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Signaling defects associated with insulin resistance in non-diabetic and diabetic individuals and modification by sex

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Abstract

Insulin resistance is present in one-quarter of the general population, predisposing to a wide-range of diseases. Our aim was to identify cell-intrinsic determinants of insulin resistance in this population using IPS cell-derived myoblasts (iMyos). We found that these cells exhibited a large network of altered protein phosphorylation in vitro. Integrating these data with data from type-2-diabetic iMyos revealed critical sites of conserved altered phosphorylation in IRS-1, AKT, mTOR and TBC1D1, in addition to changes in protein phosphorylation involved in Rho/Rac signaling, chromatin organization and RNA processing. There were also striking differences in the phosphoproteome in cells from males versus females. These sex-specific and insulin resistance defects were linked to functional differences in downstream actions. Thus, there are cell-autonomous signaling alterations associated with insulin resistance within the general population and important differences in males and females, many of which are shared with diabetes, and contribute to differences in physiology and disease.
Introduction

Insulin resistance is a major risk factor in the development of metabolic syndrome, type 2 diabetes (T2D) and cardiovascular disease (1-4). Indeed, the cardiometabolic syndrome currently affects 20%-30% of westernized populations, and its prevalence continues to increase worldwide with differing presentations in age and sex-specific manners (5). Although the impact of insulin resistance on glucose homeostasis and metabolic syndrome is well-studied, 20-30% of the non-diabetic people within the general population also have a significant level of insulin resistance, and the molecular determinants underlying the insulin resistance in this population remains elusive (6, 7). In individuals with a family history of T2D, insulin resistance precedes and predicts a high risk of developing the disease (4), whereas in individuals without a family history of diabetes, insulin resistance appears to be linked to increased risk for hyperlipidemia and accelerated atherosclerosis (8), but not necessarily diabetes (9).

At the cellular level, insulin signaling is initiated by ligand binding leading to conformational change and trans-autophosphorylation of the insulin receptor (IR) which leads to activation of the receptor and phosphorylation of insulin receptor substrates, such as the IRS proteins and Shc. As a result, two major downstream signaling cascades are initiated: the Ras-MAP kinase pathway and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (10, 11). Insulin signaling also activates mTORC1 to regulate protein translation and cell growth (12), stimulates glucose transport (13), inactivates glycogen synthase kinase 3 (GSK3) regulating glycogen synthesis (14), activates atypical PKCs mediating lipid metabolism (15), and leads to phosphorylation of FoxO1 and FoxK1/FoxK2 (16, 17), which serve as transcriptional regulators of insulin action. The insulin signaling events play an important role in the regulation of cellular metabolism and growth in the classical insulin-responsive tissues, such as the muscle, liver, and
adipose tissue (10, 18). Given that skeletal muscle is the largest organ in the body and the primary site of glucose disposal, skeletal muscle insulin resistance largely contributes to dysregulation of the whole-body glucose homeostasis (reviewed in (19)). The goal of the current study was to investigate the cell-autonomous determinants of insulin resistance and phosphorylation-mediated signaling within the non-diabetic population using myoblasts derived from induced pluripotent stem cells (iPSCs) in vitro.

In this study, we show that iPSC-derived myoblasts (iMyos) from non-diabetic individuals in the highest quintile of insulin resistance show defective insulin-stimulated glucose uptake as compared to iMyos from the most insulin sensitive quintile, thus replicating the in vivo insulin resistance defects in vitro. Quantitative global phosphoproteomic analysis of these cells reveals a large network of proteins whose phosphorylation is altered in association with insulin resistance in both the basal and insulin-stimulated states. A significant fraction of these alterations is also found in iMyos from T2D patients, thus defining an important core set of defects in cellular signaling in insulin resistance in both non-diabetic and diabetic individuals. Surprisingly, we also observed phosphorylation differences in the iMyos taken from the male versus female subjects, which were reflected in sex-dependent differences in downstream cellular effects. These findings provide novel insights into the cell autonomous mechanisms associated with insulin resistance and demonstrate that these differences are further modified by sex, and can be observed in vitro, in the absence of hormones and circulating extrinsic drivers of insulin resistance.
Results

Insulin resistant non-diabetics iPSCs cohort metabolic characterization

Human iPSCs and myoblasts derived from these iPSCs taken from patients with insulin receptor mutations and T2D have revealed the power of this system to identify genetic and cell-autonomous components of insulin-resistance *ex vivo*, i.e., in the absence of the multiple circulating factors that induce insulin resistance (20-23). In the present study, we have utilized this approach to study the cell autonomous determinants of insulin resistance within the general population. iPSCs from 10 individuals in the top quintile of insulin sensitivity (I-Sen) and 10 non-diabetics individuals in the bottom quintile, i.e., most insulin resistant (I-Res), previously identified by population screening using the steady-state plasma glucose approach (SSPG) (24), were used (Figure 1A). Both the I-Sen and I-Res cohorts were equally divided between males and females (Suppl. Data Figure 1A) and had an average age of ~60 (range 41-79 years) (Figure 1B). I-Res subjects had slightly higher BMIs and fasting plasma glucose levels than the I-Sen subjects (Figure 1B, C). The most striking difference, however, was the differential insulin resistance status as assessed by SSPG, with I-Res subjects having a mean steady-state plasma glucose of 216±10.3 mg/dL as compared to 69± 6.5 mg/dL in the I-Sen subjects (Figure 1C).

The iPSCs were derived from blood cells of these individuals using non-integrative Sendai virus (24) and converted to iPSC-derived myoblasts (iMyos) using a two-stage cocktail approach (25). Myogenic differentiation capacity was not affected by the insulin resistance status or sex of the donor as shown by comparable levels of MyoD1 nuclear staining (Suppl. Data Figure 1B). In addition, mRNA analysis of the iMyos showed changes in gene expression with differentiation across all cell lines, with marked downregulation of the pluripotency markers *NANOG, SOX2, DNMT3B*, and *POU5F1*, and significant upregulation of the myoblast markers *PAX7, PAX3,*
MYOG, and MYOD1 (Suppl. Data Figure 1C). Assessment of glucose uptake using 2\(^{14}\)C (U)-
deoxy-D-glucose (2-DOG) showed a ~30% increase in insulin-stimulated glucose uptake in the
I-Sen iMyos, typical of cultured myoblasts (26), and this was significantly impaired in I-Res
myoblasts (Figure 1D, and Suppl. Data Figure 1D). Western blot analysis of iMyos following
insulin stimulation revealed comparable increases in phosphorylation of IR\(\beta^{Y1150/1151}\) and
AKT\(^{S473}\) in the I-Sen and I-Res myoblasts, but significant 10-38% reduction in insulin stimulated
phosphorylation of GSK3\(\alpha/\beta^{S21/9}\) in I-Res males and females (Figure 1E, F). Taken together,
these data demonstrate that differentiated iMyos are insulin responsive and that cells from insulin
resistant individuals mirror the presence of mild to moderate insulin resistance in vitro.

