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ORIGINAL ARTICLE

Insulin activates the insulin receptor to downregulate the PTEN tumour suppressor

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Insulin and insulin-like growth factor-1 signaling have fundamental roles in energy metabolism, growth and development. Recent research suggests hyperactive insulin receptor (IR) and hyperinsulinemia are cancer risk factors. However, the mechanisms that account for the link between the hyperactive insulin signaling and cancer risk are not well understood. Here we show that an insulin-like signaling inhibits the DAF-18/(phosphatase and tensin homolog) PTEN tumour suppressor in *Caenorhabditis elegans* and that this regulation is conserved in human breast cancer cells. We show that inhibiting the IR increases PTEN protein levels, while increasing insulin signaling decreases PTEN protein levels. Our results show that the kinase region of IRβ subunit physically binds to PTEN and phosphorylates on Y27 and Y174. Our genetic results also show that DAF-2/IR negatively regulates DAF-18/PTEN during *C. elegans* axon guidance. As PTEN is an important tumour suppressor, our results therefore suggest a possible mechanism for increased cancer risk observed in hyperinsulinemia and hyperactive IR individuals.

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INTRODUCTION

Insulin and insulin-like growth factor (IGF)-1 signaling are conserved signaling pathways that have roles in various developmental and physiological processes, such as ageing, neuronal development, germline proliferation, glucose metabolism and cell size control.[1–4] However, excessive insulin signaling caused by hyperinsulinemia or overexpression of insulin receptor (IR) has been linked to cancer progression.[5] Recent epidemiological and experimental studies have demonstrated that hyperinsulinemia is a major cancer risk factor among many type 2 diabetes mellitus patients and obese individuals.[5,6] Type 2 diabetes mellitus patients who get metformin treatment, a drug that functions to sensitize insulin uptake and therefore lower insulin levels, have a 25–40% lower risk of cancer than those who get insulin therapy or drugs that stimulate insulin secretion.[7,8] Moreover, activated IR has been linked to breast cancer and poor survival.[9] However, the mechanism that accounts for the link between the hyperactive insulin signaling and cancer risk is not well understood.

Phosphatase and tensin homolog (PTEN) functions as a lipid phosphatase dephosphorylating the lipid phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) and inhibits the insulin signaling pathway by antagonizing phosphatidylinositol 3′-kinase (PI3K) function.[10] PTEN is the second most frequently mutated tumour-suppressor gene (after P53) found in cancer patients.[11] PTEN mutations predominantly affect one copy of the gene suggesting that PTEN is haploinsufficient for the prevention of certain malignancies.[10] Strikingly, the loss of PTEN expression has been observed in nearly 50% of breast cancer patients;[12] however, $<5\%$ of sporadic breast tumours contain mutations that affect the PTEN gene sequence. These findings suggest that PTEN levels are critical for tumour suppression and further substantiate the importance of the PTEN regulation in cancer progression. Phosphorylation has an important role in regulating PTEN stability at the post-translational level. Although phosphorylation on serine and threonine residues has been studied extensively,[10] recent findings suggest that phosphorylation on tyrosine residues also affects PTEN stability.[13,14]

daf-18 encodes the ortholog of PTEN in *Caenorhabditis elegans*.[15] Previously, Solari *et al.*[16] showed that human PTEN can functionally replace DAF-18 in *C. elegans*, suggesting that human PTEN and DAF-18 are functionally similar and that the regulation of PTEN is highly conserved in *C. elegans*.

Here, we report that insulin and IR are negative regulators of PTEN in both *C. elegans* and human cells. We show that IR physically associates with PTEN and suggest that IR inhibits PTEN through tyrosine phosphorylation. A mutual antagonism may exist between IR and PTEN, as we show that the PTEN tyrosine phosphatase can dephosphorylate IR. Furthermore, our genetic results show that IR and PTEN homologs in *C. elegans* (DAF-2 and DAF-18, respectively) function in axon guidance and have roles independent of the canonical AGE-1/PI3K and DAF-16/FOXO insulin signaling pathway. Our results provide mechanistic insight into why individuals with hyperinsulinemia or hyperactive IR have a higher cancer risk.

RESULTS

DAF-28/Insulin and DAF-2/IR negatively regulate DAF-18/PTEN protein levels in *C. elegans*

To identify PTEN regulators, we hypothesized that the IR is a negative regulator of PTEN. We proposed that IR could function in a dual manner to ensure high PIP3 levels, first through its

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well-known role in the activation of PI3K signaling cascade, and second by inhibiting PTEN, a lipid phosphatase that dephosphorylates PIP3 to PIP2.

As the insulin signaling pathway is highly conserved in *C. elegans* and humans,[17] we first used this well-studied model to test our hypothesis. If IR downregulates PTEN, we would expect to see an increased level of PTEN protein in mutants that lack the IR. In *C. elegans*, the *daf-2* and *daf-18* genes encode an insulin-like receptor and PTEN homolog, respectively.[15,18] The endogenous levels of DAF-18/PTEN are low; however there is a significant increase in DAF-18/PTEN protein levels in both the *daf-2 (e1370)* and *daf-2 (e1368)* IR mutants, which are kinase- and ligand-binding-deficient alleles, respectively[19] (Figure 1a). This suggests that the regulation is both kinase dependent and ligand-activation dependent.

We further investigated the role of insulin in PTEN regulation. The *C. elegans* genome encodes 40 insulin-like peptides,[20] among which, DAF-28 is one of the most well-studied ligands of the DAF-2/IR signaling pathway. *daf-28 (sa191)* mutants enter the dauer stage and have an extended lifespan, similar to *daf-2/IR* mutants.[21] We therefore chose the *daf-28/insulin (sa191)* mutants to test the regulation in this study. Immunoblots show an increased DAF-18/PTEN level, albeit not as dramatic as *daf-2/IR* mutants (Figure 1a). Taken together, these results are consistent with our hypothesis that the *C. elegans* insulin and IR downregulate DAF-18/PTEN.

