Analytical comparison of absolute quantification strategies to investigate the insulin signaling pathway in fat cells

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Abstract
So far, mass spectrometry-based targeted proteomics is the most sensitive approach to answer and address specific biological questions in an accurate and quantitative fashion. However, the data analysis design used for such quantification varies in the field leading to discrepancies in the reported values. In this study, different quantification strategies based on calibration curves were evaluated and compared. The best accuracy and coefficient of variation was achieved by ratio to ratio calibration curves. We applied the ratio to ratio quantification approach to analyze very low abundant insulin signaling proteins such as PIK3RA (0.10–0.93 fmol/μg), AKT1 (0.1–0.39 fmol/μg), and the insulin receptor (0.22–2.62 fmol/μg) in a fat cell model and demonstrated the adaptation of this pathway at different states of insulin sensitivity.

KEYWORDS
cell biology, differentiation, MRM/SRM, targeted proteomics, technology

1 INTRODUCTION

Knowledge about protein concentration is crucial to investigate the stoichiometry of cellular complexes [1], to establish mathematical models [2], and to validate biomarkers. In order to achieve this level of comparability, many efforts have been made to establish and improve standardized sample preparation and operating procedures in proteomics. This includes the addition of isotope labeled peptides [3, 4], proteins [5], or the use of engineered concatenated fusion proteins [6, 7] at various steps of sample preparation. These isotope coded molecules are then subsequently used for quantification, target validation, normalization, or the recalibration of non-endogenous reference material [8]. With these efforts, it is ultimately possible to evaluate instrumental fluctuations and the performance and reproducibility of many steps in proteomics. Having such possibilities of quality control, targeted proteomics can overcome limitations of other immunology-based technologies such as Western blots, ELISA, and cytometry, which suffer from a narrow linear dynamic range and the limitations of antibody specificity and availability [9].

Isotope dilution mass spectrometry is an approach to calculate the amount of endogenous molecules based on different concentrations of the added isotope encoded molecules. The basic principle can be applied to all variants of protein quantification.

Nevertheless, calculated concentrations of endogenous proteins can vary considerably depending on the values that are used to generate the calibration curve, leading to discrepancies in the calculated results between different labs. We recently demonstrated in different...
studies the beneficial use of targeted proteomics approaches for the investigation of fat cell differentiation and insulin resistance [10–15]. Here, the correct reporting of protein concentrations is crucial due to the fact that the process is mainly driven by the change of protein abundances [12]. The fat cell model used here (OP9 mouse cell line) is a bone marrow-derived adipocyte model and has been verified by others and us to have similar functional adipocyte characteristics in comparison with 3T3-L1 cells, although OP9 cells do differentiate more rapidly [11, 16]. The cells represent late-stage preadipocytes and thus are more advanced in the differentiation process to become adipocytes [16]. The key regulator of adipogenesis is the peroxisome proliferator-activated receptor gamma (PPARG), a lipid-activated nuclear receptor and transcription factor. PPARG is considered to be both necessary and sufficient for adipogenesis [17]. Secondary regulators that are able to bypass this master regulator have not been identified yet. However, PPARG in feedback with multiple different proteins (e.g., CEBPA, CEBPB, FABP4) is driving adipogenesis in a concentration dependent manner. PPARG is also in feedback with the insulin signaling pathway which positively influences fat cell differentiation in the late stage and is likely to control lipogenesis and therefore lipid droplet formation in adipocytes [11]. However, this positive feedback can be broken by tumor necrosis factor-α (TNFA) [14] inducing insulin resistance (IR) and blunt differentiation in cell culture and is thought to play an important role in IR in visceral fat [14].

In this study, we are going to establish a parallel reaction monitoring (PRM) assay to investigate such concentration dependent changes fine tuning the insulin signaling pathway. For this purpose, we will evaluate different quantification strategies to understand their advantages and disadvantages on the basis of their performance in regard to linear range, recovery factor, reproducibility, and limit of quantification (LOQ). To the end, we use the most accurate design tested in a targeted experiment to establish an absolute quantitation workflow for proteins in the insulin signaling pathway with purpose-based evaluation during IR.