**Insulin regulated phosphoproteome profile**

To identify the full spectrum of cellular signaling changes associated with the differences
in insulin sensitivity, we performed a global phosphoproteomics analysis of the I-Sen and I-Res
iMyos with or without insulin stimulation using LC-MS/MS (Suppl. Data Figure 2A). Between
14,000 and 16,000 phosphosites with a localization probability > 75% (average=97%) could be
quantitated in each of the cell lines (Suppl. Data Figure 2B). Principal component analysis of the
entire dataset demonstrated a clear separation based on two factors with Component 1 (the
largest driver of variance in the data) being the sex of the cell donor and Component 2 (the
second largest driver) being their insulin sensitivity status (Figure 2A). Interestingly, in males,
insulin resistance status shifted the relative coordinates upwards and to the left (filled squares vs.
open squares), whereas in females, insulin resistance shifted the coordinates towards the right
(filled circles vs. open circles) in the PCA plot, suggesting an interaction between insulin
sensitivity and sex on the phosphoproteome (Figure 2A).
Analysis of insulin-regulated phosphoproteome data using hierarchical clustering revealed 242 insulin-regulated phosphosites, with 186 phosphosites on 85 proteins showing increased phosphorylation upon insulin stimulation (Class I) and 56 phosphosites on 33 proteins showing decreases by insulin stimulation (Class II) (Figure 2B, Suppl. Data Table 1). Within Class I, we could identify four subclasses based on different phosphorylation patterns, as determined using a variable cut height approach (27). Class IA included 119 phosphosites which were equally upregulated by insulin stimulation in both I-Sen and I-Res males and females, as exemplified by AKT1S473 (Figure 2B, C). Class IB represented 14 insulin-stimulated phosphosites that showed enhanced phosphorylation in the I-Res iMyos when compared to I-Sen iMyos, especially in cells from males, for example STMN1S25 (Figure 2B, C). Class IC included 33 insulin-stimulated phosphosites, which were hyper-responsive in I-Res myoblasts versus I-Sen myoblasts, with the effect being dominant in cells from females, e.g., AKT1S1T246 (Figure 2B, C). Finally, Class ID represented 20 insulin-stimulated phosphosites that were hyper-responsive in the I-Sen iMyos, especially in female subjects, as exemplified by NDRG2S332 (Figure 2B, C).

Insulin downregulated phosphorylations (Class II) could also be divided into two subcategories. Class IIA included 27 phosphosites that were approximately equally down-regulated upon insulin stimulation in both I-Res males and females, for example SH3BP2S444 (Figure 2B, C). Class IIB, on the other hand, was composed of 29 insulin-stimulated phosphosites that were down-regulated by insulin in both I-Sen and I-Res myoblasts, but only in cells female subjects, e.g., SOWAHBS508 (Figure 2B, C).

Thus, in Classes I and II, i.e., the insulin up- and down-regulated sites, about two-thirds of the sites were unaffected by the insulin sensitivity status of the donor (Classes IA and IIA), while about one-third were altered by differences in insulin sensitivity (Classes IB, IC, ID and IIB),
with some of these changes seen in both males and females, and others being present in cells from donors of only one sex. Reactome pathway analysis of the phosphosites in Class I showed significant enrichment for pathways regulated by receptor tyrosine kinases (RTK), mTOR and ERK, while Class II sites were enriched in pathways related to nuclear events, muscle contraction and deubiquitination (Suppl. Data Figure 2C).

**Integrated phosphoproteomic changes in non-diabetics and type 2 diabetics**

In our previous study of iMyos from patients with insulin resistance due to type 2 diabetes, we noted that in addition to the changes in insulin stimulation, there were also significant changes in the level of basal, i.e., non-insulin stimulated, phosphorylation in iMyos from T2D versus control individuals. Indeed, this involved 732 phosphosites on 561 proteins (20). To better understand changes in phosphorylation-mediated signaling associated with insulin resistance, we sought to overlap the data from the current study of insulin resistance in the non-diabetic population with the previous study in T2D to identify common alterations, both in the insulin-stimulation ratio for each of the phosphorylation events and in the insulin-independent (basal) phosphorylation changes. Of a total of 7803 phosphosites identified in both studies, we found 389 phosphosites that showed significant changes in insulin-stimulation ratio shared in both non-diabetics and T2D, and an additional 197 sites that showed alterations in basal phosphorylation, independent of insulin stimulation, in both studies (FDR<0.05, Figure 3A). Hierarchical clustering analysis of the 389 protein phosphorylation changes of the insulin stimulation ratio revealed 214 phosphosites on 181 proteins whose ratio was up-regulated in I-Res and T2D and 175 phosphosites on 129 proteins whose stimulation-ratio was down-regulated in I-Res and T2D (FDR<0.05, Figure 3B, Suppl. Data Table 2). The up-regulated cluster was exemplified by
CHAMP1$^{S173}$ and STMN1$^{S38}$; and the down-regulated cluster, i.e., with significantly lower stimulation ratio in both I-Res and T2D, was exemplified by SRRM2$^{S876}$ and PHF3$^{S1722}$ (Figure 3C). Similarly, hierarchical clustering analysis of the 197 basal protein phosphorylation changes revealed 90 phosphosites on 70 proteins up-regulated in cells from both I-Res non-diabetics and T2D and 107 phosphosites on 76 proteins down-regulated in I-Res and T2D cells (FDR<0.05, Figure 3D, Suppl. Data Table 3). The phosphosites showing significantly higher phosphorylation in I-Res and T2D are exemplified by mTOR$^{S2481}$ and LIMCH1$^{S72}$; those significantly down-regulated in both I-Res and T2D independent of insulin stimulation are exemplified by CDK1$^{Y15}$ and SLC38A10$^{S802}$ (Figure 3E).

An integrated signaling map showing the altered sites of phosphorylation which were present in both iMyos from I-Res non-diabetic and T2D is shown in Figure 4, and the biological process (GO) analysis of these insulin resistance changes is presented in Suppl. Data Figure 3. In the signaling map, basal upregulated and downregulated phosphosites identified in both studies are shown by purple and blue text, respectively, and the insulin stimulated ratio upregulated and downregulated phosphosites are shown in red and green text, respectively.

At proximity to the insulin receptor, of the eight Ser/Thr phosphosites identified on IRS-1 in both studies, only one showed consistent alteration in insulin resistance, and this was an upregulation of basal phosphorylation at IRS-1 Ser$^{1101}$. Increased Ser$^{1101}$ phosphorylation has been observed in cells after treatment by TNFα (28) and in liver of obese mice (29). In addition, there was upregulation of basal phosphorylation of AKT Ser$^{124}$ and mTOR Ser$^{2481}$. On the other hand, insulin regulated GSK3A Ser$^{282}$ and basal TBC1D1 Ser$^{237}$ sites were downregulated. Interestingly, insulin regulated sites on FOXO3 and FOXK2 were upregulated, and phosphorylation of FoxK1 showed basal up and downregulation on multiple sites, whereas
neither of the FOXO1 phosphosites, Ser287 and 319, were consistently altered by insulin resistance.

