proper formulation is dependent upon the route of administration chosen.

[0268] Suitable routes of administration include systemic, such as orally or by parenteral administration such as subcutaneous, intramuscular, intravenous and intradermal routes. For example, for oral administration, the pharmaceutical composition may be formulated by combining the active compounds, i.e. the disclosed inhibitor alone or in combination with any of the disclosed compounds, with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, lozenges, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient. Pharmaceutical preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding other suitable auxiliaries if desired, to obtain tablets or dragee cores. Useful excipients are as outlined above and, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol, cellulose preparations such as, for example, maize starch, wheat starch, rice starch and potato starch and other materials such as gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinyl-pyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid. For parenteral administration, the active compounds can be combined with sterile aqueous or organic media to form injectable solutions or suspensions. For example, solutions in sesame or peanut oil, aqueous propylene glycol and the like can be used. The injectable solutions prepared in this manner can then be, administered intravenously, intraperitoneally, subcutaneously, or intramuscularly.

[0269] While the inhibitor of PERK gene may be any one of the inhibitors disclosed above, the present invention also contemplates other inhibitors of PERK gene. To aid in the discovery of other inhibitors of PERK gene, there is provided, in one embodiment, a method of identifying a compound that modulates expression of PERK gene in a cell, the method comprising:

[0270]   a. exposing cells expressing the PERK gene with a test compound;

[0271]   b. determining the expression level of the PERK gene in the cells which were exposed to the test compound under (a);

[0272]   c. comparing the level of expression of the PERK gene determined under (b) with the expression of the PERK gene in control cells which were not exposed to the test compound; wherein a difference in the expression level between the cells under (b) compared to the control cells identifies the compound that modulates expression of the PERK gene in a cell.

[0273] The difference in the expression level may be a positive difference. That is, the expression level of the PERK gene of the control cells is higher than the expression level of the PERK gene determined under step (b) above, indicating that the test compound is an inhibitor of the PERK gene and down-regulates the expression of the PERK gene.

[0274] Conversely, the difference in the expression level may be a negative difference. That is, the expression level of the PERK gene of the control cells is lower than the expression level of the PERK gene determined under step (b) above, indicating that the test compound does not exhibit inhibition of the PERK gene and therefore does not down-regulate the expression of the PERK gene.

[0275] The disclosed method of identifying a compound that modulates expression of PERK gene in a cell may permit the screening of potential inhibitors of the PERK gene.

[0276] While the inhibitor of PERK protein may be any one of the inhibitors disclosed above, the present invention also contemplates other inhibitors of PERK protein. To aid in the discovery of other inhibitors of PERK protein, there is provided, in one embodiment, a method of identifying a compound that modulates the amount or activity of PERK protein comprised in a cell, the method comprising:

[0277]   a. exposing cells expressing PERK protein with a test compound;

[0278] b. determining the amount or activity of PERK protein in the cells which were exposed to the test compound under (a);

[0279] c. comparing the amount or activity of PERK protein determined under (b) with the activity of PERK protein in control cells not exposed to the test compound; wherein a difference in the amount or activity of PERK protein between the cells under (b) compared to the control cells identifies the compound that modulates the amount of PERK protein in the cells.

[0280] In another embodiment, there is also provided a method of identifying a compound that modulates the amount of PERK protein comprised in a cell, the method comprising:

[0281] a. exposing cells expressing PERK protein with a test compound;

[0282] b. determining the amount of PERK protein in the cells which were exposed to the test compound under (a);

[0283] c. comparing the amount of PERK protein determined under (b) with the amount of PERK in control cells not exposed to the test compound; wherein a difference in the amount of PERK protein between the cells under (b) compared to the control cells identifies the compound that modulates the amount of PERK protein in the cells.

[0284] The difference in the expression level may be a positive difference. That is, the expression level of the PERK protein of the control cells is higher than the expression level of the PERK protein determined under step (b) above, indicating that the test compound is an inhibitor of PERK protein and down-regulates the amount or activity of the PERK protein.

[0285] Conversely, the difference in the expression level may be a negative difference. That is, the expression level of the PERK protein of the control cells is lower than the expression level of the PERK protein determined under step (b) above, indicating that the test compound does not exhibit inhibition of the PERK protein and therefore does not down-regulate the amount or activity of the PERK protein.

[0286] The disclosed method of identifying a compound that modulates the amount or activity of PERK protein in a cell may permit the screening of potential inhibitors of the PERK protein.

[0287] The activity of PERK protein may be determined by using any one of the following methods:

[0288] a. in vitro kinase assays, wherein the in vitro kinase assay is optionally using a known substrate such as the identified PERK phosphorylation sites on PERK (T980) on eIF2a (S51), and/or the novel site(s) identified in *Drosophila* FOXO (as shown in Table 1 below) or in human Foxo1 (S303);

[0289] b. a cell based assay for FOXO activity, wherein the cell based assay optionally includes luciferase assays using a FOXO reporter transgene in human or animal cells;

[0290] c. a cell based assay for nuclear localization of FOXO, such as a cell based assay referred to in Example 1 below;

[0291] d. a cell based assay based on expression of known FOXO target genes, such as a cell based assay referred to in Example 2 or Examples 7A and 7B below;

[0292] e. a cell based or in vitro assay based on PERK protein activity optionally measured by a phospho-specific antibody to the auto-phosphorylation site on PERK (T980), to the PERK site S51 on eIF2a, or to S303 on human Foxo1 (or optionally any of the other novel PERK sites identified or predicted on any of the human FOXO proteins).

[0293] In vitro kinase assays and cell based assays provide a high throughput method for screening for inhibitors of PERK protein and are suitable for an initial screen of candidate inhibitors. An in vitro kinase assay is a lab-based technique to study the activity of a kinase of interest bound to an antibody with a target substrate. A cell based assay is commonly used to refer to any assay based on some measurement of a living cell.

[0294] Known FOXO target genes are, for example, 4E-BP (a regulator of overall translation levels in cells), CCAAT/enhancer binding protein epsilon (CHOP), Bim (anti- or pro-apoptotic regulators) and Growth Arrest and DNA Damage gene (GADD45).

[0295] While there is provided inhibitors of PERK gene and inhibitors of PERK protein that are useful in the treatment or prevention of insulin resistance syndrome, the disclosed inhibitors may not be effective in certain individuals. Accordingly, to aid in predicting whether a subject is receptive to the disclosed treatment, there is provided, in one embodiment, a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises identifying and determining the PERK activity in the patient, wherein an increased PERK activity indicates that the person may be receptive for a treatment with a PERK inhibitor.