2 | RESULTS

2.1 | Evaluation of the calibration type setting

Based on the summary of recent literature, we identified three common used possibilities to generate calibration curves (Supporting information). Type 1 is created with the peak areas of the corresponding external calibration using heavy isotope coded peptides on the Y-axis and the corresponding amount of the external calibrant on the X-axis [15, 18, 19]. This calibration curve was generated by spiking an OP9 peptide matrix with different concentration of synthetic internal standard (SIS) peptides and later quantifying the amount of a certain peptide species in a different experiment using this external calibration curve (Figure 1B). Type 2 is generated with the ratio of the peak area of the SIS peptide to its corresponding endogenous peptide on the Y-axis and corresponding SIS amount on the X-axis [20]. Here, the external calibration curve and an internal standard for the ratio on the Y-axis is used. Type 3 is established by using the same ratio as for type 2 on the Y-axis and the ratio of the SIS peptide amount to its corresponding endogenous peptide on the X-axis (Figure 1B) [21]. Here, the information of an external calibration curve and for both axis the information on the internal standard is applied (Figure 1B, right). To assess the extent by which these three types differ and to determine the degree of variation, we calculated the absolute amounts of the proteins involved in the insulin signaling pathway for each type by examining their regulation after different stimuli. In addition, and to complete our setup, we also performed a one-point calibration for each chosen peptide to compare this approach with the other three listed above.

For illustration purposes, we selected the peptide ALTDLVSEGR from the insulin receptor substrate 2 (IRS2) (Figure 1A). As observable from Figure 1B, each calibration curve displays a different equation. By comparing all four types of quantification approaches, the calculated amounts for the same peptide displayed significant differences (Figure 1C). These differences cannot only lead to conflicting results but also making it difficult to $M_{calib}$ compare and integrate data across laboratories and platforms. Currently, there is no comprehensive summary or standard procedure for calculating absolute quantities of proteins. Therefore, this study investigated the above-mentioned calibration curve designs and the different methods for the calculation of absolute quantities associated with them according to different analytical aspects. Ultimately, the aim was to work out which of the above-mentioned calibration curves provides the best results in terms of linear range, recovery factor, reproducibility, and LOQ (Supporting information).

In order to compare the different methods, 113 peptides of 47 proteins from the STAMPS database were used for evaluation (Table S1 [22].

2.1.1 | Correlation coefficient

One purpose of calibration curves is to evaluate the linear range of the analyte. This is needed to determine the detection limits of the used instrument platform. A reliable quantification can only be performed in the linear range with a correlation coefficient ($R^2$) close to 1. To see, if the different calibration curves have varying $R^2$-values, a linear regression was carried out (Figure 2A). Although $R^2$ varied in a small range, no significant differences were observed across all 113 utilized peptides with $R^2$ values (0.99 in average). This is indicating that across the largest proportion of the chosen linear dynamic range all three calibration curve types perform similarly and no significant information is lost.
FIGURE 1  Quantification example for different approaches. Peptide ALTDLVSEGR of the IRS2 protein was selected to evaluate different strategies. (A) Top three fragments of the peptide ALTDLVSEGR were used to calculate the peak area before and after fat cell differentiation. (B) Three calibration curves of the same peptide were generated from different methods comprising the peak area ratio (H/L) to amount ratio (H/L); peak area (H) to amount (H); peak area ratio (H/L) to amount (H). (C) Absolute peptide amount from DMSO and Rosiglitazone treated samples were calculated from: one-point calibration, amount to area calibration curves, amount to ratio calibration curves, and ratio to ratio calibration curves. Error bars represent the SD of the mean and are derived from three technical for the depicted calibration curves (B) and three biological replicates were used in the control (DMSO) and Rosiglitazone treatment (C). DMSO, dimethyl sulfoxide; SD, standard deviation

2.1.2 Accuracy

The comparison of the calculated amount with the theoretical amount is used to evaluate the accuracy. The recovery factor $RF_{(p)}$ is designed to assess accuracy and is defined in Equation (1). In this study, $M_{cal(p)}$ is the calculated amount of peptide from its corresponding calibration curve, $M_{(p)}$ is the actual amount of peptide:

$$RF_{(p)} = \frac{M_{cal(p)}}{M_{(p)}} \times 100$$

When the calculated amount is equal to the actual amount, the RF is equal to 100. This means that the more accurate the value is, the closer it is to 100. The RF of type 3 achieves an average accuracy of 100 with the smallest variation (Figure 2B). Accuracy values calculated on the basis of type 1 and 2 calibration curves achieved across all peptides average accuracy values of 82.6 and 79.6. This indicates that using the calibration curve of type 3 delivers the closest values to the reference values. Off note the one-point calibration achieved $RF = 80$ since the spiked-in concentrations were adapted to the signal intensity of endogenous peptides (Figure S1).