Simultaneous analysis of both insulin resistance in iMyos of non-diabetics and T2D also identified a large network of insulin regulated and basal protein phosphorylation changes related to outside the proximal insulin signaling cascade. These included alterations in multiple regulators of Rab, Rac and Rho GTPase signaling, including changes in phosphorylation of TBC1D10A and B, ARHGAP5 and 12, and ARHGEF2 and 12, as well as many of their downstream targets involved in cytoskeleton organization. Phosphorylation of several proteins related to muscle contraction were also downregulated in the basal state. Even more striking, the phosphoproteomic analysis uncovered a novel signature of phosphorylation defects in various proteins in the nucleus. This included increased basal and insulin-regulated changes in phosphorylation of proteins related to gene expression. Similarly, phosphosites on proteins involved in DNA/chromatin organization and RNA splicing/processing showed both up- and down-regulated phosphorylation changes in the basal and/or stimulated states. Taken together, these analyses demonstrate that insulin resistance changes not only point to critical nodes of alteration in the classical insulin signaling cascade, but also demonstrate a network of phosphorylation changes in proteins related to Rab/Rho/Rac signaling, actin remodeling/cytoskeleton organization, as well as a novel nuclear signature in cells from both non-diabetics and diabetics ex vivo.

Sex-specific phosphoproteome fingerprint

PCA analysis of the phosphoproteomics data from non-diabetic individuals indicated that in addition to insulin resistance, the sex of the cell donor may be an important modulator of cell
signaling. Indeed, hierarchical clustering analysis of insulin-independent protein phosphorylation, i.e. sites whose phosphorylation was not changed following insulin stimulation, revealed striking 3,784 phosphosites that differed significantly between male and female cells (FDR<0.05) (Figure 5A, Suppl. Data Table 4), with 1,965 phosphosites on 584 proteins showing significantly higher phosphorylation in males as compared to females (Class III) and 1,455 phosphosites on 682 proteins showing higher phosphorylation in females compared to males (Class IV). There were two subclasses within each of these classes. Class IIIA was composed of phosphosites showing significantly higher phosphorylation in males than females that were not further altered by insulin resistance, as exemplified by CDC37S13 (Figure 5A, B), whereas Class IIIB consisted of phosphosites with greater phosphorylation in males than females that were also upregulated in the male by insulin-resistance, for example SRRM1T406 (Figure 5A, B).

Conversely, Class IVA represented phosphosites that were lower in males versus females, for example MARCKS S167 (Figure 5A, B). Finally, Class IVB were phosphosites that showed lower protein phosphorylation in males versus females, and this difference was magnified in insulin-resistant individuals, e.g., APAF1 Interacting Protein APIPS87 (Figure 5A, B). The sexual dimorphic patterns of phosphorylation of CDC37S13 in Class IIIA and MARCKSS167 in Class IVA were confirmed by western blotting using phosphosite-specific antibodies (Figure 5C).

Given the unexpected strong sexually dimorphic nature of changes in protein phosphorylation changes, we compared our current results to the phosphoproteomic changes in the T2D study but focusing on only the iMyos from the non-diabetic subjects that served as controls (20). Principal component analysis of the combined data again demonstrated a clear separation of the phosphoproteome with the sex of the cell donor being the strongest component in both study cohorts (Suppl. Data Figure 4A). Likewise, hierarchical clustering analysis
revealed 902 phosphosites that differed significantly between male and female cells in both cohorts (FDR<0.05), with 400 phosphosites showing significantly higher phosphorylation in females as compared to males and 502 phosphosites showing the opposite pattern (Suppl. Data Figure 4B, C). Thus, in addition to any differences associated with insulin sensitivity status, protein phosphorylation exhibits unique patterns specific to males and females, even in vitro in the absence of added sex hormones.

Reactome pathway analysis revealed a small number of highly linked biological pathways underlying the sexual dimorphism in phosphoproteomic changes (Suppl. Data Figure 5A and Figure 5D). For example, cells from both males and females showed sex-specific differences in phosphorylation of proteins involved in Rho GTPase signaling, but these occurred largely on different proteins in this pathway depending on sex. Likewise, males dominated over females in phosphorylation of proteins involved in mRNA processing/splicing versus, while females had higher phosphorylation of proteins involved in the cell cycle and DNA metabolism. Significant sex differences were also observed in proteins involved in SUMOylation versus SUMO E3 ligases and ubiquitination, and in transcriptional regulation by TP53 versus overall changes in transcription and gene expression (Figure 5D). As a result, even though similar pathways were regulated in cells of both males and females, the protein phosphorylation pattern showed sex specific differences, with only a small fraction of the proteins in these pathways showing equal phosphorylation in male and female cells (highlighted in white in the center of Figure 5D).

To identify potential upstream drivers of these sexually dimorphic phosphorylation differences we performed kinase-substrate enrichment analysis (Suppl. Data Figure 5B). Phosphosites in Class IIIA which showed higher levels of phosphorylation in male cells were potential targets of the casein kinases (CK2A2, CK2A) and the serine/threonine kinase ATR,
whereas members of the MAP kinase family (ERK1/2, P90RSK, MEK1, mTOR, ROCK1) were potential upstream drivers of Class IIIB phosphorylations, i.e., sites higher in males than females and upregulated in by insulin-resistance (Suppl. Data Figure 5B). On the other hand, sites of upregulation of protein phosphorylation in female cells in Classes IVA and IVB were predicted targets for the cyclin-dependent kinases (CDK and CDC2) and CAMK2 and MARK2 kinases, respectively (Suppl. Data Figure 5B). Again, it is important to emphasize that the sexual dimorphic differences in protein phosphorylation occurred in vitro and in the absence of sex hormones.