[0296] Subjects that may be receptive may also benefit from inhibitors of ER stress, which include PERK inhibitors.

[0297] The term "prognosis", or grammatical variants thereof, as used herein refers to a prediction of the probable course and outcome of a clinical condition or disease. A prognosis of a patient is usually made by evaluating factors or symptoms of a disease that are indicative of a favorable or unfavorable course or outcome of the disease. The term "prognosis" does not refer to the ability to predict the course or outcome of a condition with 100% accuracy. Instead, the term "prognosis" refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a patient exhibiting a given condition, when compared to those individuals not exhibiting the condition.

[0298] By the term "suffering from insulin resistance syndrome" is meant that the patient has already been diagnosed, or is suspected to be suffering from insulin resistance syndrome.

[0299] The increased PERK protein activity may be determined by a phospho-specific antibody to the auto-phosphorylation site on PERK protein (T980), or to the PERK phosphorylation site S51 on eIF2α, or to the PERK phosphorylation site S303 on human Foxo1 (or optionally any of the other novel PERK sites identified or predicted on any of the human FOXO proteins).

[0300] The present inventors have now found that PERK-dependent phosphorylation of FOXO drives FOXO into the nucleus, thereby leading to the conclusion that the ratio of AKT-site phosphorylation to PERK-site phosphorylation is informative.

[0301] Accordingly, in one embodiment, there is provided a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises

measuring activity of a protein kinase AKT and/or PI3Kinase activity in a subject, wherein in comparison to a control a lowered AKT activity and/or lowered PI3Kinase activity indicates that the patient may be receptive for a treatment with a PERK inhibitor.

[0302] The control may be the AKT or PI3K activity in a non-diabetic individual.

[0303] In one embodiment, there is provided a prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises measuring the relative levels of phosphorylation on the AKT site and on the PERK site(s), wherein a lower ratio of AKT site phosphorylation to PERK site phosphorylation indicates that the patient is receptive for a treatment with a PERK inhibitor.

[0304] The determination whether the ratio of AKT site phosphorylation to PERK site phosphorylation is lowered may be carried out by comparing the measured ratio with the ratio determined in a healthy non-diabetic population.

[0305] In one embodiment, there is provided a kit for use in treating or preventing insulin resistance syndrome in a patient, said kit comprises one of the following selected from the group consisting of a siRNA as disclosed herein, an antibody as disclosed herein, an organic molecule as disclosed herein and a pharmaceutical composition as disclosed herein.

[0306] In one embodiment, there is provided a kit for determining whether a patient suffering from insulin resistance syndrome is receptive for a treatment with a PERK inhibitor, wherein the kit comprises:

[0307] a. antibodies specific to one or more of the AKT phosphorylation site(s) on one or more of the human FOXO proteins; and

[0308] b. antibodies specific for one or more of the PERK phosphorylation site(s) on one or more of the human FOXO proteins or for one or more of the PERK phosphorylation site(s) on PERK protein or on eIF2a.

[0309] The antibodies may be used to determine the ratio of AKT/PERK phosphorylation on FOXO proteins. Assays include, but are not limited to, immunofluorescence and immunoblotting, or enzyme-linked immunosorbent assay (ELISA), or other immobilized antibody method based on antigen capture on samples of patient material. Other methods such as mass spectrometry are possible, but may not be practically useful in a clinical setting. The ratio of AKT/PERK phosphorylation on FOXO proteins may be compared to normal non-diabetic controls. A ratio lower than the controls would indicate that the patient may be a good candidate for treatment with a PERK inhibitor.

[0310] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and

that such modifications and variations are considered to be within the scope of this invention.

[0311] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0312] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[0313] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

[0314] The following Examples were carried out based on the fact that the insulin signaling pathway is conserved throughout the animal kingdom. It is therefore possible to perform genetic screens in *Drosophila* to identify new regulatory mechanisms. Accordingly, the following Examples demonstrate the insulin signaling mechanisms in *Drosophila*, thereby identifying corresponding mechanisms in mammals.

[0315] *Drosophila* has one FOXO gene. Through a genetic screen in *Drosophila*, the endoplasmic reticulum stress pathway (ER stress) was identified as an inducer of FOXO activity. On the other hand, ER stress has three effector pathways in mammals: activating transcription factor 6 (ATF6), IRE1 and PERK (also known as eIF2α K_3). ATF6 is an ER stress-regulated transmembrane transcription factor that activates the transcription of ER molecules. IRE1 is a ser/thr protein kinase that possesses endonuclease activity and is important in altering gene expression as a response to ER stress signals.

Example 1

[0316] This example demonstrates that ER stress induces nuclear localization of FOXO in a PERK-dependent manner. A cell-based assay was used to show that ER stress counteracted the effects of insulin signaling, thereby increasing nuclear localization of *Drosophila* FOXO.

[0317] A FOXO-green fluorescent (GFP) fusion protein was expressed in *Drosophila* S2 cells by transient transfection. The *Drosophila* cells were grown in serum free medium and deprived of insulin. The transfected cells were treated with 10 μg/ml of insulin for 30 min and the subcellular localization of FOXO-GFP was scored by obtaining images of the FOXO-GFP expressing cells. The insulin treated cells were named sample (b). A control sample (a) was obtained by having no insulin treatment to the transfected cells. In sample (c), ER stress was induced by RNA interference (RNAi) to disrupt endoplasmic-reticulum-associated-protein degradation (ERAD) machinery. The disruption of ERAD machinery was done by treatment of dsRNA to deplete HMG-CoA reductase degradation protein 3 (Hrd3), thereby inducing ER stress. Thereafter, the cells were treated with insulin 4 days after the dsRNA treatment. In sample (d), ER stress was induced by dsRNA treatment to deplete Hrd3. PERK protein

was also depleted using dsRNA treatment. Thereafter, the cells were treated with insulin 4 days after the dsRNA treatment.