2.1.3 Reproducibility

Ensuring reproducibility is a very crucial task. One measure for assessing reproducibility (also referred to as replicability) is the coefficient of variation (CV). It is calculated by dividing the standard deviation by the mean. Here, we used the CV of three technical replicates to evaluate the reproducibility (Figure 2C). As expected, the CVs of the type 1 curves (amount to area) was in average 7.7% and varied from 1% to 80% due to the lack of isotope labeled internal standards that would help to mitigate the influence on quantification results due to variances across different measurements. Whereas the range of CVs of the type 3 curves (ratio to ratio) was the narrowest among different methods and ranged from 0.1% to 28% and was in average 6.2% whereas type 2 curves have had an average CV of 6.4%.

2.1.4 Lower limit of quantification

The LOQ here referred as lower LOQ or LLOQ is one of the important confidence indicators in quantitation. According to the US Food and Drug Administration (FDA), its value is determined as the lowest
FIGURE 2  Evaluation of quantification strategies. To calculate major analytical parameters such as $R^2$ derived from the linear regression, the accuracy of quantification, the standard deviation in percent, or the lower limit of quantification (LLOQ) for all calibration curves, all peptides derived from the 58 proteins were considered including their biological and technical replicates ($n = 3; m = 3$). (A) Correlation coefficients of the differently generated calibration curves. (B) Accuracy of the calculated absolute amount. (C) Reproducibility of different strategies. (D) Estimation of LLOQ values from calibration curves.

bound on the calibration curve which is still in the linear range with an accuracy (ACC) within 20% of nominal concentration as well as a CV below 20% [23]. However, most of the time, especially when assessing medium to high abundant peptides, the linear range covering the measured quantities is more useful than the precise knowledge of the LLOQ for absolute quantification. Nevertheless, by comparing the type 1–3 strategies to each other the following average limits of quantification were achieved: type 1 amount to area, 28.1 fmol; type 2 amount to ratio, 24.2 fmol; and type 3 ratio to ratio, 6 fmol (Figure 2D). This indicates also here the type 3 calibration curve as the method of choice to achieve the best analytical output, which is of utmost importance if low protein amounts have to be determined as it is often the case for signaling pathways [2, 12, 18].

2.2  Quantitative mapping of the insulin signaling pathway

Based on the results of the method comparison, the ratio to ratio method (type 3) provides the best accuracy and reproducibility and was chosen for the absolute quantification of the insulin signaling pathway.