**Functional implications of the phosphoproteome**

Given the striking effects of insulin resistance and sex on the phosphoproteome, we assessed potential functional implications in two important pathways: the DNA damage/checkpoint pathway and the Rho GTPase pathway. DNA damage has been implicated in diabetes complications (30) and can be assessed by the number of apurinic/apyrimidinic (AP) sites in DNA, i.e. sites that have neither a purine nor a pyrimidine base (31). An important driver of the DNA damage response is the protein TP53BP1, and this protein showed higher basal phosphorylation of Ser\(^{1430}\) in females versus males and a significant reduction in phosphorylation specifically in iMyos from I-Res males (Figure 6A). This reduction in phosphorylation correlated with a significantly reduced number of AP sites in DNA in the cells of I-Res males, but not females, indicative of differences in DNA damage repair in these cells (Figure 6B). The Rho GTPase pathway, on the other hand, is involved in the cytoskeleton remodeling required for normal regulation of glucose metabolism (32). As noted above, there was altered phosphorylation of both ARHGAPs and ARHGEFs, which differed based on both sex and
insulin resistance status. Likewise, basal phosphorylation on Thr\(^{230}\) and Ser\(^{2}\) of PAK1, a Ser/Thr kinase linked to RhoA, Cdc42 and Rac1 activation, showed distinct and opposite differences in phosphorylation in cells of males and females, with a significant >50% reduction in PAK1\(^{T230}\) phosphorylation in cells from I-Res males, while cells from I-Res females showed a trend to increased PAK phosphorylation at this site, whereas PAK2\(^{S2}\) site was highly male dominant (Figure 6C). RhoA activation assessed in these same iMyos using a pull-down strategy showed differences in stoichiometry, which paralleled these phosphorylation differences. Thus, iMyos from females showed lower levels of active RhoA, which increased in the insulin resistant cohort, while male iMyos showed higher levels of RhoA activation, which decreased in the insulin resistant cohort (Figure 6D, E). Taken together, these data demonstrate how the phosphoproteomic changes are reflected in functional differences in the DNA damage response and actin cytoskeleton remodeling and are affected by both insulin resistance status and sex of the donor.
Discussion

Insulin resistance is a complex metabolic phenotype and is central to pathophysiology of a variety of diseases including obesity, T2D and metabolic syndrome. In diseases like diabetes, insulin resistance can be driven by a variety of circulating extrinsic factors, which are altered in disease pathogenesis, including free fatty acids, ceramides, cytokines, adipokines and even circulating exosomal miRNAs, which have been shown to alter both upstream and downstream aspects of insulin signaling (reviewed in (33)). Recently, we have shown that IPS cells from type 2 diabetic patients differentiated into myoblasts in vitro demonstrate insulin resistance and a large network of altered signaling, indicating T2D intrinsic or cell autonomous defects (20). Consistent with this, insulin resistance can be identified in offspring of T2D parents many years prior to disease (4). Insulin resistance is also present in a significant fraction of the general population where it has been shown to predispose to metabolic syndrome (7). In the present study, we have explored the cell autonomous determinants of insulin resistance in the general population using iMyos generated from non-diabetic individuals over the range of insulin sensitivity. Our data show that these cells exhibit large differences in their phosphoproteome based on insulin resistance status and that many of these overlap with the alterations observed in cells from T2D patients, thus highlighting key steps through which to target insulin resistance. In addition, we find that the sex of the cell donor further modifies intracellular signaling and that these changes can be reflected in differences in downstream biological responses in these cells.

Changes in phosphoproteome related to insulin resistance reveal several important categories of proteins. In the proximal insulin signaling cascade, we find increased basal phosphorylation of IRS-1 Ser1101 in cells from both non-diabetic and diabetic individuals. This site has been previously shown to be increased in phosphorylation in insulin resistance in obese
mice (29) and after treatment of cells by TNFα (28), and has been ascribed to be a target of PKCθ (28), IKK and S6 kinase (29). Increased serine/threonine phosphorylation of IRS-1 has been shown to modulate stability and tyrosine phosphorylation of the protein, thus creating feedback regulation in both physiological and pathological states (reviewed in (34)). Treatment of cells with L-citrulline reduces phosphorylation of Ser^{1101} in IRS-1 and is associated with improved insulin signaling (35).

Another consistent finding in insulin resistance in the cells from the non-diabetics and T2D patients is increased phosphorylation of AKT1 at Ser^{124} and mTOR at Ser^{2481}. Phosphorylation of AKT1 at Ser^{124} has previously been shown to regulate the extent of AKT activation independent of PI3K (36, 37). This site has also been shown to be phosphorylated by the novel PKCζ, as well as regulated by the atypical PKC-interaction protein, Par-4, leading to reduced Akt enzymatic activity (38). Ser^{2481} in mTOR is a site of autophosphorylation and associated with activation of the mTORC activity (39), a kinase which has many downstream targets affecting protein translation. In addition to its physiological roles, mTOR has been postulated to play an important role in the development of insulin resistance, via its activation by branched chain amino acids (40) and obesity (reviewed in (41)). Another important alteration is decreased phosphorylation of TBC1D1 at Ser^{237} in both non-diabetics and T2D iMyos. This site has been shown to be induced with exercise and correlate with the activity of AMPK, and thus is normally positively associated with glucose transport (42).

In addition to changes in the canonical insulin signaling cascade, there are many alterations in signaling which are conserved in cells from non-diabetic insulin resistant individuals and cells from T2D patients. These occurred in pathways involved in regulation of Rab/Rho/Rac GTPase signaling, actin/cytoskeletal organization, regulation of chromatin structure, gene expression, and
RNA processing and splicing. Among transcriptional regulators, it is interesting to note that
while no alterations in FOXO1 phosphorylation were observed in both populations of cells, there
were consistent changes in one site on FOXO3 (Ser\textsuperscript{12}), and multiple sites in FOXK1 and FOXK2
phosphorylations. FOXK1 showed downregulated basal phosphorylation of Ser\textsuperscript{213,223} and
upregulated phosphorylation of Ser\textsuperscript{416,420}, i.e., changes in the opposite direction of the
phosphorylations induced by insulin action (17). These sites are thought to be target sites of
GSK3 and mTOR and have been shown to impact on the translocation of FOXK1 in and out of
the nucleus (17, 43). Also of note were the family of ZNF transcription factors, such as ZNF106,
ZNF608, ZNF711, ZNF768, along with various other proteins involved in regulation of gene
expression, whose phosphorylation was upregulated in basal and insulin stimulated states in non-
diabetic insulin resistant and T2D iMyos.

Another interesting set of proteins whose phosphorylation was altered by insulin resistance
were proteins involved in mRNA splicing and processing. Previous studies in rodents have
shown that insulin regulates the expression of several genes encoding proteins involved in
constitutive and alternative mRNA splicing in muscle (44). Altered spliceosome function due to
down-regulation of the splicing factor SFRS10 has also been observed in liver and muscle
biopsies of obese humans (45). The altered phosphorylation network also included multiple
proteins involved in chromatic organization and DNA repair. Clinical studies have provided
evidence that pathophysiological alterations in diabetes, such as hyperglycemia,
hyperinsulinemia, advanced glycation end-products, and free fatty acids can contribute to DNA
damage including DNA strand break and base oxidation (46). In addition, we observed changes
in RhoA activation based on insulin sensitivity status and sex of the individual. It remains to be
determined how alterations in the activity of Rho signaling pathway might contribute to insulin
resistance in a sex-dependent manner in iMyos. Overall, this comprehensive map of the overlapping changes in non-diabetics and T2D in our study demonstrate large network of altered critical nodes associated with insulin resistance.