To study whether this regulation is at the transcriptional level, we performed a quantitative real-time reverse transcriptase–PCR analysis and showed that the *daf-18/PTEN* transcript level is unaffected in the *daf-2/IR (e1370)* mutants, suggesting that this regulation is likely to be post transcriptional (Figure 1b). This is also consistent with several *daf-2* microarray and serial analysis of gene expression (SAGE) experiments.[22–24]

DAF-28/Insulin and DAF-2/IR negatively regulate DAF-18/PTEN in neurons and intestine

To gain insight into what tissues the DAF-28/Insulin and DAF-2/IR negatively regulate DAF-18/PTEN, we performed an *in situ* antibody staining on these mutants. Consistent with our immunoblots, we found that wild-type animals have low levels of DAF-18/PTEN and is not detected in neurons or intestinal cells.[14] In contrast, we see increased DAF-18/PTEN protein levels in the neurons and the intestine in both the *daf-2/IR* and the *daf-28/insulin* mutants (Figure 1c). Interestingly, the increased intestinal DAF-18/PTEN levels were localized to nuclei. These results further support our hypothesis that DAF-28/Insulin and DAF-2/IR negatively regulate DAF-18/PTEN.

daf-2/IR negatively regulates *daf-18/PTEN* in axon guidance

If the loss of DAF-2/IR causes an increase in DAF-18/PTEN levels, we would expect *daf-18/PTEN* mutants to genetically suppress the phenotype of *daf-2/IR* and *daf-28/insulin* mutants. Consistent with this notion, research from the Ruvkun, Kenyon and Thomas groups have shown that *daf-18/PTEN* mutants are able to suppress the *daf-2/IR* and *daf-28/insulin* mutants in the dauer-constitutive and longevity phenotypes, the two main phenotypes used in studying the *C. elegans* insulin signaling pathway.[15,25,26] Although this suppression has long been interpreted as the lack of inactivation of the PI3K and AKT, it is also consistent with our model that DAF-18/PTEN levels are increased.

To look at PTEN regulation in a different developmental process, we studied the function of these genes in axon guidance, as both *daf-2/IR* and *daf-18/PTEN* have been reported to function in axon guidance.[14,27–29] We used a mechanosensory neuron reporter (*Pmec-4::gfp)* strain to examine axon guidance in the posterior lateral microtubule (PLM) neuron.[30] In the wild-type adult worms, the PLM axon terminates in the middle of the animal near the vulva area.[31] We found that 35% of the PLM axons overextend in

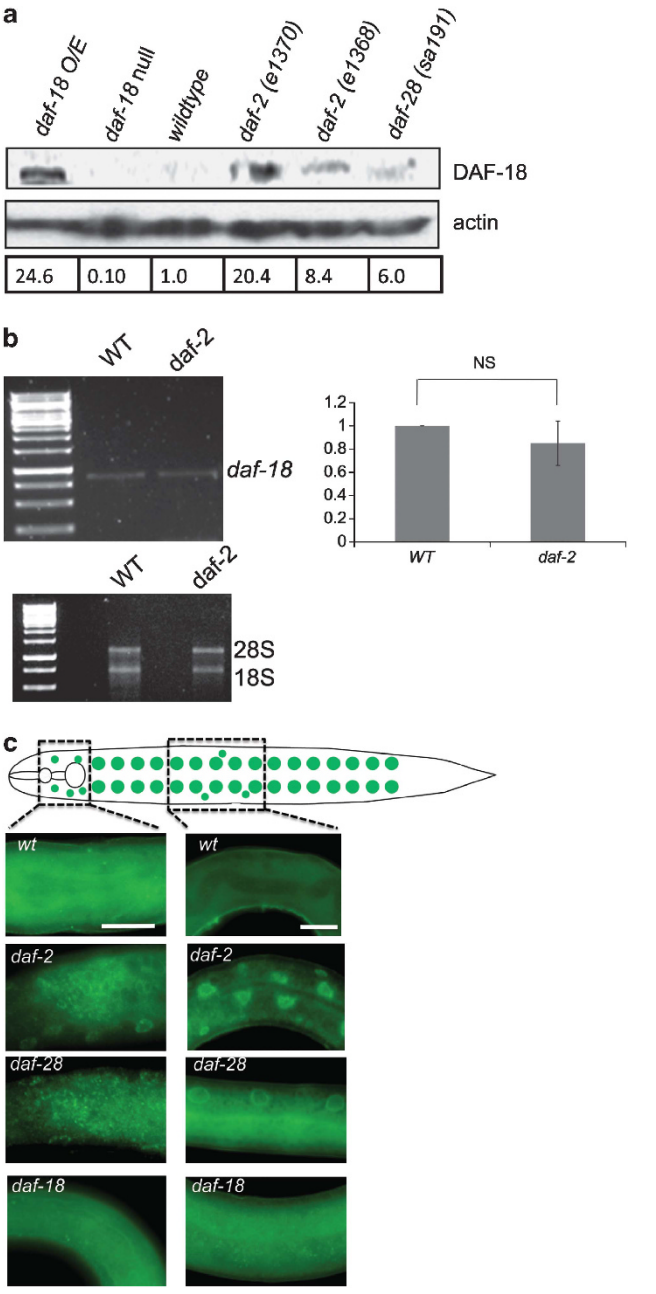

Figure 1. *daf-2/IR* and *daf-28/insulin* mutants have increased DAF-18/PTEN protein. (**a**) Immunoblot with DAF-18/PTEN antibodies detects DAF-18 protein in mixed stage worms. *daf-18* O/E (overexpression; *quIs18 genomic rescue construct*), *daf-18(ok480)* is a null allele, *daf-2(e1370)* is a kinase inactive allele, and *daf-2(e1368)* is a ligand-binding-deficient allele. Relative intensities are shown below, and wild-type (WT) DAF-18/PTEN expression was standardized as relative intensity 1.0. (**b**) The regulation of DAF-18/PTEN by DAF-2/IR is not at the transcript level. WT and *daf-2 (e1370)* have equal *daf-18* transcript levels, as determined by reverse transcriptase–PCR (left); total RNA level (28S and 18S ribosomal subunits) are shown below. *daf-18* transcript levels are not significantly different, as determined by real-time PCR (right). Three replicates were performed. Error bar represents the s.e.m. Student's *t*-test: NS, not significant. (**c**) DAF-18/PTEN antibodies do not detect endogenous levels of DAF-18, but *daf-2/IR* and *daf-28/insulin* mutants show a significant DAF-18/PTEN protein in head neurons and intestinal cells (left and right, respectively). The increased intestinal PTEN is predominantly nuclear. A schematic diagram is shown on the top of the panel and the boxed region corresponds to the region in the photo. Anterior to the left, scale bar = 30 μm.