[0318] The results are shown in FIG. **2**, where N means predominantly nuclear, C means predominantly cytoplasmic and CN means both nuclear and cytoplasmic. In other words, FIG. **2** shows the percentage of cells in which FOXO-GFP was found primarily in the nucleus, primarily in the cytoplasm or found in both. It can be seen in the control sample in FIG. **2**(*a*) that the FOXO-GFP protein was predominantly nuclear, while it is seen in FIG. **2**(*b*) that the location of FOXO-GFP is cytoplasmic in about 70% of cells and both nuclear and cytoplasmic in about 30% of cells. Accordingly, this evidences that insulin treatment shifts FOXO out of the nucleus and into the cytoplasm. From sample (c) in FIG. **2**(*c*), it can be seen that ER stress (Hrd3 depletion) counteracted the effect of insulin in moving FOXO-GFP out of the nucleus because FOXO activity is now both nuclear and cytoplasmic in about 70% of cells, predominantly cytoplasmic in about 25% of cells and predominantly nuclear in about 5% of cells. However from sample (d) in FIG. **2**(*d*), it can be seen that the depletion of PERK protein using PERK RNA interference even during ER stress retains FOXO predominantly in the cytoplasm in about 70% of cells and FOXO in both the nucleus and cytoplasm in about 30% of cells. Comparing N, CN and C categories of 3 independent experiments for Hrd3 depletion (sample (c)) and co-depletion of Hrd3 and PERK (sample (d)) yielded p-values of $p<0.05$ for sample (c) and $p<0.001$ for sample (d). PERK depletion had no effect alone (results not shown).

[0319] Accordingly, it is evidenced that depletion of PERK blocked the effect of ER stress on FOXO nuclear localization. It also can be concluded from the results that PERK activity is required to mediate the effects of ER stress to increase nuclear FOXO activity and suggests that PERK activity promotes FOXO activity by promoting nuclear localization of FOXO. On the other hand, the depletion of IRE1 had no effect on the in a comparable experiment (data not shown), showing that in response to ER stress, depletion of IRE1 does not affect the IRE1 effector pathway.

Example 2

[0320] This example demonstrates that PERK potentiates FOXO activity in vivo in *Drosophila*.

Example 2A

[0321] Quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to measure expression of a known FOXO target, 4E-BP (a regulator of overall translation levels in cells), in RNA samples derived from a *Drosophila* tissue expressing the nub-Gal4 transgene. This control sample is denoted as "Nub". Overexpression of an upstream activation sequence (UAS)-PERK transgene in transgenic flies was done by crossing with flies expressing a nub-Gal4 transgene and this sample is denoted as "Nub>PERK". The RNA was extracted from wing discs of wandering 3[rd] instar larvae, and treated with DNAse to eliminate genomic DNA contamination. Oligo-dT primers were used for reverse transcription. Results were normalized to Kinesin mRNA levels and to the level of the test RNAs in the nub-Gal4 control samples.

[0322] The results are shown in FIG. **3**A as fold change relative to the "Nub" control. As seen in FIG. **3**A, the levels of 4E-BP are much higher in "Nub>PERK" than in "Nub". The levels of PERK mRNA shown in FIG. **3**A are relative levels normalized to a control mRNA and show the magnitude of increase in PERK mRNA levels resulting from overexpression of the UAS-PERK gene in "Nub>PERK". It can be seen that the levels of PERK mRNA are also much higher in "Nub>PERK" than in "Nub". Accordingly, the experimental conditions in this Example positively demonstrate that the overexpression of UAS-PERK in "Nub>PERK" flies increased 4E-BP levels of about 130 fold above the baseline as compared to the "Nub" control flies. Hence, it is evidenced that overexpression of PERK protein in vivo in *Drosophila* results in an increase in expression of the FOXO target, 4E-BP, and enhances the effects of FOXO overexpression. It thus can be concluded that PERK promotes FOXO activity.

Example 2B

[0323] In this example, relative eye size was used as a measure of the effects of FOXO activity in the control of tissue growth.

[0324] The control sample were flies expressing a GMR-Gal4 transgene and is denoted as "ctrl". Depletion of PERK was accomplished by RNA interference (RNAi) and this sample is denoted as "PERK RNAi". Overexpression of an upstream activation sequence (UAS)—FOXO transgene in transgenic flies was done by crossing with the control flies and this sample is denoted as "FOXO". Depletion of PERK was accomplished by coexpression of a UAS RNAi transgene, which expresses a double strand RNA (dsRNA) sequence to target PERK for depletion under Gal4 control, together with UAS-FOXO and GMR-Gal4. This sample is denoted as "FOXO,PERK RNAi". Digital images of the eye sizes of each sample were taken using a microscope under a standardized magnification. The total area of affected eyes of each genotype sample was measured in pixels using ImageJ, a Java-based image processing program.

[0325] The results are shown in FIG. **3**B which plots the average eye area in arbitrary units including standard deviation for the samples. As seen in FIG. **3**B, when "FOXO" was compared with "FOXO,PERK RNAi", it is evidenced that depletion of PERK counteracted the effects of FOXO overexpression, as evidenced by an increase in relative eye size. However, when "ctrl" was compared with "PERK RNAi", there was no effect on relative eye size. Accordingly, it can be concluded that depletion of PERK activity in vivo in *Drosophila* counteracts the effects of excess FOXO activity. Specifically, depletion of PERK offsets the effects of FOXO overexpression, resulting in an increase in eye area ($p<0.05$, using Student's T-Test).

[0326] From Examples 2A and 2B, it can be concluded that PERK promotes FOXO activity. Further, from both Examples 1 and 2, it can likely be concluded that the promotion of FOXO activity is done by an increase in the movement of FOXO into the nucleus where it can act as a transcription factor.

Example 3

[0327] This example demonstrates that human PERK protein promotes human Foxo1 and Foxo3a activity.

[0328] FOXO proteins are transcription factors. To measure the effects of human PERK on human FOXO activity, FOXO luciferase reporters transfected into Michigan Cancer Foundation-7 (MCF-7) cells (a breast cancer cell line) were

used. The FOXO luciferase reporters determine FOXO gene expression as measured by luciferase levels.

Example 3A

[0329] In Example 3A, control cells were transfected to express a human Foxo3a firefly luciferase reporter alone, denoted as "4FRE". The control cells were further cotransfected to express Foxo3a (denoted as "+Foxo3a"), PERK (denoted as "+PERK"), or both Foxo3a and PERK together (denoted as "+Foxo3a+PERK"). A renilla luciferase reporter was co-transfected to assess transfection efficiency. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cell transfection was done using FuGENE® 6 reagents (Roche Applied Science, Germany). Cells were lysed and luciferase assays performed 3 days after transfection using Promega Dual-Luciferase® assay kit (Promega Corporation, Wisconsin, United States of America) in accordance with the manufacturer's instructions. Normalized luciferase levels of the samples were measured and the results are shown in FIG. 4A. As seen in FIG. 4A, expression of Foxo3a increased reporter gene expression as measured by an increase in luciferase levels in the sample "+Foxo3a" as compared to "4FRE" control. PERK expression alone had little effect in the sample "+PERK". However, co-expression of PERK potentiated the effects of Foxo3a in the sample "+Foxo3a+PERK".