In addition to the insulin resistance-related changes, the most striking finding of this study is the unexpected differences in protein phosphorylation in cells from male versus female subjects. Indeed, we identified a network of 3,420 phosphosites in 1,143 unique proteins, which exhibited significantly differential phosphorylation based on sex, with almost 2,000 phosphosites showing higher phosphorylation in iMyos from male vs. female donors and over 1,450 sites which showed the opposite pattern. Like the insulin resistance changes, these were confirmed in a second independent cohort of cell lines from control individuals (20). Importantly, these sex-dependent differences in phosphorylation occurred in vitro in a setting in which male and female cells were in identical tissue culture media and without addition of sex hormones, thus showing a cell autonomous sex-specific phosphorylation changes. Most of the proteins involved in these differences were in a limited number of complementary pathways. Thus, male cells show enhanced phosphorylation of proteins associated with transcription, Rho GTPases, SUMOylation, mRNA splicing, and membrane trafficking, while proteins whose phosphorylation showed female dominance include those involved in cell cycle, chromatin organization, gene expression, Rho GTPase cycle, and protein ubiquitination. To our knowledge, such sexual dimorphism in signaling has not been previously reported, although recently Oliva et al. identified sex differences in the transcriptome and its genetic regulation across a range of human tissues in vivo (47).

We also found an interaction between the sex effects and the insulin resistance effects, which could be observed at the level of phosphorylation, as well in functional downstream
readouts of these pathways. In the U.S., men have a slightly higher prevalence of diabetes compared with women (14.0 vs 12.0%) (48), despite having a significantly lower prevalence of obesity (49). Consistent with this, women have higher insulin-stimulated glucose uptake in skeletal muscle in vivo as compared with men, despite lower lean mass (50). Also, weight loss yielded greater reduction in risk for diabetes in men than in women in the participants of the diabetes prevention program (DPP) (51), and sex was identified as one of the significant predictors of the progression of non-diabetic offspring of parents with T2D into prediabetes in the pathobiology of prediabetes in a biracial cohort (POP-ABC) study (52). Furthermore, impaired fasting glucose is more prevalent in men than women in some populations, whereas impaired glucose tolerance is more pronounced in women than men (53). Independent of diabetes, it also is well known that the risk of development of coronary heart disease is much greater in men than in premenopausal women (54), although this difference disappears after menopause, suggesting that it is due to differences in hormonal milieu rather than cell-autonomous differences between males and females. Sex-specific differences have also been observed in the risk of stroke related to T2D, with the relative risk being higher in women than in men (55). Therefore, even though the prevalence of diabetes is only modest skewed toward males vs. females, the progression from normal glycemia to prediabetes, as well as metabolic alterations linked to insulin resistance and skeletal muscle physiology can have sexually dimorphic phenotypes.

The novel sexually dimorphic protein phosphorylation pattern observed in our study may be linked to several potential upstream regulators. Although only ~4% of the proteins involved in differential phosphorylation between males and females are encoded on the X chromosome, the X-chromosome encodes several protein kinases that might play a role in some of these protein
phosphorylation differences either directly or secondarily by regulation of autosomally-encoded protein kinases or phosphatases. Indeed, in the study noted above exploring sex differences in gene expression using databases containing information on 44 human tissues, 37% of all genes had sex-biased expression changes in at least one tissue (47). Finally, sex-hormone dependent epigenetic modification could also play a role in exerting male- and female-specific phosphoproteome signature, although iPSCs reprogramming is known to erase most epigenetic marks (56). Another point to be noted is that the BMI of both the I-Sen and I-Res individuals falls within the overweight to slight obese range. Whether this might exert any effect on insulin signaling via epigenetic imprinting remains to be determined; however, as noted above, iPSC reprogramming erases most epigenetic marks.

In summary, phosphoproteomic analysis of human iPSC-derived myoblasts demonstrates a large network of dysregulated phosphorylations linked to differences in insulin resistance/insulin sensitivity and the sex of the cell donor. An important fraction of these is shared with cells from patients with type 2 diabetes, indicating a more primal role in cell intrinsic, possibly genetically programmed, insulin resistance. These include altered phosphorylation of IRS1 Ser\(^{1101}\), AKT Ser\(^{124}\), mTOR Ser\(^{2481}\), and TBC1D1 Ser\(^{237}\), all phosphorylations which can negatively influence insulin sensitivity. In addition, insulin resistance is marked by altered phosphorylation in a wide array of processes involving DNA repair, mRNA processing, cellular organization, protein translation, and cellular signaling outside the insulin action pathway. These latter alterations are further modified by the sex of the donor. For at least two of these pathways, DNA repair and RhoA activation, we find functional differences in cellular response, which parallel the changes in upstream signaling. Importantly, all of these differences in the phosphoproteome related to gender and state of insulin sensitivity are retained in cells studied in vitro and after genetic
reprogramming, indicating the cell-autonomous or intrinsic nature of these defects. Further studies will be needed to identify which kinases/phosphatases link the different pathways in this network and how this sexually dimorphic nature of the phosphoproteome impacts normal physiology and the risk of different metabolic diseases between males and females. Nonetheless, these findings point to important critical nodes in insulin resistance, which can serve as sites for future therapeutic development.
Methods
Study subjects, SSPG, and iPSC reprogramming

The iPSC lines used in this study were generated from 20 study subjects that had been recruited and assessed for insulin sensitivity using steady state plasma glucose (SSPG) obtained from the modified insulin suppression test (57) at the Stanford Clinical and Translation Research Unit as part of the NIH-sponsored GENESiPS project. Briefly, the SSPG method involves an overnight fast followed by simultaneous infusion of octreotide, insulin, and glucose at fixed doses followed by blood collections at 10 min intervals from 150 to 180 mins of the infusion. This combined infusion allows glucose and insulin to both be in steady state leading to the steady state plasma glucose or SSPG.

iPSC lines were generated as described previously (24), and those used in the study were chosen from 10 in the upper quintile of insulin sensitivity (I-Sen) and 10 in the lowest quintile of insulin sensitivity (I-Res), matched age, sex and race/ethnicity based on the SSPG (24).

iPSC culture and myogenic differentiation

The iPSCs were culture on plates coated with hESC-qualified Matrigel (Corning) using the mTeSR1 media containing the 5X complement (StemCell Technologies) and passaged as aggregates using ReLeSR (StemCell Technologies). For differentiation into myoblasts, a modified version of the two-step differentiation protocol was used based on (25). First, approximately 7x10^3 iPSCs/cm^2 were seeded onto collagen I-coated plates (Biocoat, Fisher) in skeletal muscle cell growth basal medium (Lonza) containing 5 % horse serum (HS), 50 μg/mL fetuin, 3 μM CHIR99021, 2 μM Alk5 inhibitor, 1 ng/mL bFGF, 10 ng/mL human recombinant epidermal growth factor (hr- EGF), 10 μg/mL insulin, 0.4 μg/mL dexamethasone, 10 μM Y27632 (Rock inhibitor) and 200 μM ascorbic acid with media change every two days,
which resulted in myogenic precursor/satellite-like (SC-like) cells within 10 days. The SC-like cells were then trypsinized and plated at ~7x10^3 iPSCs/cm^2 onto collagen I-coated plates (Biocoat, Fisher) in skeletal muscle cell growth basal medium (Lonza) containing 5 % horse serum (HS), 50 µg/mL fetuin, 10 µg/mL insulin, 0.4 µg/mL dexamethasone, 10 µM Y27632 (Rock inhibitor), 10 ng/mL hr-EGF, 20 ng/mL hr-hepatocyte growth factor (HGF), 10 ng/mL hr-platelet-derived-growth factor (PDGF-AB), 10 ng/mL Oncostatin M, 20 ng/mL bFGF, 10 ng/mL insulin-like growth factor 1 (IGF1), 2 µM SB431542, and 200 µM ascorbic acid with media change every two days. This resulted in myoblasts (iMyos) within another 10 days.