the *daf-2/IR (e1370)* mutants (Figures 2a and e). The *daf-18(ok480)* null mutant is able to fully suppress this overextension defect (Figure 2e).

However, it is still possible that DAF-2/IR regulates axon guidance solely through the canonical PI3K/AKT pathway, and the suppression by *daf-18* mutants could be attributed to the lack of inactivation of PI3K/AKT at the PIP2/PIP3 level. To test this possibility, we studied the role of DAF-16/FOXO, a well-known downstream transcription factor in the insulin signaling, in this developmental process. If DAF-2/IR functions only through the PI3K/AKT pathway, then *daf-16/FOXO* should suppress the axon guidance defect in *daf-2/IR* mutants similar to *daf-18/PTEN*. Interestingly, *daf-16/FOXO* null mutation *daf-16(mu86)* was only able to partially suppress the *daf-2* defect (Figure 2e). In addition, the *age-1/PI3K* null mutant phenotype is weaker than the *daf-2/IR* in the PLM axon guidance (Supplementary Figure S1), suggesting that the DAF-2/IR has PI3K-independent roles. Furthermore, consistent with the genetic analysis, the increase in DAF-18/PTEN levels in the *daf-2/IR* mutants is not dependent on *daf-16* (Supplementary Figure S2). These results collectively support that DAF-2/IR has both a PI3K-dependent role, through DAF-16/FOXO, and a PI3K-independent role, through negatively regulating DAF-18/PTEN, to regulate PLM axon guidance.

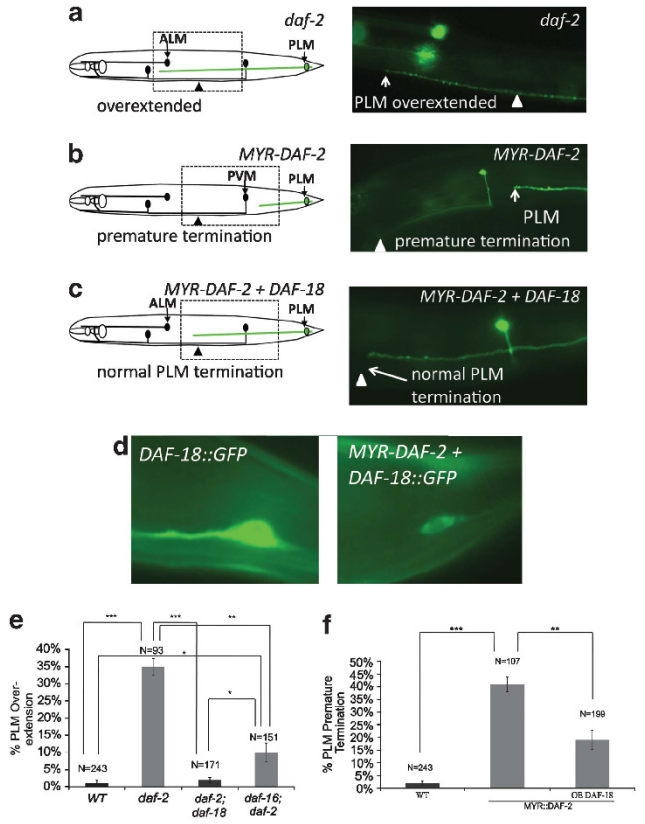

Figure 2. *daf-2/IR* and *daf-18/PTEN* have roles in axon guidance. (**a**–**c**) The PLM mechanosensory axon termination point is shown. A schematic diagram is shown on the left of each panel and the boxed region corresponds to the region in the photo. The mechanosensory neurons are visualized using *Pmec-4::gfp*. In wild-type adults, the PLM axons terminate near the vulva (triangle) region and never extend past the anterior lateral microtubule (ALM) neurons. (**a**) *daf-2/IR (e1370)* animals have PLM axons that overextend past the vulva and ALM neurons. (**b**) Constitutively active MYR-DAF-2 causes a premature termination phenotype (opposite to the reduction-of-function *daf-2/IR* mutants). (**c**) Overexpressing DAF-18/PTEN suppresses the premature termination phenotype caused by MYR-DAF-2. (**d**) Overexpression of DAF-2 decreases DAF-18/PTEN. DAF-18::GFP was expressed in the touch neurons and has bright fluorescence, PLM shown (left). MYR-DAF-2 can reduce the DAF-18::GFP expression (right); exposure settings were identical for both micrographs. (**e**) *daf-18/pten (ok480)* loss-of-function fully suppresses the overextension defect of the PLM axons of *daf-2/IR (e1370)* animals, while *daf-16/foxo (mu86)* loss-of-function partially suppresses. (**f**) Constitutively active MYR-DAF-2 causes a premature termination phenotype. Three independent transgenic lines were examined and the line with the highest penetrance is shown here. The same line was used to test for suppression by overexpressing (OE) DAF-18. Two independent overexpressing lines were examined, and combined results were graphed and subjected to statistical analysis. Overexpression of DAF-18/PTEN suppressed the premature termination defect caused by overexpressing MYR::DAF-2. N refers to the number of axons scored. WT, wild type. Error bar represents the s.e.m. Student's *t*-test: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