Example 3B

[0330] In Example 3B, control cells were transfected to express a human Foxo1 luciferase reporter alone, denoted as "IRS". The control cells were further cotransfected to express Foxo1 (denoted as "+Foxo1"), PERK (denoted as "+PERK"), or both Foxo1 and PERK together (denoted as "+Foxo1+PERK"). A renilla luciferase reporter was co-transfected to assess transfection efficiency. Cells were grown in DMEM media supplemented with 10% FBS. Cell transfection was done using FuGENE® 6 reagents. Cells were lysed and luciferase assays performed 3 days after transfection. Normalized luciferase levels of the samples were measured and the results are shown in FIG. 4B. As seen in FIG. 4B, PERK expression increased reporter activity, presumably acting on endogenous Foxo1. Addition of Foxo1 increased reporter activity in the sample "+Foxo1" as compared to "IRS" control. Co-expression of PERK with Foxo1 increased reporter activity and potentiated the effect of Foxo1 on the Foxo1 luciferase reporter in the sample "+Foxo1+PERK".

[0331] From Examples 3A and 3B, it can be concluded that increased PERK activity potentiated the effects of human FOXO.

Example 4

[0332] This example demonstrates that depletion of PERK reduces FOXO activity in human AGS cells.

[0333] The expression of certain mRNAs was measured by quantitative reverse transcription polymerase chain reaction (RT-PCR). In this example, the mRNA was extracted and purified using RNeasy Mini Kit (Qiagen, Netherlands) with DNase to eliminate genomic DNA contamination. Reverse transcription to synthesize the first strand utilized oligo-dT primers. The mRNAs measured are known FOXO targets: CCAAT/enhancer binding protein epsilon (CHOP), Bim (anti- or pro-apoptotic regulators), Growth Arrest and DNA

Damage gene (GADD45) and PERK. Bip2, which is not a FOXO target, was also measured as a control. Results were normalized to Kinesin mRNA.

Example 4A

[0334] Dimethyl sulfoxide (DMSO)-treated human AGS cells were used as a control, denoted as "ctrl". The human AGS cells were grown in Roswell Park Memorial Institute (RPMI) media supplemented with 10% FBS, and treated with 10 µg/ml tunicamycin dissolved in DMSO at 1 mg/ml for 4 hours to induce ER stress. The tunicamycin treated cells are denoted as "TM".

[0335] Quantitative RT-PCR was performed to show the relative level of the indicated human mRNA targets. The results are shown in FIG. 5A where normalized levels of mRNA transcripts were measured for the "TM" cells compared to the "ctrl" cells. It can be seen from FIG. 5A that the "TM" cells had significantly higher levels of Bip2, CHOP, Bim and GADD45 as compared to the "ctrl" cells. It can be concluded that ER stress induces the expression of Bip2, CHOP, Bim and GADD45.

Example 4B

[0336] The tunicamycin treated ("TM") cells were further treated with siRNA treatment to deplete PERK from human AGS cells. The siRNA sequence used was 5'-CAAACU-GUAUAACGGUUUATT-3' (SEQ ID NO: 1). The siRNA was transfected into cells using HiPerFect reagent (Qiagen, Netherlands) for 3 days before tunicamycin treatment. The PERK-depleted cells are denoted as "PERK RNAi+TM".

[0337] Quantitative RT-PCR was performed to show the relative level of the indicated human mRNA targets. The results are shown in FIG. 5B where normalized levels of the target mRNA transcripts were measured for the "TM" cells compared with the "PERK RNAi+TM" cells. The levels of these target mRNAs in PERK-depleted ("PERK RNAi+TM") cells were normalized to the levels of tunicamycin-treated ("TM") control cells.

[0338] It can be seen from FIG. 5B that the PERK levels decreased more than 80% in the "PERK RNAi+TM" cells as compared to the "TM" cells. As previously concluded in Example 1, ER stress induces nuclear FOXO activity and that PERK promotes FOXO activity. Hence, in this example, PERK depletion down-regulates FOXO activity, thereby reducing the degree of induction of Foxo1 targets CHOP, Bim and GADD45 when comparing "PERK RNAi+TM" cells with "TM" cells. However, the levels of Bip2 transcripts were comparable between both sample cells. This is because Bip2 is not a FOXO target and therefore was not affected by the depletion of PERK.

[0339] Examples 4A and 4B thus demonstrate that reduction of PERK activity can reduce FOXO activity in humans. Accordingly, inhibition of PERK provides a novel mechanism to control FOXO activity.

Example 5

[0340] In this example, the hypothesis that PERK phosphorylates FOXO to promote nuclear entry was tested.

[0341] A *Drosophila* FOXO-GFP fusion protein was expressed in *Drosophila* cells. The cells were treated with 10 µg/ml tunicamycin for 4 hours to induce ER stress, which in turn induces PERK activity. The control cells were not treated with tunicamycin. The purified FOXO-GFP protein was then

examined by mass spectrometry to identify sites of phosphorylation that were induced by ER stress.

[0342] Table 1 below lists the results of this example. *Drosophila* FOXO amino acid residues showing increased phosphorylation in response to ER stress was identified. Specifically, the first phosphorylation site is at the amino acid serine at position 66 (S66), the second phosphorylation site is at the amino acid threonine at position 222 (T222), the third phosphorylation site is at the amino acid serine at positions 226 and 227 (S226/S227), the fourth phosphorylation site is at the amino acid serine at position 243 (S243) and the fifth phosphorylation site is at the amino acid serine at position 263 (S263).

TABLE 1

Site number	*Drosophila*
1	Serine 66
2	Threonine 222
3	Serine 226
3	Serine 227
4	Serine 243
5	Serine 263

Example 6

[0343] This example tests the functions of the identified phosphorylation sites in *Drosophila*.

[0344] The identified Serine or Threonine residues were individually mutated to Alanine in *Drosophila* FOXO and the mutant proteins were tested for ER stress induced nuclear localization. The phosphorylation sites 2, 3 and 5 mutants, labeled as "T222A", "S263A" and "S226A/S227A", behaved like the native FOXO, i.e. as T222, S263 and S226/S227 respectively. S66A (site 1) and S243A (site 4) mutant proteins were refractory to ER stress induced nuclear localization, i.e. there was no induced nuclear localization. This indicates that the presence of a phosphorylatable Serine residue at positions 66 and 243 is required for ER stress induced movement of *Drosophila* FOXO into the nucleus. The other sites do not appear to be required.

[0345] The assay described in Example 1 was repeated here, except that the predominantly nuclear (N) and both nuclear and cytoplasmic (NC) data were combined in this example. Example 1 was also repeated to test the mutant proteins expressed in *Drosophila* cells.