**DNA, RNA isolation and qPCR**

DNA was isolated using DNAzol (ThermoFisher), and the samples were processed further for the AP site ELISA assay as described below. Total RNA from all the cell types was isolated using TRIzol (ThermoFisher) following the chloroform/isopropanol/ethanol extraction method. Complementary DNA (cDNA) was synthesized from 400ng of RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and the resulting cDNA was used for the qPCR reaction with iQ SybrGreen Supremix (Bio-Rad, catalog 1708884) performed on a C1000 Thermal Cycler (BioRad, catalog CFX384). TATA box binding protein (Tbp) was used as housekeeping gene to normalize gene expression. Primer sequences used are Tbp (Forward: TGATGCCTTATGGCAGCTGGACTGA, Reverse: CTGCTGCCCTTTGTGCTCTTCCAA), Nanog (Forward: TCCAACATCCTGACTCCCTCAG, Reverse: GACTGGATGTCTGGGTCTG), Sox2 (Forward: GCCGAGTGAAACTTTTGTCG, Reverse: GGCAGCGTGTACTTATCCTT), DNMT3B (Forward: ATAAAGTCGAAGGTGCGTCTCGT, Reverse: GGCAACATCTGAAGCCATT), POU5F1/Oct4
Insulin signaling, protein extraction, immunoblotting, and immunostaining

For insulin stimulation, the iMyos were washed twice with PBS and incubated with starvation media containing Ham’s F10 and 0.1% BSA) for 4-6hrs before stimulation with 100nM insulin for 10 mins. Cells were washed twice with ice-cold PBS and lysed using RIPA buffer supplemented with protease and phosphatase inhibitor. Equal amounts of proteins (~10μg) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Following incubation in phosphate-buffered saline containing 0.01% Tween-20 (PBS-T) and 10% dry milk or 5% BSA, membranes were probed with the following antibodies: pIRβ Y1150/1151 (#3024, Cell Signaling), IRβ (sc-711, Santa Cruz), AKT S473 (#9271, Cell Signaling), AKT (#4685, Cell Signaling), p-GSK3α S21/β S9 (#8566, Cell Signaling), GSK3α (#9338, Cell Signaling), CDC37 S13 (#13248, Cell Signaling), MARCKS S167 (#8722, Cell Signaling), and Vinculin (#MAB3574, Millipore Sigma). Membranes were then incubated with appropriate HRP-conjugated secondary antibodies. Signal was detected by chemiluminescence using the ChemiDoc Touch Imaging System (Bio-Rad) and quantified with ImageLab software (Bio-Rad).
For immunofluorescence analysis of MyoD1, iMyos grown on 24-well plates were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, blocked with 10% normal donkey serum, and incubated with the MyoD1 primary antibody (#13812, Cell Signaling) overnight at 4°C. The following day, cells were washed, incubated with Alexa Fluoro 488 donkey anti-rabbit secondary antibody for 1-2hr at room temperature. The cells were also stained with Dapi (1:1000, ThermoFisher) for 10 mins at room temperature and then imaged using an Olympus IX51 inverted fluorescence microscope.

Glucose uptake, quantification of the apurinic/apyrimidinic sites (AP sites) in DNA, and Active Rho pull down assays

For the glucose uptake assay, iMyos grown in a 96-well plate were serum starved (DMEM F/12 + 0.25% BSA) overnight, washed, and incubated with Krebs-Ringer bicarbonate HEPEs (KRBH) buffer (120 mM NaCl, 10 mM NaHCO₃, 4 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 30 mM HEPES) for 1hr at 37°C, then stimulated with 100 nM insulin for 30 min. Following insulin stimulation, 13 µL reaction mixture containing 0.1 µCi 2-[14C(U)]-deoxy-D-glucose (2-DOG, Perkin Elmer) and 200 µM non-radiolabeled 2-DOG (Sigma) was added to each well and incubated at for 10 mins at 37°C. Glucose uptake reaction was stopped by the addition of 26 µL 200 mM non-radiolabeled 2-DOG and immediate transfer of the plates to an ice bath. Cells were washed three times with PBS and lysed with 100µL RIPA buffer followed by scintillation counting.

For the functional assays including the quantification of the number of apurinic/apyrimidinic sites (AP sites) in DNA through Eliza (#STA-324, Cell BioLabs) and
active RhoA pull down assay (#8820, Cell Signaling), differentiated iMyos were processed according to each of the manufacturer’s protocol.

**Statistical analysis**

Data analysis was performed using appropriate unpaired or paired two-tailed Student’s t test (GraphPad Prism Software version 8.4.3), and P < 0.05 was considered to be significant.

**Phosphoproteomic analysis of iMyos**

1. **Lysis and digestion**

The basal and insulin stimulated iMyos were processed as previously described (58). In brief, cells were washed with ice cold PBS, lysed in SDC digestion buffer containing 4% SDC, 100 mM Tris pH8.5 and were snap frozen. The samples were boiled, sonicated for 20 cycles in Biorupter plus (Diagenode), vortexed, and protein concentration was determined by BCA assay. For each sample, 750 μg of protein was alkylated with 10 mM CAA and reduced with 40mM TCEP by incubating for 20 min on ice in dark. Then, the samples were mixed with LysC and Trypsin (1:100 ratio) proteases and incubated overnight at 37°C, 1200 rpm in ThermoMixer.

2. **Phosphopeptide enrichment**

The resulting digested peptides were further mixed in ThermoMixer for 30 seconds at 1500 rpm along with 750 μl ACN and 250μl TK buffer (36% TFA and 3mM KH₂PO₄). Any debris was cleared by centrifugation at 13,000 rpm for 15 min and the supernatant was transferred to 2 ml Deep Well Plate (Eppendorf). For the phosphopeptide enrichment step, TiO₂ beads (prepared in 80%ACN, 6%TFA buffer) were added (1:10 ratio protein/beads) and incubated at 40°C, 2000 rpm, for 5 min in ThermoMixer. The TiO₂ bound phosphopeptides were pelleted by
centrifugation, transferred to clear tubes, and washed 4 times using a wash buffer containing 60% ACN, 1% TFA to remove nonspecific or non-phosphorylated peptides. The beads were suspended in a transfer buffer (80% ACN, 0.5% Acetic acid). Then, transferred on top of single layer C8 Stage Tips (stop-and-go-extraction tips) and centrifuged until dryness. The phosphopeptides were eluted with elution buffer (40% ACN, 20% NH4OH) and concentrated in a SpeedVac for 20 min at 45°C. This was followed by phosphopeptides acidification by the addition of 100 μl of 1% TFA. The acidified peptides were loaded on to equilibrated styrene divinylbenzene–reversed phase sulfonated (SDBRPS) Stage Tips for desalting and further cleanup. These SDBRPS StageTips were washed once in isopropanol/1% TFA and twice with 0.2% TFA. Finally, the desalted phosphopeptides were eluted with 60 μl of elution buffer (80%, 1.25% NH4OH). The dried elutes were resuspended in MS loading buffer (3% ACN, 0.3% TFA) and stored at -20°C until MS measurement.