Overexpressing DAF-18/PTEN suppresses the axonal premature termination defect caused by overexpressing DAF-2/IR

To further confirm that DAF-2/IR has a negative influence on DAF-18/PTEN, we performed gain-of-function experiments. To cause a hyperactive DAF-2/IR, we expressed a myristoylation-tagged DAF-2 intracellular region under the *mec-4* promoter (*Pmec-4::myr-daf-2*) and observed 41% premature termination defect in the PLM neurons (Figures 2b and e). We reasoned that if this defect is caused through downregulation of DAF-18/PTEN, in addition to activating the canonical PI3K pathway, then overexpressing DAF-18/PTEN should suppress this phenotype. Consistent with this idea, overexpressing DAF-18/PTEN significantly suppressed the premature termination defect (Figures 2c and e). Taken together, both of our loss-of-function and gain-of-function experiments support the model that DAF-2/IR negatively regulates DAF-18/PTEN in PLM axon guidance.

Overexpression of DAF-2/IR decreases DAF-18::GFP in the touch neurons

We used an antibody-independent assay to confirm that DAF-2/IR downregulates DAF-18/PTEN. We expressed DAF-18::GFP under control of the *mec-4* promoter. Green fluorescent protein (GFP) expression was visible in all the touch neurons, including the PLM (Figure 2d). However, when MYR-DAF-2 was also expressed in the touch neurons, the DAF-18::GFP levels were significantly reduced, and this usually resulted in an early termination of the PLM axon, further supporting that DAF-2/IR could downregulate DAF-18 (Figure 2d). In some cases, we also observed high DAF-18::GFP expression even when MYR-DAF-2 was present. In these cases, the PLM axon grew normally and occasionally overextended (data not shown). This is also consistent with the previous result that overexpressing DAF-18 could rescue the premature termination defect caused by MYR-DAF-2.

Human insulin downregulates PTEN and is dependent on IR

We postulated that if this regulation is conserved in humans, then it could serve as one of the mechanisms that accounts for the correlation between hyperactive insulin signaling and increased cancer risk. To test this hypothesis, we first examined whether the addition of insulin could cause a decrease in PTEN. To mimic the hyperinsulinemia, we used two concentrations of insulin (100 nM and 2 μM) to stimulate MDA-MB-231 breast cancer cells, which is known for expressing a high level of IR beta subunit (IRβ).[20] A 10- or 30-min insulin treatment was sufficient to cause a significant decrease in the PTEN levels (Figures 3a and Supplementary Figures S3 and S4). We also used siRNA to knock down IRβ in MDA-MB-231 cells, and this resulted in an increased PTEN level (Figure 3b). We further applied insulin treatment to the siRNA-treated cells and found that the reduction of PTEN upon insulin treatment was prevented (Figure 3c), suggesting PTEN levels are dependent on IR signaling. Furthermore, we overexpressed the myristoylated IRβ intracellular region (MYR-IRβ) in HEK293 cells to

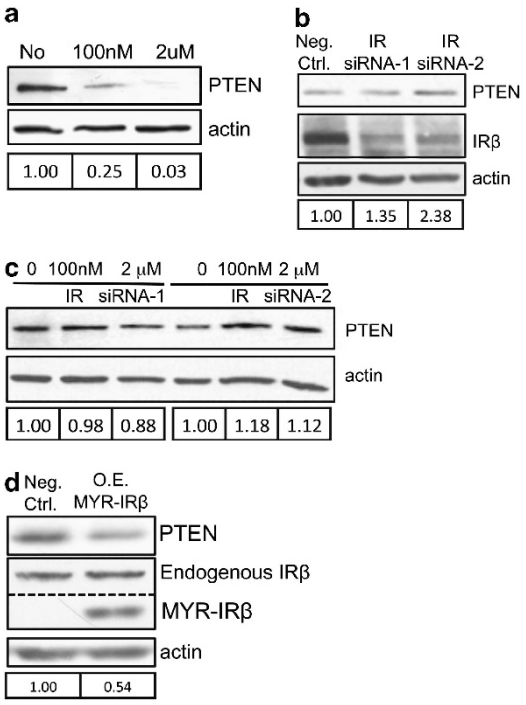

Figure 3. Human insulin signaling downregulates PTEN. (**a**) Insulin stimulation downregulates PTEN in MDA-MB-231 cell line. Two concentrations of insulin both caused a significant decrease in PTEN level. (**b**) Knocking down IRβ upregulates PTEN in MDA-MB-231 cell line. Two independent siRNA treatments were both able to knock down the expression of IRβ and increase PTEN protein level. (**c**) Downregulation of PTEN by insulin treatment is dependent on IR. (**d**) Overexpressing MYR-IRβ downregulates PTEN in HEK293 cell line. Relative intensities were shown below each blot, and no insulin treatment or negative controls (Neg ctrl) were standardized as relative intensity 1.00.

mimic the high levels of IRβ found in cancer patients. We observed a 46% decrease in the PTEN levels compared with the empty vector control (Figure 3d). These results are consistent with our finding in *C. elegans* that human insulin activates IR to downregulate PTEN.

DAF-2/IR and DAF-18/PTEN physically interact

As DAF-2/IR negatively regulates DAF-18/PTEN, we asked whether they physically bind each other. Through deletion analysis, we have determined that the DAF-2/IR kinase region was able to bind the C-terminal region of DAF-18/PTEN (aa298–962) (Figures 4a and b).

We further tested whether human IR and PTEN could physically interact. When individually expressed in bacteria as N-terminal and C-terminal fragments, these PTEN pieces are insoluble (data not shown), thereby excluding binding tests. Nonetheless, we used the full-length PTEN, which is soluble, and confirmed its binding with IR intracellular region (Figure 4c).

DAF-18/PTEN has a longer C-terminal tail than PTEN (Figure 4d), which is less conserved compared with the functional phosphatase domain. However, we did an alignment using the regions that bind DAF-2/IR (aa298–962) with PTEN and found four conserved motifs (Figures 4d and e), which might act as the binding regions to DAF-2/IR.