[0346] The results are shown in FIG. 6. This example reinforced that insulin treatment (sample denoted as "Control+insulin") promoted cytoplasmic localization of FOXO-GFP, while ER stress (sample denoted as "ER stress") counteracted the effects of insulin to promote nuclear localization. The FOXO-GFP mutant proteins "S66A" and "S243A" were treated with RNAi to disrupt ERAD machinery and to induce ER stress but were refractory to the effects of ER stress. Accordingly, ER stress did not substantially counteract the effects on insulin in "S66A" and "S243A" and thus, there was little movement from the cytoplasm into the nucleus, as compared to the "ER stress" sample. It can thus be concluded that *Drosophila* FOXO phosphorylation sites 1 and 4, i.e. S66 and 5243, are required to induce movement of *Drosophila* FOXO into the nucleus.

Example 7

[0347] In this example, the mutant phosphorylation site in human Foxo1 was validated.

[0348] Residue S303 in human Foxo1 was mutated to Alanine (corresponding to phosphorylation site 4, S243, in *Drosophila* FOXO). The assay described in Example 3B was repeated here for samples "IRS+Foxo1" and "+PERK" and was repeated for the mutant human Foxo1 (denoted as "IRS+Foxo1 S303A" and "+PERK").

[0349] Activity of human Foxo1 S303A was compared to Foxo1 using the luciferase reporter assay. The results are shown in FIG. 7. As shown in FIG. 7, "IRS+Foxo1S303A" was slightly less effective than the native Foxo1 protein "IRS+Foxo1" ($p<0.05$) when expressed on its own. When Foxo1 is co-expressed with PERK, the activity of human Foxo1 was enhanced by about 2 fold, when comparing "IRS+Foxo1" with "+PERK". However, the effect of PERK was attenuated when co-expressed with Foxo1 S303A compared to the native Foxo1 protein ($P<0.05$), evidenced by the lower levels of normalized luciferase in the "+PERK" samples.

[0350] This indicates that S303 in human Foxo1 plays an important role in mediating the effects of PERK on Foxo1 activity. The fact that PERK can still induce activity of Foxo1 S303A suggests that there may be additional sites for PERK-dependent phosphorylation.

Example 8

[0351] H1299 cells (a human non-small cell lung carcinoma cell line derived from the lymph node) were transfected to express V5-epitope tagged Foxo1 or Foxo3 as Foxo1-V5 or Foxo3-V5 for 2 days and subjected to ER stress by treatment with 10 μg/ml of tunicamycin for 4 hours in DMEM.

[0352] The V5 tagged proteins were immunopurified and examined for phosphorylation. Mass spectrometric analysis identified 8 phosphorylated sites in Foxo1 and 12 sites in Foxo3 in the ER-stress induced H1299 cells. The DNA sequences of Foxo1 and Foxo3 are shown in FIG. 8 with phosphorylated sites shown in red and the corresponding phospho-peptides are shaded in grey. The full DNA sequences of the human Foxo1 and Foxo3 gene are represented as SEQ ID NO: 2 and SEQ ID NO: 3 respectively in the sequence listing.

[0353] The cDNA of the human Foxo1 gene has an accession number of CCDS9371.1. The gene encoding the human Foxo1 protein has an accession number of Q12778. The cDNA of the human Foxo3 gene has an accession number of CCDS5068.1. The gene encoding the human Foxo3 protein has an accession number of 043524.

Example 9

[0354] The phosphorylated residues S298 in Foxo1 and S294/T296/S297 in Foxo3 map to a region of the proteins corresponding to the region of S243 in *Drosophila* FOXO.

[0355] To test its function, Serine 298 was converted to Alanine in Foxo1 ("S298A") and tested for responsiveness to PERK. H1299 cells were transfected to express natural Foxo1 ("FOXO1") or the S298A mutant version of Foxo1 ("S298A") and each were co-transfected to overexpress PERK ("FOXO1+PERK" and "S298A+PERK"). This Foxo1 luciferase reporter determines Foxo1 protein activity as measured by luciferase levels and the induction of the Foxo1 luciferase reporter was assayed.

[0356] The results are shown in FIG. 9 where normalized luciferase levels were measured for the samples. The levels of luciferase were normalized to the levels of FOXO1 alone. It can be seen from FIG. 9 that the activity of the S298A mutant form of Foxo1 ("S298A") was comparable to that of the normal intact form of Foxo1 ("FOXO 1") in the reporter assay without added PERK. However, "S298A" showed a lower response to PERK overexpression as compared to the normal Foxo1, when comparing "S298A+PERK" with "FOXO1+PERK".

[0357] It can be concluded that S298 is one of the sites on human Foxo1 that contributes to mediating the effects of PERK on Foxo1 activity.

Example 10

[0358] Human H1299 cells were transfected to express human Foxo1 ("FOXO1") and co-transfected to express PERK ("FOXO1+PERK"). The transfected H1299 cells were treated with 1 μM PERK kinase inhibitor ("FOXO1+inhibitor" and "FOXO1+PERK+inhibitor"). The PERK kinase inhibitor has the chemical formula: 5-Bromo-N_4-2-pyridinyl-N_2-[3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine. The activity of the Foxo1 luciferase reporter gene was then measured in terms of normalized IRS-luciferase levels.

[0359] The results are shown in FIG. 10. It can be seen from FIG. 10 that PERK potentiates the effect of Foxo1, when comparing "FOXO1" with "FOXO1+PERK". The PERK inhibitor did not affect the activity of Foxo1 alone, when comparing "FOXO 1" with "FOXO1+inhibitor". However, when comparing "FOXO1+PERK" with "FOXO1+PERK+inhibitor", it can be seen that the partial inhibition of PERK activity reduced the level of Foxo1 activity.

[0360] Accordingly, it can be concluded that the PERK kinase inhibitor partially inhibits PERK activity, thereby down-regulating Foxo1 activity.

Example 11

[0361] To test the effects of PERK inhibition on endogenous Foxo1 targets, the cells were serum starved to remove growth factors and treated with the PI3K inhibitor LY294002 (50 μM) to further reduce AKT activity ("serum starved+LY") for 4 hours. This treatment can be viewed as a model for extreme insulin resistance, in that it removes the capacity of the cell to respond insulin signaling by activating AKT. Control cells were grown in DMEM supplemented with 10% FBS ("control with serum"). The endogenous human FOXO targets are BIM gene (a pro-apoptotic member of the BCL-2 protein family), Cyclin G2 gene (encoding a cyclin that blocks cell cycle entry), insulin receptor substrate 2 gene (IRS-2; encoding a cytoplasmic signaling molecule that mediates effects of insulin), p27KIP1 gene (encoding a cell cycle regulatory protein), pyruvate dehydrogenase lipoamide kinase isozyme 4 gene (PDK4; encoding a protein that inhibits the pyruvate dehydrogenase complex), phosphoenolpyruvate carboxykinase 2 (PCK2; encoding a mitochondrial enzyme), p21 gene (encoding a cyclin-dependent kinase inhibitor) and insulin receptor gene (INSR). The rp132 gene (a ribosomal protein gene) and the mActin gene (encoding a muscle-specific type actin) are not FOXO targets and were used as controls.