In total, we quantified 58 proteins covering a dynamic range of over two orders of magnitude with KAP3 being with 31 fmol/μg the most abundant and PK3CA with 0.2 fmol/μg the least abundant signaling component (Table 1). The CV across the analyzed technical replicates was 6.2%. In comparison to previously published targeted assays [19], 58 proteins of the insulin signaling pathway were for the first time absolutely quantified and 10 proteins among them such
| No. | Protein name | Description | Uniprot ID | Representative peptide | Amount (fmol/µg) | CV (%) |
|-----|--------------|-------------|------------|------------------------|-----------------|--------|
| 1   | PK3CA        | Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform | P42337     | SLWVINSALR              | 0.2             | 102    |
| 2   | AKT1         | RAC-alpha serine/threonine-protein kinase | P31750     | DEVAHTLTENR             | 0.3             | 28     |
| 3   | B RAF        | Serine/threonine-protein kinase B-raf | P28028     | GLIPECCAVYR             | 0.4             | 13     |
| 4   | CIP4         | Cdc42-interacting protein 4 | Q8CJ53     | ELVAESLGIR              | 0.4             | 13     |
| 5   | MP2K1        | Dual specificity mitogen-activated protein kinase 1 | P31938     | IPEQILGK                | 0.5             | 119    |
| 6   | SH2B2        | SH2B adapter protein 2 | P9JID9     | VSIPLSAIIEVR             | 0.6             | 78     |
| 7   | PK3CA        | Phosphatidylinositol 3-kinase regulatory subunit alpha | P26450     | NESLAQYNPK              | 0.8             | 12     |
| 8   | PDPK1        | 3-Phosphoinositide-dependent protein kinase 1 | Q9Z2A0     | SQTPEGSSPGPSGVSR        | 1.0             | 13     |
| 9   | KAPCB        | cAMP-dependent protein kinase catalytic subunit beta | P68181     | ILQAVEFPFLVR             | 1.0             | 15     |
| 10  | KS6B2        | Ribosomal protein S6 kinase beta-2 | Q9Z1M4     | LVLPPYTPDAR             | 1.1             | 9      |
| 11  | IKKB         | Inhibitor of nuclear factor kappa-B kinase subunit beta | O88351     | YLNQFENCCGLR             | 1.2             | 16     |
| 12  | CALM1        | Calmodulin-1 | P0DP26     | DGNGYISAELR             | 1.3             | 28     |
| 13  | RAF1         | RAF proto-oncogene serine/threonine-protein kinase | Q99N57     | AAHEDINACTLTTSPR        | 1.3             | 10     |
| 14  | IF4E         | Eukaryotic translation initiation factor 4E | P63073     | IIVGYQSHADTATK           | 1.4             | 5      |
| 15  | ARAF         | Serine/threonine-protein kinase A-Raf | P04627     | QFYHSIQDLSGGSR          | 1.5             | 7      |
| 16  | CRKL         | Crk-like protein | P47941     | ILYDTTTLIEPAPR          | 1.5             | 13     |
| 17  | RPTOR        | Regulatory-associated protein of mTOR | Q8K4Q0     | LYSLLSDPIPEVR            | 1.6             | 6      |
| 18  | KS6B1        | Ribosomal protein S6 kinase beta-1 | Q8B5K8     | FSPGDFWGR                | 1.6             | 13     |
| 19  | IRS1         | Insulin receptor substrate 1 | P35569     | SVSAPQIIINPIR            | 1.6             | 10     |
| 20  | SHIP2        | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 | Q6P549     | SALLPNPLEQPPR           | 1.6             | 7      |
| 21  | SHC1         | SHC-transforming protein 1 | P98083     | ELFDPSYVINQNLDK          | 1.7             | 13     |
| 22  | AAKP2        | 5′-AMP-activated protein kinase catalytic subunit alpha-2 | Q8BRK8     | GGVFYIPDYLNR            | 1.8             | 17     |
| 23  | RASK         | GTPase KRas | P32883     | SFEDIHYR                  | 1.9             | 26     |
| 24  | CRK          | Adapter molecule crk | Q64010     | ILYDTTTLIEPVAR           | 2.0             | 10     |
| 25  | PTN1         | Tyrosine-protein phosphatase non-receptor type 1 | P35821     | FSYLAVIEGAK              | 2.1             | 13     |
| 26  | BAD          | Bcl2-associated agonist of cell death | Q61337     | SAPPNLWAAQR             | 2.1             | 8      |
| 27  | PP1G         | Serine/threonine-protein phosphatase PP1-gamma catalytic subunit | P63087     | NVQLQENEIR              | 2.2             | 21     |
| 28  | GRB2         | Growth factor receptor-bound protein 2 | Q60631     | ESESAPGDFSLSVK          | 2.3             | 10     |
| 29  | IF4E2        | Eukaryotic translation initiation factor 4E type 2 | Q8BMB3     | FQEDIISIWNK              | 2.4             | 11     |
| 30  | GSK3B        | Glycogen synthase kinase-3 beta | Q9VW60     | DIKPQNLILLDPDTAVLK       | 2.5             | 7      |
| 31  | FOXO1        | Forkhead box protein O1 | Q9R1E0     | LPSDLGDMFIER             | 2.5             | 18     |
| 32  | AAKP1        | 5′-AMP-activated protein kinase catalytic subunit alpha-1 | Q5EG47     | HTLDELNPQK              | 2.6             | 11     |
| 33  | MK01         | Mitogen-activated protein kinase 1 | P63085     | VADPDHDHTGFLTEYVATR     | 2.6             | 7      |

(Continues)
### TABLE 1 (Continued)