IR and PTEN are substrates for each other

How does DAF-2/IR exert its negative regulation on DAF-18/PTEN? Our lab previously showed that the VAB-1/Eph receptor tyrosine kinase downregulates DAF-18/PTEN through phosphorylation.[14] As a *daf-2/IR* kinase-deficient mutant has increased protein levels of DAF-18/PTEN (Figures 1a and b), we reasoned that the kinase activity is pivotal for this regulation. To test whether PTEN is an IR substrate, we performed an *in vitro* kinase assay using the active IR kinase (SignalChem, Richmond, BC, Canada) and bacterially expressed PTEN. The active IR tyrosine kinase region indeed phosphorylated PTEN. Additionally, a dual phosphatase inactive version, PTEN (C124S),[32] was strongly phosphorylated, further suggesting that wild-type PTEN may have auto-phosphatase activity or inhibits IR tyrosine kinase activity (Figure 5a).

Our results suggest that IR could phosphorylate PTEN on certain tyrosines to make it vulnerable for degradation, possibly through conformational change. If this is true, then somatic mutations of such tyrosines to negatively charged amino acids (aspartic acid or glutamic acid) might mimic the tyrosine phosphorylation and make it unstable; and we should thus expect to find such mutations in cancer patients. We searched for somatic mutations in PTEN from an online database COSMIC (Catalogue Of Somatic Mutations In Cancer; Sanger Institute, Cambridge, UK).[33] We found four tyrosines that were mutated to aspartic acids (Y27, 65, 138, 174) and among which Y27 and 174 are conserved in the worm homolog DAF-18 (Figure 5b). We mutated these two tyrosines individually to see whether the phosphorylation is decreased in the kinase assay. As IR phosphorylates phosphatase inactive PTEN (C124S) at a much stronger level than wild-type PTEN (Figure 5a), we chose to mutate candidate tyrosines in this sensitized background. Consistent with our rationale, both Y27F and Y174F decreased PTEN (C124S) phosphorylation (Figure 5c). Taken together, our results show that PTEN is a substrate for IR tyrosine kinase, and IR downregulates PTEN possibly through tyrosine phosphorylation on Y27 and 174.

PTEN has been shown to have protein tyrosine phosphatase activity, but its protein phosphatase roles are not well understood.[34–36] As IR and PTEN physically interact, we therefore tested whether IR is a protein substrate of PTEN. Similar to the kinase assay, we used active IR kinase (SignalChem) and bacterially expressed PTEN in a phosphatase assay. Our results show that wild-type PTEN could dephosphorylate IR but not the tyrosine phosphatase inactive PTEN (C124S; Figure 5d) and therefore suggest that PTEN and IR are substrates for each other and have mutual inhibitory roles on each other.

DISCUSSION

Our data support a model in which insulin activates IR to downregulate the PTEN tumour suppressor (Figure 6). We show that this regulation functions in the *C. elegans* neuronal development and also validated this negative regulation in human cell lines.

Our finding that the addition of insulin or overexpressing IR reduces PTEN protein levels is intriguing, as hyperactive insulin signaling and loss of PTEN have individually been observed in human cancers. Recent studies suggest that hyperinsulinemia or overexpression of IR is frequently found in cancers, such as breast and colon.[5] Impaired PTEN expression has been estimated at 35–50% in breast and colon cancers that do not harbour PTEN mutations.[12,37] Moreover, activated IR has been linked to breast cancer and poor survival,[9] and loss of PTEN protein is also associated with poor outcome in breast cancer.[12] On the other hand, we show that downregulating IR increases PTEN. Recent research shows that downregulation of IR inhibits cell proliferation, angiogenesis and metastasis in cancer cells and xenografts,[38] in which PTEN has also been implicated to have an important role.[10,39] Although it is beyond the scope of this study to validate this model in clinical samples, our model provides a promising mechanistic base for cancer biologists to correlate these observations in cancer patients.