[0362] It is to be noted that in this example, the cells were not transfected to overexpress PERK, to prove that endogenous PERK activity contributes to FOXO activation under conditions where the inhibitory effects of insulin/AKT signaling are removed.

Example 11A

[0363] The serum starved and PI3K inhibited cells were further treated with PERK kinase inhibitor 5-Bromo-N_4-2-pyridinyl-N_2— [3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine, denoted as "serum starved+LY+PERK inhibition".

[0364] The results are shown in FIG. 11A. It can be seen from FIG. 11A that reduction of AKT activity generally increased Foxo1 activity. Further, PERK inhibition generally counteracted the effects of AKT reduction, thereby reducing the target genes expression in Michigan Cancer Foundation-7 (MCF-7) cells (a breast cancer cell line).

Example 11B

[0365] Example 11A was repeated with HEPG2 hepatocyte cells instead.

[0366] The results are shown in FIG. 11B where PERK inhibition also counteracted the effects of AKT reduction and reduced the target genes expression. In contrast, PERK inhibition did not reduce the expression of rp132 and mActin as they are not FOXO target genes.

Example 12

[0367] This example demonstrates that the inhibition of PERK overcomes insulin resistance.

[0368] Resistance to insulin is a hallmark of Type 2 diabetes, and obesity-related metabolic syndrome is accompanied by an acquired insulin resistance.

Example 12A

[0369] HEPG2 liver cells were treated with 0.75 mM of palmitate, a saturated fatty acid, for 17 hours to induce insulin resistance, and the effects of PERK inhibition were assessed by monitoring Foxo1 target gene expression. The Foxo1 target genes are pyruvate dehydrogenase lipoamide kinase isozyme 4 gene (PDK4; encoding a protein that inhibits the pyruvate dehydrogenase complex) and phosphoenolpyruvate carboxykinase 1 (PCK1; encoding a mitochondrial enzyme).

[0370] The results are shown in FIG. 12A, where the data was normalized to Kinesin mRNA levels and to the level of the rp132 control mRNAs. It can be seen that palmitate treated cells had substantially higher levels of expression of the Foxo1 targets PDK4 and PCK1 (measured by quantitative real time PCR). However, the levels of rp123 and mActin, which are not Foxo1 targets, were not substantially altered.

[0371] It can thus be concluded that saturated fatty acids induces Foxo1 activity as a consequence of induced insulin resistance.

Example 12B

[0372] The palmitate treated cells were further treated by inhibiting PERK kinase inhibitor 5-Bromo-N_4-2-pyridinyl-N_2-[3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine.

[0373] The results are shown in FIG. 12B, where the data was normalized to Kinesin mRNA levels and to the level of the rp132 control mRNAs. It can be seen that PERK inhibition decreased the expression of the Foxo1 targets, PDK4 and PCK1. However, the levels of rp123 and mActin, which are not Foxo1 targets, were not substantially altered.

[0374] It can thus be concluded that a reduction of Foxo1 activity is a consequence of PERK inhibition in cells induced with insulin resistance.

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1. A method of treating or preventing insulin resistance syndrome in an animal body by administering an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

2. A method of reducing activity of transcription factors of the FOXO family (Foxo1, 3a, 4 and 6) by administering an inhibitor of protein kinase RNA-like endoplasmic reticulum kinase (PERK) gene, or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

3. The method of claim **1**, wherein the method is used for treating any one of the following conditions which are caused by insulin resistance syndrome: insulin resistance, hypertension, dyslipidemia, Type 2 diabetes or coronary artery disease.

4. (canceled)

5. The method of claim **1**, wherein the inhibitor of any of the genes referred to in any one of the preceding claims comprises at least one oligonucleotide or at least one antibody or at least one inorganic molecule or at least one organic molecule.

6. The method of claim **5**, wherein the oligonucleotide is an interfering ribonucleic acid, or PNA (protein nucleic acid) or LNA (locked nucleic acid).

7. The method of claim **6**, wherein the interfering ribonucleic acid is a small interfering ribonucleic acid (siRNA) or small hairpin ribonucleic acid (shRNA) or micro ribonucleic acid (miRNA).

8. (canceled)

9. The method of claim **7**, wherein the siRNA comprises the following sequence: 5'-CAAACUGUAUAACGGU-UUAUU-3', or functional variants thereof.

10. (canceled)

11. The method of claim **5**, wherein the organic molecule is selected from the group consisting of:

5-bromo-N4-2-pyridinyl-N2-[3-(1,2,3,6-tetrahydro-4-py-ridinyl)-1H-indol-5-yl]-2,4-pyrimidinediamine;

1-[5-(4-Amino-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-2,3-dihydro-1H-indol-1-yl]-2-[3-fluoro-5-(trifluo-romethyl)phenyl]ethanone;

1-methyl-3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-1H-pyra-zolo[3,4-d]pyrimidin-4-amine;

7-methyl-5-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(3-py-ridinyl)thieno[3,2-c]pyridin-4-amine;

1-methyl-4-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-indazol-3-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(4-py-ridinyl)thieno[3,2-c]pyridin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-pyridinyl)thieno[3,2-c]pyridin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-3-yl)thieno[3,2-c]pyridin-4-amine;

4-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-indazol-3-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(1H-pyrazol-4-yl)thieno[3,2-c]pyridin-4-amine;

7-(1-methyl-1H-pyrazol-4-yl)-3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

3-{1-[(2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

1-methyl-3-{1-[(2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

1-methyl-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-[1-(phenylacetyl)-2,3-dihydro-4H-indol-5-yl]-7-(1,2,3,6-tetrahydro-4-pyridinyl)thieno[3,2-c]pyridin-4-amine;

3-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)thieno[3,2-c]pyridin-4-amine;

3-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

3-(1-{[3-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-in-dol-5-yl)thieno[3,2-c]pyridin-4-amine;

3-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-in-dol-5-yl)thieno[3,2-c]pyridin-4-amine;

3-[1-(2-naphthalenylacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyridin-4-amine;