3. LC-MS/MS measurement

The phosphopeptides were analyzed using Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fischer Scientific) coupled to a nanoflow EASY-nLC1000 HPLC (Thermo Fisher Scientific). The phosphopeptides were loaded onto a 50cm C18 column with a 75 μM inner diameter with the temperature maintained at 50°C by an in-house made column oven. The phosphopeptides were separated in a duration of a 140-minute gradient with two mobile phase system buffer A (0.1% formic acid) and buffer B (60% ACN plus 0.1% formic acid) at a flow rate of 300 mL/min. Furthermore, the electro sprayed peptides were analyzed by the Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fischer Scientific) in a data dependent mode, with one survey scan at a target of 3x10^6 ions (300-1650
m/z, R=60,000 at 200 m/z), followed by Top10 MS/MS scans with HCD (high energy collisional
dissociation) based fragmentation (target 1x105 ions, maximum filling time 120ms, Isolation
window 1.6 m/z, and normalized collision energy 27%), detected in the Orbitrap (R=15,000 at
200 m/z). Apex trigger (4 to 7s), charge exclusion (unassigned, 1, 5, -8 & >8), and dynamic
exclusion 40s were enabled.

4. Phosphoproteomics data analysis

The acquired raw data files were processed using Maxquant (59) software environment
(version 1.5.5.2) with the built in Andromeda search engine for identification and quantification
of phosphopeptides. The data were searched using a target-decoy approach with a reverse
database against Uniprot Human (August 2016 version) reference proteome fasta file with a false
discovery rate of less than 1% at the level of proteins, peptides, and modifications. A few minor
changes to the default settings used are as follows: oxidized methionine (M), acetylation (protein
N-term), and in the case of phosphopeptide search phospho (STY) was selected as variable
modifications and carbamidomethyl (C) as a fixed modification. A maximum of 2 missed
cleavages was allowed, a minimum peptide length of seven amino acids and enzyme specificity
was set to Trypsin. In addition, the match between the run algorithm was enabled. The Maxquant
output phospho (STY) table was processed using Perseus (60) (version 1.5.2.11) software suite.
Prior to the analysis, contaminants marked as potential contaminants and reverse hits were
filtered out. Phosphopeptides that had more than 80% valid values in at least one group were
selected for downstream analysis. Missing values were replaced by random numbers that were
drawn from normal distributions with means that are 1.6 times sample standard deviations
downshifted from the sample means and standard deviations that are 0.6 times the sample
deviations. Values were log2-transformed and further normalized to make all samples have the same median. The statistical significance of phosphopeptides was assessed with empirical Bayesian linear modeling using the limma package with default priors (61). P-values were corrected using the Benjamini-Hochberg false discovery rate (FDR). Hierarchical cluster analysis was performed using a variable cut height approach based on the Euclidean distance of the significant phosphopeptides (27). Clusters were defined according to the hierarchical tree. The human kinase substrates analysis was performed from the PhosphositePlus (62) and RegPhos (63) and were tested using the Fisher exact test.

Comparison of phosphoproteome data from two independent studies was performed by adjusting for the batch effect using the surrogate variable analysis (64). Furthermore, the overlapping sites in non-diabetics and T2D iMyos were then identified through comparison of differential phosphosites in the control groups of each study (I-Sen & Ctlrs) and case groups of each study (I-Res & T2D) using a single combined data file containing both I-Res and T2D set of the phosphoproteomics raw data. The phosphoproteomics data set generated for this study are deposited in the PRIDE database.

All phosphosites were determined using Maxquant software. For those proteins, where the identified phosphosite was in a specific isoform, we adjusted the amino acid numbering system in Figure 4 to match the major isoform identified in Uniport. These included GSK3A S219, Y216, TBC1D4 S749, ARHGEF2 S695, PHACTR4 S448, and SF1 S205, S207, which are now designated as GSK3A S282, Y279, TBC1D4 S588, ARHGEF2 S696, PHACTR4 S291, and SF1 S80, S82 respectively.
**Author contributions**

N.H. designed and performed all the experiments, analyzed all the data, designed the figures, and wrote the paper. J.L. designed the study and prepared samples for phosphoproteomics. A.K.J. designed the study, analyzed, and performed the phosphoproteomics. Co-first authorship was assigned based on the listed author contributions. T.M.B. contributed to experiments and critical reading of the paper. H.P. and J.D. performed bioinformatics analysis. I.C. and J.K. provided the I-Sen and I-Res iPS cell lines and contributed to the study design and critical reading of the paper. M.M. supervised the phosphoproteomics work. C.R.K. conceived the study, helped with data analysis and interpretation, reviewed and edited the manuscript, and supervised the project. All the authors read, reviewed and edited the manuscript.
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Conflict of interest statement

The authors have declared that no conflict of interest exists.
References


Figure 1: Metabolic characterization of I-Sen and I-Res iPSCs-derived myoblasts.
A) Studies performed over the spectrum of insulin resistance changes B) Age, Body mass index (BMI) C) Fasting plasma glucose, and steady state plasma glucose (SSPG) data is shown for each I-Sen (green bars) and I-Res (red bars) subject (male = blue □, female = red ○). Data are means ± SEM, n = 10/group. **** P < 0.0001, unpaired t test D) 2-DOG glucose uptake assay in iMyos stimulated with 100nM of insulin for 30 mins. Data are means ± SEM, n = 9/group, * P < 0.05 basal vs insulin, and Insulin I-Sen vs I-Res, two-way ANOVA followed by correction for multiple comparison by controlling the FDR. E) Insulin signaling western blotting in I-Sen and I-Res iMyos from males and females showing phosphorylation of IRβ Y1150/1151, AKT S473, GSK3α/β S21/S9, and their respective total protein levels. F) Quantification of the insulin signaling experiments normalized by the total protein expression. Data are means ± SEM. * P <0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001 basal vs insulin, and I-Sen vs I-Res, two-way ANOVA followed by correction for multiple comparison by controlling the FDR.
Figure 2: Overview of the global phosphoproteome data and highlight of the insulin regulated changes.