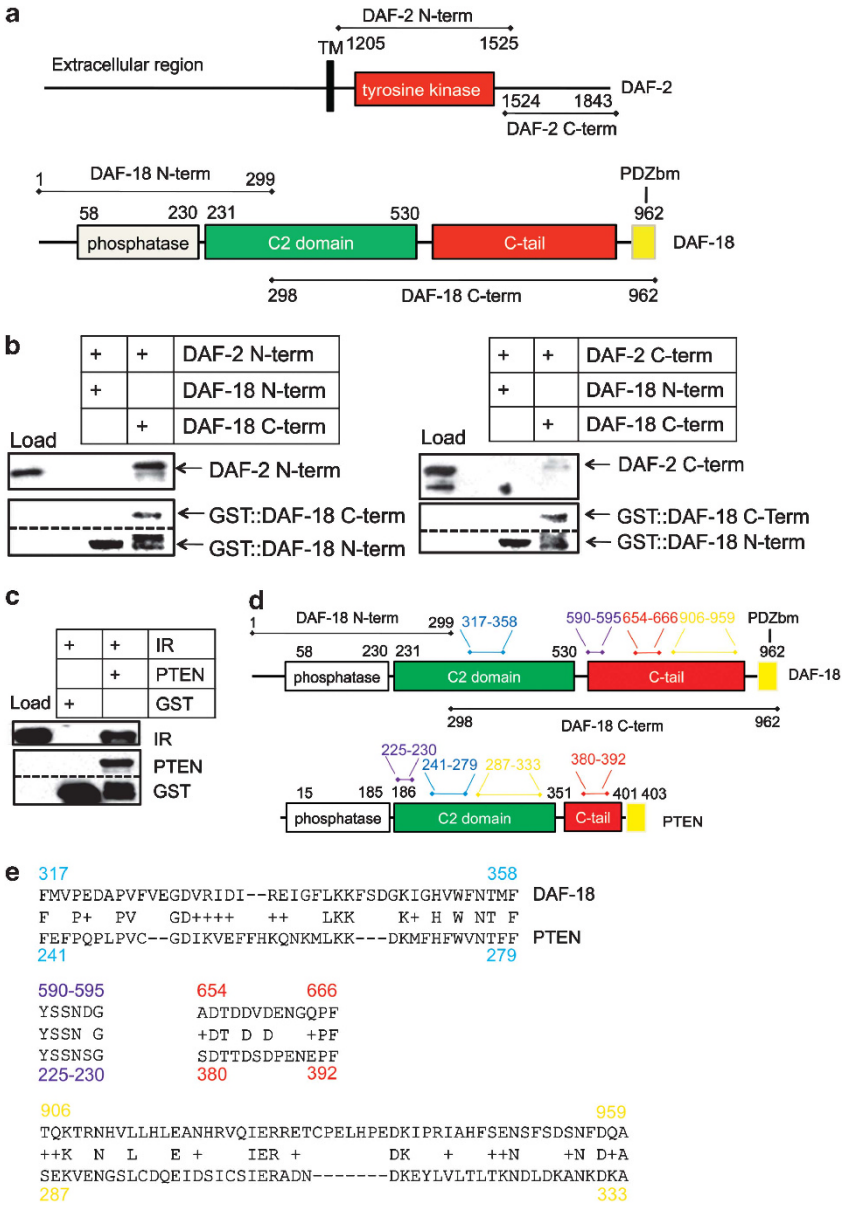

Figure 4. DAF-2/IR binds DAF-18/PTEN. (**a**) Schematic diagram of the DAF-2/IR and DAF-18/PTEN proteins and other regions tested for binding. Numbers indicate the corresponding amino acids. PDZbm, PDZ binding motif. (**b**) DAF-2 kinase region binds the DAF-18 C-terminal but not the N-terminal in GST pull-down assays. DAF-18/PTEN fragments were tagged with GST and DAF-2/IR fragments were tagged with MBP. The DAF-2 N-terminal binds specifically to the DAF-18 C-terminal end, whereas DAF-2 C-terminal does not bind DAF-18. (**c**) Human IR specifically binds GST-PTEN in the GST pull-down assay. (**d**) Alignment between DAF-18 and PTEN. The regions indicated in blue, purple, red and orange are the conserved motifs between DAF-18 and PTEN. The regions with the same colour are conserved to each other. Only the conserved motifs from DAF-18 (298–962) are indicated. (**e**) Amino-acid sequences of the conserved motifs are shown. Numbers indicate the corresponding amino acids, and the colours match the corresponding regions in panel (**d**).

Post-translational modification has an important role in regulating PTEN stability for its tumour suppression.[10] Phosphorylation on Ser/Thr has been extensively studied. Recently, research from our lab also shows that tyrosine phosphorylation in DAF-18/PTEN regulates its stability in the Eph RTK signaling pathway.[14] In the current study, we discovered that IR also downregulates PTEN through tyrosine phosphorylation and suggest that Y27 and 174 are the two key tyrosines. More importantly, these tyrosines are mutated to aspartic acid in some cancer patients.[33] This is consistent with our model that IR phosphorylates PTEN on certain tyrosines to make it susceptible for degradation, as the negatively charged aspartic acid mimics the phosphorylation. Similar to various Ser/Thr phosphorylation, in which both stabilization and destabilization effects have been reported,[10] research from the Lin Lab shows that Rak tyrosine kinase phosphorylates Y336 and stabilizes PTEN.[13] Interestingly, we also found somatic mutation of Y336 to phenylalanine, which is structurally similar to tyrosine but cannot be phosphorylated, in some cancer patients in the COSMIC database (unpublished observation). This suggests that PTEN tyrosine phosphorylation may also help to stabilize PTEN and thus serves as an important post-translational modification to regulate PTEN levels in the cell.

Our research in *C. elegans* suggests that DAF-2/IR and DAF-18/PTEN have PI3K-independent functions in PLM axon guidance. Human PTEN also has roles independent of the canonical PI3K/AKT signaling pathway, for example, in the nucleus.[40]

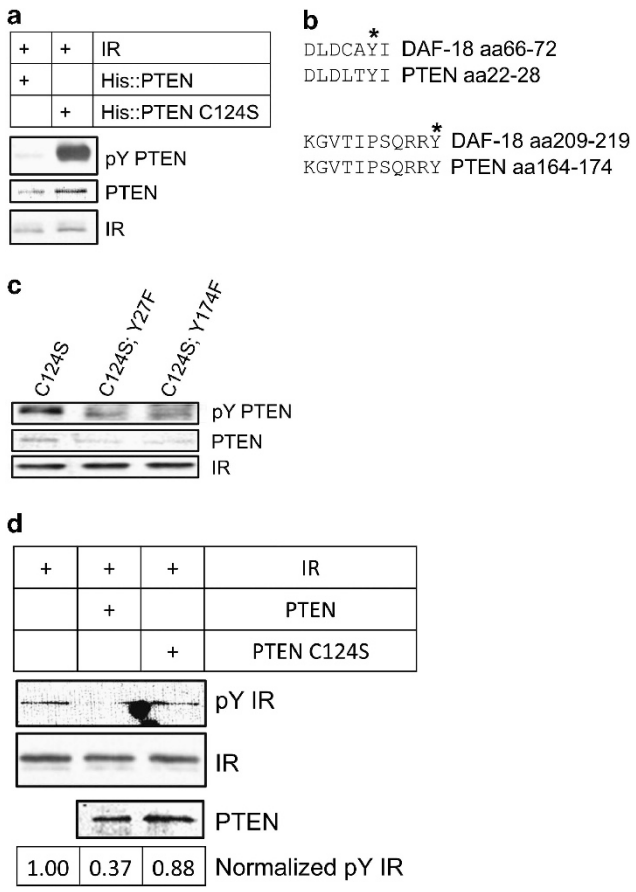

Figure 5. IR and PTEN are substrates for each other. (**a**) Human IR phosphorylates PTEN *in vitro*. Anti-phosphotyrosine antibodies (4G10) were used to probe tyrosine phosphorylation status (pY). IR weakly phosphorylates wild-type PTEN but strongly phosphorylates a phosphatase inactive PTEN (C124S). Relative protein amounts are shown below the blot. (**b**) Y27 and Y174 are conserved in DAF-18 and PTEN. Asterisks indicate the two conserved tyrosines. (**c**) Y27F and Y174F decrease tyrosine phosphorylation by IR. (**d**) PTEN dephosphorylates IR *in vitro*. Activated IR was incubated with wild-type PTEN or phosphatase inactive PTEN C124S.