3-[1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-(4-pip-eridinyl)thieno[3,2-c]pyridin-4-amine;

7-{3-[(dimethylamino)methyl]phenyl}-3-[1-(pheny-lacetyl)-2,3-dihydro-1H-indol-5-yl]thieno[3,2-c]pyri-din-4-amine;

3-{1-[(2,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-4H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[2,3-d]pyrimidin-4-amine;

3-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

7-methyl-5-{1-[(2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

7-methyl-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[3,2-c]pyridin-4-amine;

3-{2-[5-(4-aminothieno[3,2-c]pyridin-3-yl)-2,3-dihydro-1H-indol-1-yl]-2-oxoethyl}benzonitrile;

3-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(2,3-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

1-methyl-3-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

7-methyl-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-methyl-5-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

1-methyl-3-(1-{[3-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

7-methyl-5-(1-{[3-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

1-methyl-3-(1-{[2-(methyloxy)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

1-methyl-3-{1-[(2,3,5-trifluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(2,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-4-yl)furo[3,2-c]pyridin-4-amine;

3-{1-[(3,5-dichlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1H-pyrazol-4-yl)thieno[3,2-c]pyridin-4-amine;

3-{1-[(3,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methyl-4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}thieno[2,3-d]pyrimidin-4-amine;

3-{1-[(3-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

3-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

3-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

1-methyl-3-{1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-chlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

5-{1-[(2,3-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2-fluoro-3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2-fluoro-5-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2-fluoro-3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-fluoro-2-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methyl-4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-chloro-4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(3-chloro-4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(3-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(2,3-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

1-(1-methylethyl)-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

2-(4-amino-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)ethanol;

5-{1-[(3,5-dimethylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(4-piperidinyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

1-ethyl-3-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methylfluro[3,2-c]pyridin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-(1-methylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

5-{1-[(3,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-methyl-5-{1-[(2,3,5-trifluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3,5-dichlorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-(3-azetidinyl)-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-N-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(4-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-methyl-5-{1-[(4-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-chloro-2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-(1-{[3-fluoro-5-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-[(methyloxy)methyl]-5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-methyl-5-{1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(1-methylethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(5-chloro-2-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(4-morpholinyl)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(3,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

phenylmethyl[2-(4-amino-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-7-yl)ethyl]carbamate;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-methylbutyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(dimethylamino)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(6-chloro-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(3-chloro-2,4-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine;

7-(2-aminoethyl)-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

4-amino-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridine-7-carbonitrile;

5-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-[4-fluoro-1-(phenylacetyl)-2,3-dihydro-1H-indol-5-yl]-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{4-fluoro-1-[(1-methyl-1H-pyrrol-2-yl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-4-fluoro-2,3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)furo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-chloro-5-fluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

5-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}furo[2,3-d]pyrimidin-4-amine;

5-(1-{[3-fluoro-5-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)furo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(4-piperidinyl)ethyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-methyl-5-{1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-(1-{[4-fluoro-3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-(3-oxetanyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-1H-indol-5-yl}-7-[2-(dimethylamino)ethyl]furo[3,2-c]pyridin-4-amine;

7-methyl-5-(1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-(3-oxetanyl)-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-[2-(4-morpholinyl)ethyl]-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine;

7-(1-methylethyl)-5-(1-{[3-(trifluoromethyl)phenyl]
acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]
pyrimidin-4-amine;

7-(3-methylbutyl)-5-(1-{[3-(trifluoromethyl)phenyl]
acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]
pyrimidin-4-amine;

4-{1-[(3-methylphenyl)acetyl]-2,3-dihydro-1H-indol-5-
yl}-1H-pyrazolo[3,4-c]pyridin-3-amine;

7-chloro-3-{1-[(2,5-difluorophenyl)acetyl]-2,3-dihydro-
1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

7-(3-azetidinyl)-5-(1-{[3-(trifluoromethyl)phenyl]
acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]
pyrimidin-4-amine;

7-(1-methyl-3-azetidinyl)-5-(1-{[3-(trifluoromethyl)phe-
nyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,
3-d]pyrimidin-4-amine;

7-[2-(dimethylamino)ethyl]-5-(1-{[3-(trifluoromethyl)
phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo
[2,3-d]pyrimidin-4-amine;

5-(4-fluoro-1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-di-
hydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-d]pyri-
midin-4-amine;

5-{4-fluoro-1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihy-
dro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyri-
midin-4-amine;

5-(4-fluoro-1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-
2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-
d]pyrimidin-4-amine;

5-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-4-fluoro-2,
3-dihydro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]
pyrimidin-4-amine;

5-(4-fluoro-1-{[4-fluoro-3-(trifluoromethyl)phenyl]
acetyl}-2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyr-
rolo[2,3-d]pyrimidin-4-amine;

3-{1-[(2,5-difluorophenyl)acetyl]-4-fluoro-2,3-dihydro-
1H-indol-5-yl}furo[3,2-c]pyridin-4-amine;

5-{4-fluoro-1-[(4-fluorophenyl)acetyl]-2,3-dihydro-1H-
indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-
amine;

4-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-
1H-indol-5-yl)-1H-pyrazolo[3,4-c]pyridin-3-amine;

1-methyl-4-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-
dihydro-1H-indol-5-yl)-1H-pyrazolo[3,4-c]pyridin-3-
amine;

7-(3-azetidinyl)-5-{1-[(2,5-difluorophenyl)acetyl]-2,3-di-
hydro-1H-indol-5-yl}-7H-pyrrolo[2,3-d]pyrimidin-4-
amine;

7-[2-(4-piperidinyl)ethyl]-5-(1-([3-(trifluoromethyl)phe-
nyl)acetyl]-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,
3-d]pyrimidin-4-amine;

7-(2-aminoethyl)-3-{1-[(2,5-difluorophenyl)acetyl]-4-
fluoro-2,3-dihydro-1H-indol-5-yl}furo[3,2-c]pyridin-
4-amine;

3-{1-[(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-2,3-dihy-
dro-1H-indol-5-yl}-1-methyl-1H-pyrazolo[3,4-d]pyri-
midin-4-amine;

5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-
1H-indol-5-yl)-1H-pyrrolo[2,3-d]pyrimidin-4-amine;

5-{4-chloro-1-[(6-methyl-2-pyridinyl)acetyl]-2,3-dihy-
dro-1H-indol-5-yl}-7-methyl-7H-pyrrolo[2,3-d]pyri-
midin-4-amine; and

5-(4-chloro-1-{[6-(trifluoromethyl)-2-pyridinyl]acetyl}-
2,3-dihydro-1H-indol-5-yl)-7-methyl-7H-pyrrolo[2,3-
d]pyrimidin-4-amine;

and salts thereof, including pharmaceutically acceptable
salts thereof.