A) PCA plot showing the separation of the phosphoproteome data by subject sex (blue: males, red: females) and insulin sensitivity status (open shape: insulin sensitive, filled shape: insulin resistant). A basal (lighter shade) and insulin stimulated (darker shade) pair for each cell line is shown (dotted line connecting basal and stimulated pair). B) Hierarchical clustering of the phosphopeptides showing insulin-regulated effects of insulin resistance. Rows represent Z-scores of the log2-transformed intensity of phosphosites for each sample labeled in the column. C) Quantification of representative phosphosites from each of the different clusters (Class IA-D and Class IIA-B). AKT serine/threonin kinase 1 (AKT1), Stathmin (STMN1), Proline-rich AKT1 substrate 1 (AKT1S1), NMYC downstream-regulated gene 2 (NDRG2), SH3 domain-binding protein 2 (SH3BP2), Sosondowah Ankyrin Repeat Domain Family Member B (SOWAHB). Data are means ± SEM of phosphosites intensity values (x10^-5). * P <0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001 basal vs insulin or I-Sen vs I-Res, #P<0.05, ## P<0.01, ### P < 0.001, #### P<0.0001 male vs female, two-way ANOVA followed by correction for multiple comparison by controlling the FDR.
A. Insulin resistance in T2D and Non-diabetics. 7803 phosphosites identified in both studies with FDR<0.05.

B. Insulin stimulated ratio comparison between This Study and Batista, T. et al.'s study.

C. Phosphosite intensity values for CHAMP1_S173, STMN1_S38, SRRM2_S876, PHF3_S1722, mTOR_S2481, LIMCH1_S72, CDK1_Y15, SLC38A10_S802.

D. Basal Insulin/basal ratio comparison:
- Up-regulated in I-Res and T2D (214 phosphosites)
- Down-regulated in I-Res and T2D (175 phosphosites)

E. Log intensity Z-score plots for selected phosphosites.
**Figure 3: Insulin resistance non-diabetics phosphoproteome changes shared with T2D.**

A) Overview of the analysis to compare overlapping phosphoproteome changes in I-Res and T2D (FDR<0.05).

B) Hierarchical clustering of the insulin/basal ratio of the phosphopeptides showing shared phosphoproteome changes in I-Res and T2D iMyos. Rows represent Z-scores of the log2-transformed intensity of phosphosites for each sample labeled in the column.

C) Quantification of representative phosphosites from each of the up- and down-regulated clusters from B). Chromosome alignment-maintaining phosphoprotein 1 (CHAMP1), Stathmin (STMN1), Serine/arginine repetitive matrix protein 2 (SRRM2), PHD finger protein 3 (PHF3). Data are means ± SEM of the phosphosites intensity values. * P <0.05, ** P <0.01, I-Sen vs I-Res or T2D vs Ctrls, unpaired t test.

D) Hierarchical clustering of the phosphopeptides showing shared phosphoproteome changes in I-Res and T2D iMyos at the basal state. Rows represent Z-scores of the log2-transformed intensity of phosphosites for each sample labeled in the column.

E) Quantification of representative phosphosites from each of the up- and down-regulated clusters from D). Male and females were combined for clearer data presentation. Serine/threonine protein kinase mTOR (mTOR), LIM and calponin homology domains-containing protein 1 (LIMCH1), Cyclin-dependent kinase 1 (CDK1), Putative sodium-coupled neutral amino acid transporter 10 (SLC38A10). Data are means ± SEM of the phosphosites intensity values. * P <0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001 basal vs insulin or I-Sen vs I-Res or T2D vs Ctrls, two-way ANOVA followed by correction for multiple comparison by controlling the FDR.
Figure 4: Signaling map highlighting critical nodes of phosphoproteome alterations overlapping non-diabetics and T2D.

Signaling map showing shared protein phosphorylation changes in the basal state (upregulated in purple, downregulated in blue) and insulin stimulation ratio (upregulated in red, downregulated in green) not changed shown in black. The pathway shown are significantly enriched based on biological GO analysis (FDR<0.05) and the related phosphosites show significant differences between I-Sen vs I-Res and/or T2D vs Ctrls (p<0.05). The map is drawn using Adobe Illustrator 2020.
Figure 5: Insulin independent protein phosphorylation alterations highlighting the sexual dimorphism.
A) Hierarchical clustering of the phosphopeptides showing sexual dimorphism and insulin resistance changes. Rows represent Z-scores of the log2-transformed intensity of phosphosites for each sample labeled in the column.
B) Quantification of representative phosphosites from each of the different clusters (Class IIIA-B, and Class IVA-B). Hsp90 co-chaperone Cdc37 (CDC37), serine and arginine repetitive matrix 1 (SRRM1), myristoylated alanine-rich C-kinase substrate (MARCKS), methylthioribulose-1-phosphate dehydratase (APIP). Data are means ± SEM of phosphosites intensity values (x10^(-5)). * P <0.05, ** P <0.01, *** P < 0.001 I-Sen vs I-Res or #P<0.05, # # P<0.01, # # # # P<0.0001 male vs female, two-way ANOVA followed by correction for multiple comparison by controlling the FDR.
C) Validation of a few phosphosites by immunoblotting in I-Res and I-Sen iMyos from males and females at the insulin stimulated state.
D) Map representation of the most enriched biological pathways and the related proteins in males (blue) and females (pink). The map is drawn using the Cytoscape software (3.8.0) and Adobe Illustrator 2020.
Figure 6: Functional implications of the phosphoproteomics alterations.
A) DNA damage response overview and quantification of TP53BP1 phosphosite. Tumor suppressor p53-binding protein 1 (TP53BP1). Data are means ± SEM of the basal phosphosites intensity values (×10-5). * P < 0.05, I-Sen vs I-Res or # P < 0.05, #### P < 0.0001 males vs females, one way ANOVA followed by correction for multiple comparison by controlling the FDR. B) Quantification of the number of apurinic/apyrimidinic sites (AP sites) for equal amount of each of the DNA samples from iMyos as per Eliza’s manufacturer instruction. Data are means ± SEM. *** P < 0.001, I-Sen vs I-Res, one way ANOVA followed by correction for multiple comparison by controlling the FDR. C) Quantification of PAK1 and PAK2 phosphosites. Serine/threonine-protein kinase (PAK). Data are means ± SEM of the basal phosphosites intensity values (×10-5). * P < 0.05, I-Sen vs I-Res or ## P< 0.01, #### P < 0.0001 Males vs Females, one way ANOVA followed by correction for multiple comparison by controlling the FDR. D) Western blot of the I-Sen and I-Res iMyos from females and males cell lysates processed through the active RhoA pull down (PD) experiment and the total cell lysates (TCL). E) Quantification of the raw PD western blot showing active form of RhoA as well as the TCL showing total RhoA levels. Vinculin is used as a loading control. Data are means ± SEM. * P< 0.05, ** P <0.01, I-Sen vs I-Res or # # P<0.01, # # # P < 0.001 males vs females, one way ANOVA followed by correction for multiple comparison by controlling the FDR.