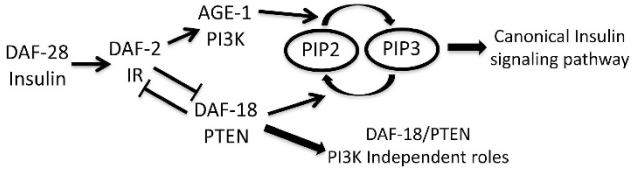

Figure 6. Working model. DAF-28/Insulin activates DAF-2/IR to implement dual functions. One function is to activate the canonical AGE-1/PI3K pathway to synthesize more PIP3, and the other is to phosphorylate and downregulate DAF-18/PTEN to prevent the dephosphorylation of PIP3. In addition, PTEN has roles independent of PI3K (for example, in the nucleus), therefore insulin/IR also has a direct role in regulating the PI3K-independent PTEN functions. PTEN dephosphorylates IR, thus these two enzymes are substrates for each other and are mutually antagonistic.

Consistent with this, our immunohistochemistry results show that inhibiting insulin signaling increased nuclear DAF-18/PTEN. Moreover, research from our lab and the Hajnal Lab suggest that DAF-18/PTEN regulates mitogen-activated protein kinase in oocyte maturation and vulva development independent of canonical PI3K/AKT pathways.[14,41] DAF-18/PTEN was also shown to have roles independent of DAF-16/FOXO as DAF-2/IR regulates the nuclear localization of SKN-1/Nrf2 transcription factor, which is dependent on DAF-18/PTEN but not on DAF-16/FOXO.[24]

Recently, the importance of PTEN protein phosphatase activity has been correlated to tumour suppression independent of PI3K and AKT.[36] Thus our model expands insulin/IR to a role in regulating PTEN function independent of PI3K pathways and also extends the traditional holding that PTEN antagonizes IR signaling only through its lipid-phosphatase activity.

Although numerous studies have shown that insulin may act through IR to affect tumour progression,[42,43] we cannot rule out the possibility that IGF-1 and IGF1-R might downregulate PTEN in a similar fashion as proposed for insulin and IR. Also, IR has been shown to form hetero-receptor complexes with IGF receptor, which could be activated by other insulin-like growth factors as well.[44] Taken together, our results suggest a direct negative regulation of PTEN by insulin and IR. Although this is certainly not the sole mechanism that accounts for the higher cancer risk found in hyperinsulinemia or IR overexpressing individuals, our model provides a promising foothold that cancer biologists should consider when designing drugs for targeted cancer therapy.

MATERIALS AND METHODS

Genetics and culture conditions

All strains were cultured and manipulated at 20 °C as described by Brenner,[45] unless otherwise noted. We used N2 Bristol as the wild-type strain. Mutants used in this study were as follows: LGI: *zdls5 [Pmec-4::GFP]* (gift of S. Clark);[30] *daf-16 (m26, mu86)*; LGIII: *daf-2 (e1368, e1370)*; LGIV: *daf-18 (ok480)*; and LGV: *daf-28 (sa191)*. Not mapped: *quIs18 (daf-18 genomic overexpressing line; pRF4)*.[14]

Constructs and transgenic lines

To generate *Pmec4::myr-daf-2*, we incorporated the c-Src N-terminal myristoylation (MYR) signal (MGSSKS) using a PCR-based approach. A 1.9-kb cDNA encoding the intracellular region of *daf-2* was fused in frame to myristoylation signal and cloned into a *Pmec-4*-expressing vector[31] to generate pIC758.This construct was co-injected with *odr-1::RFP* (both 30 ng/μl) into *mec-4::gfp(zdls5)* to generate *quEx362*. pIC176 (*Pmec-4::daf-18*) was co-injected with *Pttx-3::gfp* marker (both 30 ng/μl) into *quEx362* to generate two independent lines *quEx527* and *quEx528*. To generate *Pmec-4::daf-18::gfp*, we used fusion PCR approach to fuse *daf-18* genomic DNA in frame to *gfp* and cloned into a *Pmec-4*-expressing vector to generate pIC897. This construct was co-injected with *Pttx-3::gfp* (both 30 ng/μl) into *Pmec-4-MYR-DAF-2* (*quEx362)* to generate a strain carrying two extrachromosomal arrays *Pmec-4-MYR-DAF-2* (*quEx362) and Pmec-4-DAF-18::GFP (quEx512)*. Animals that spontaneously lost *Pmec-4-MYR-DAF-2* (*quEx362)* array were kept as *Pmec-4-DAF-18::GFP* (*quEx512)* alone.

Reverse transcription PCR and quantitative PCR assays

Mixed stage wild-type and *daf-2 (e1370)* worms were washed from unstarved plates, and total RNA was extracted using SV Total Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. First-strand cDNA was prepared from 1 μg total RNA using the Protoscript RT-PCR Kit (New England Biolabs, Ipswich, MA, USA). Region 1–853 nt, which spans introns 1 and 2, was used for *daf-18 cDNA* amplification. In addition, the real-time PCR was performed using GoTaq qPCR master mix (Promega) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Region 357–544 nt of *daf-18* cDNA, which spans intron 2 of *daf-18* genomic sequence, was amplified. Three independent samples of each genotype were tested. The ΔC_T values were determined using *cdc-42* as the internal reference, and the $\Delta\Delta C_T$ values for *daf-2 (e1370)* were calculated by comparison with the wild-type. Primer sequences are available upon request.