12. (canceled)

13. siRNA directed against the nucleic acid transcribed
from the PERK gene.

14. The siRNA of claim **13** having the sequence
5'-CAAACUGUAUAACGGUUUATT-3'.

15. An antibody, or a functional variant thereof, or a frag-
ment of the antibody capable of binding to PERK protein.

16. (canceled)

17. The method of claim **1**, further comprising administra-
tion of a peroxisome proliferator-activated receptor (PPAR)
agonist and/or an anti-diabetic agent.

18. (canceled)

19. (canceled)

20. The method of claim **1**, further comprising administra-
tion of an agent used in the treatment of diabetes, obesity or
insulin resistance syndrome.

21. (canceled)

22. (canceled)

23. (canceled)

24. (canceled)

25. (canceled)

26. (canceled)

27. A pharmaceutical composition comprising an inhibitor
of PERK gene or a functional variant thereof, or an inhibitor
of PERK protein or a functional variant thereof.

28. (canceled)

29. A method of identifying a compound that modulates
expression of PERK gene in a cell, the method comprising:

a. exposing cells expressing the PERK gene with a test
compound;

b. determining the expression level of the PERK gene in the
cells which were exposed to the test compound under
(a);

c. comparing the level of expression of the PERK gene
determined under (b) with the expression of the PERK
gene in control cells which were not exposed to the test
compound; wherein a difference in the expression level
between the cells under (b) compared to the control cells
identifies the compound that modulates expression of
the PERK gene in a cell.

30. A method of identifying a compound that modulates the
amount or activity of PERK protein comprised in a cell, the
method comprising:

a. exposing cells expressing PERK protein with a test com-
pound;

b. determining the amount or activity of PERK protein in
the cells which were exposed to the test compound under
(a);

c. comparing the amount or activity of PERK protein deter-
mined under (b) with the activity of PERK protein in
control cells not exposed to the test compound; wherein
a difference in the amount or activity of PERK protein
between the cells under (b) compared to the control cells
identifies the compound that modulates the amount of
PERK protein in the cells.

31. A method of identifying a compound that modulates the
amount of PERK protein comprised in a cell, the method
comprising:

a. exposing cells expressing PERK protein with a test com-
pound;

b. determining the amount of PERK protein in the cells
which were exposed to the test compound under (a);

c. comparing the amount of PERK protein determined under (b) with the amount of PERK in control cells not exposed to the test compound; wherein a difference in the amount of PERK protein between the cells under (b) compared to the control cells identifies the compound that modulates the amount of PERK protein in the cells.

32. A prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises identifying and determining the PERK activity in the patient, wherein an increased PERK activity indicates that the person may be receptive for a treatment with a PERK inhibitor.

33. (canceled)

34. A prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises measuring activity of a protein kinase AKT and/or PI3Kinase activity in a subject, wherein in comparison to a control a lowered AKT activity and/or lowered PI3Kinase activity indicates that the patient may be receptive for a treatment with a PERK inhibitor.

35. (canceled)

36. A prognostic method for determining the receptiveness of a patient suffering from insulin resistance syndrome for a treatment with a PERK inhibitor, wherein the method comprises measuring the relative levels of phosphorylation on the AKT site and on the PERK site(s), wherein a lower ratio of AKT site phosphorylation to PERK site phosphorylation indicates that the patient is receptive for a treatment with a PERK inhibitor.

37. (canceled)

38. A kit for use in treating or preventing insulin resistance syndrome in a patient, said kit comprises one of the following selected from the group consisting of a siRNA directed against the nucleic acid transcribed from the PERK gene, an antibody, or a functional variant thereof, or a fragment of the antibody capable of binding to PERK protein and a pharmaceutical composition comprising an inhibitor of PERK gene or a functional variant thereof, or an inhibitor of PERK protein or a functional variant thereof.

39. A kit for determining whether a patient suffering from insulin resistance syndrome is receptive for a treatment with a PERK inhibitor, wherein the kit comprises:

a. antibodies specific to one or more of the AKT phosphorylation site(s) on one or more of the human FOXO proteins; and

b. antibodies specific for one or more of the PERK phosphorylation site(s) on one or more of the human FOXO proteins or for one or more of the PERK phosphorylation site(s) on PERK protein or on eIF2α.

40. (canceled)

41. (canceled)

* * * * *

江 苏 省 科 学 技 术 厅

2016 年江苏省基础研究计划（自然科学基金）等拟立项目公示

　　为深化拓展科技创新工程，建设更高水平的创新型省份，根据《江苏省科技计划项目立项工作操作规程》，经组织申报、专家评审、厅长办公会票决审定等立项程序，现将 2016 年省基础研究计划（自然科学基金）、重点研发计划（社会发展）、科技型创业企业孵育计划等拟立项目共 1891 项名单予以公示（见附件），公示时间自 2016 年 6 月 8 日至 6 月 14 日。公示期间如对项目有异议，请向我厅书面反映，凡以单位名义反映情况的材料要加盖单位公章，以个人名义反映情况的材料需具实名并附联系方式。

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　　咨询电话：

1、省基础研究计划（自然科学基金）：省科技厅社发处 孙彦 025-83363439。

2、省重点研发计划（社会发展）：省科技厅社发处 丛兴忠 025-84215986。

3、省科技型创业企业孵育计划：省科技厅高新处 张迪 025-83379768。

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电 话：025-83350162、15951610699。

附件：1. 2016年省基础研究计划（自然科学基金）拟立项目清单

2. 2016年重点研发计划（社会发展）拟立项目清单

3. 2016年省科技型创业企业孵育计划拟立项目清单

江苏省科学技术厅

2016 年 6 月 7 日

说明：公示项目名称为申报单位所填写，合同签订时将进一步予以规范。

2016年江苏省自然科学基金青年基金获得者名单

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3	邹德成	扬州大学
4	尹万健	苏州大学
5	穆蕊	苏州大学
6	何成	南京航空航天大学
7	王佳栋	江苏大学
8	郭晓倩	中国矿业大学
9	朱茂春	江苏大学
10	郭各朴	南京师范大学
11	王奎	苏州大学
12	顾宇	南京理工大学
13	王婧	南京理工大学
14	郭玉杰	南京航空航天大学
15	顾红	南京财经大学
16	王海金	南京邮电大学
17	蔡红明	南京理工大学
18	周圣高	苏州大学
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23	尚旭东	南京师范大学泰州学院
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273	白竣文	江苏大学
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