GST (glutathione *S*-transferase) pull-down assay

Plasmids were individually transformed into *Escherichia coli* Tuner (DE3) to induce protein expression. Generally, 1 μg of MBP (maltose-binding protein) fusion proteins and 1 μg of GST fusion proteins were mixed with 40 μl of glutathione resin beads (GST-bind, Novagen, Uppsala, Sweden) and allowed for binding for 1.5 h at 4 °C. Protein bound to beads was

washed three times (25 mM Tris-Cl (pH7.5), 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.5% Triton X-100) and then resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) followed by immnunoblot analysis. MBP fusion proteins (MBP::DAF-2 and MBP::IR) were detected by using anti-MBP conjugated to horseradish peroxidase (HRP; NEB, Ipswich, MA, USA, 1:5000) followed by enhanced chemiluminescence (Pierce; Billerica, MA, USA). GST fusion proteins (GST::DAF-18 N-terminal, GST::DAF-18 C-terminal and GST::PTEN) were detected by using anti-GST conjugated to HRP (NEB, 1:5000) followed by enhanced chemiluminescence.

Kinase assays

Active IR kinase was purchased from SignalChem. His::PTEN (aa1–350) and His::PTEN (C124S, aa1–350) were bacterially expressed and purified from Nickel column as per the manufacturer's protocol. In all, 100 ng of kinase and substrate were used with Abl kinase buffer (NEB) in each kinase reaction at 30 °C for 30 min. Samples were resolved on SDS–PAGE followed by immunoblot analysis. Anti-phospho-tyrosine was detected by using 4G10 (Millipore, Lake Placid, NY, USA, 1:2500) as primary antibody, and goat anti-mouse conjugated to HRP (1:5000) was used as secondary antibody. To show relative protein levels, PTEN (wild-type) and PTEN (C124S) were detected by Coomassie blue staining, and IR was detected using anti-IR antibodies (L55B10, Cell Signaling, Danvers, MA, USA).

Phosphatase assays

IR and His::PTEN were the same as the ones used in the kinase assay. In all, 100 ng of kinase and substrate were used with phosphatase buffer (100 mM HEPES, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, (pH 7.0)) in each phosphatase reaction at 30 °C for 2 h. Samples were resolved on SDS–PAGE followed by immunoblot analysis. Antibody detection was performed in a manner similar to kinase assay.

Immunoblot analysis using total worm lysate

Unstarved mixed stage worms were washed off from plates and mixed with equal volume of 2X SDS sample loading buffer and boiled for 5 min. Samples were resolved on SDS–PAGE followed by immunoblot analysis. DAF-18 was detected by anti-DAF-18 serum (1:250)[14] as primary antibody, and goat anti rabbit conjugated to HRP (1:10 000) was used as secondary antibody.

Cell culture

HEK293 and MDA-MB-231 cells were cultured in Dulbecco's modified media (Sigma; Oakville, ON, Canada) supplemented with 10% fetal bovine serum (Sigma) and 1% Penicillin/Streptomycin (Invitrogen; Burlington, ON, Canada).

Protein extraction and quantification

Protein was extracted in modified radio-immunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 1% NP40, 10 mM EDTA, (pH7.4)) with proteinase inhibitor and quantified using the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada).

Transfections and siRNA knockdown of IR

A total of 1×10^6 HEK293 cells were plated in six-well plates and transfected with plasmid expressing myr-IR (pIC733) or an empty vector negative control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, protein was extracted and overexpression of myr-IR was verified by immunoblotting. To transiently knock down IR, siRNA duplexes-targeted IRβ subunit were purchased from Integrated DNA technologies (Coralville, IA, USA). MDA-MB-231 cells were transfected with 10 nM siRNA-1, siRNA-2 or negative control using Lipofectamine 2000 according to the manufacturer's instructions. For the best knockdown, protein was extracted 3 days post transfection. Downregulation of IR was verified by immunoblotting.

Insulin treatment

A total of 1×10^5 MDA-MB-231 cells were plated in six-well plates. The next day 100 nM or 2 μM insulin was added to the cells and incubated for 10 or 30 min followed by protein extraction. To treat cells with downregulated IR expression, MDA-MB-231 cells were transfected with siRNA-targeting IR as described above. Two days post transfection, cells were re-plated at 1×10^5 cells/well, followed by insulin treatment and protein extraction.

Immunoblot analysis using samples from cell culture

A total of $0.5 \sim 1$ μg of proteins were resolved on SDS–PAGE, followed by immunoblot analysis. Anti-PTEN (D5G7) or (D4.3) and anti-IR (L55B10) were used as primary antibodies (Cell Signaling) in a dilution of 1:1000. Goat anti-rabbit (1:10 000) and goat anti-mouse (1:5000) conjugated to HRP were used as secondary antibodies against anti-PTEN and anti-IR, respectively. Anti-beta actin (Clone C4, MP Biomedicals, Solon, OH, USA) was used as a loading control.

Immunohistochemistry

Antibody staining on mixed stage worms was carried out as described by Chin-Sang *et al.*[46] Anti-DAF-18 antibody (1:25) was visualized with secondary fluorescein isothiocyanate-anti-rabbit antibodies (1:25; Upstate/Millipore, Billerica, MA, USA).

Phenotypic analysis on PLM axons

We used *zdls5 [Pmec-4::gfp]* as a reporter to visualize the PLM axons. In wild-type adult worms, the PLM axon terminates around the vulva area. We measured the length of the PLM axon away from the vulva, and designated '+' or '−' for the axons that terminate anterior or posterior to the vulva, respectively. We defined the range from '+80' to '−40' μm as wild-type. Longer axons were regarded as overextension defect, and shorter axons were regarded as premature termination defect. Phenotypic analysis at L1 stage was performed as described in Mohamed *et al.*[47]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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