| No. | Protein name | Description | Uniprot ID | Representative peptide | Amount (fmol/µg) | CV (%) |
|-----|--------------|-------------|------------|------------------------|-----------------|--------|
| 34  | KAP0         | cAMP-dependent protein kinase type I-alpha regulatory subunit | Q9DBC7 | LTVDALEPVQFEDGQK       | 2.6             | 15     |
| 35  | INSR         | Insulin receptor | P15208 | IELQACNQDSPDER          | 2.6             | 6      |
| 36  | RASH         | GTPase HRas   | Q61411    | SFEDIHQYR               | 2.7             | 11     |
| 37  | AKT2         | RAC-beta serine/threonine-protein kinase | Q60823 | SLLAGLLK               | 2.8             | 7      |
| 38  | IRS2         | Insulin receptor substrate 2 | P81122 | SQSSGSSATHPISVPGAR      | 2.9             | 6      |
| 39  | MP2K2        | Dual specificity mitogen-activated protein kinase 2 | Q63932 | SEGEEVDFAGWLCR          | 2.9             | 8      |
| 40  | ACACA        | Acetyl-CoA carboxylase 1 | Q5SWU9 | GGSLVVIDPTINPR          | 3.1             | 17     |
| 41  | MK03         | Mitogen-activated protein kinase 3 | Q63844 | IADPEHDHTGFLTEYVATR    | 3.1             | 3      |
| 42  | PP1B         | Serine/threonine-protein phosphatase PP1-beta catalytic subunit | P62141 | ICQDIHQYTDLLR           | 3.1             | 8      |
| 43  | GLUT4        | Solute carrier family 2, facilitated glucose transporter member 4 | P14142 | TFDQIISAAFR            | 3.1             | 7      |
| 44  | MTOR         | Serine/threonine-protein kinase mTOR | Q9JLN9 | LGWQLNQGINESTPK         | 3.6             | 11     |
| 45  | PYGB         | Glycogen phosphorylase, brain form | Q8CI94 | QAVDQISSGFFSPK         | 4.0             | 13     |
| 46  | FLOT2        | Flotillin-2 | Q60634 | VDEIVVLSDNSK           | 4.3             | 16     |
| 47  | RHEB         | GTP-binding protein Rheb | Q921J2 | ALAESWNAFLESSAK        | 4.5             | 22     |
| 48  | KAPCA        | cAMP-dependent protein kinase catalytic subunit alpha | P05132 | WETPSQNTAQLDQFDR       | 5.1             | 13     |
| 49  | HXX2         | Hexokinase-2 | O08528 | ASGCEGEDVVTLKK         | 5.3             | 17     |
| 50  | FLOT1        | Flotillin-1 | O08917 | VQVQVVER               | 5.8             | 14     |
| 51  | GYS1         | Glycogen [starch] synthase, muscle | Q9Z1E4 | VTGDEWGDNYLYVPETQGVR    | 5.9             | 7      |
| 52  | FAS          | Fatty acid synthase | P19096 | SDEAVKPLGKV           | 6.3             | 11     |
| 53  | PCKGM        | Phosphoenolpyruvate carboxykinase [GTP], mitochondrial | Q8BH04 | TLIGHVPDQR            | 9.3             | 13     |
| 54  | SRBS1        | Sorbin and SH3 domain-containing protein 1 | Q62417 | TPVVDYDLPYSSPSR        | 9.4             | 7      |
| 55  | PP1A         | Serine/threonine-protein phosphatase PP1-alpha catalytic subunit | P62137 | NVQLTENEIR          | 11.6            | 15     |
| 56  | RS6          | 40S ribosomal protein S6 | P62754 | DIPGSTDVVPR           | 16.2            | 9      |
| 57  | LIPS         | Hormone-sensitive lipase | P54310 | FAIDPELR            | 26.0            | 7      |
| 58  | KAP3         | cAMP-dependent protein kinase type II-beta regulatory subunit | P31324 | AATITATPGALWGDLR       | 30.9            | 18     |

The absolute amount of 58 representative peptides in control group were sorted and showed in the table. Depicted in this table is the absolute amount of 58 proteins, each with a representative peptide. The results reported here were calculated using the control group. The corresponding standard deviation was calculated from three biological replicates.

As GYS1, ARAF, E1f4e, CRKL were detected for the first time in a targeted proteomics assay (Table S2). To the best of our knowledge, so far, no absolute amounts on the insulin signaling pathway were reported [24–27].

Next, we used the assay to monitor absolute peptide levels of key proteins in the insulin signaling pathway during the induction of insulin resistance (TNFA) and sensitivity (Rosiglitazone, Figure S2) or to monitor dropping insulin sensitivity (Rosiglitazone/TNFA). Therefore, fat cells were treated as described in the material and method part, processed and analyzed using internal peptide standards.

Although the absolute amounts of peptides of the same proteins varied, all peptides derived from the same protein displayed a similar trend in regulation. For instance, the concentration of peptides LTVDALEPVQFEDGQK and SEGEREEVDFAGWLCR in KAP0 were changed from 5.28 to 2.59 fmol/µg and 2.87 to 1.17 fmol/µg after treated with Rosiglitazone, respectively, however the regulation trend of both peptides displayed an upregulation (Table S4). As expected, we found a strong regulation of proteins involved in fatty acid formation (FASN, LIPE) and glucose metabolism (GLUT4, HK2) during insulin sensitization (Table S3). Most interestingly, a group of 22 proteins including...
INSR, IRS-1,-2, AKT2, KAP3, and PRKAR2B were found to be significantly upregulated (p value < 0.05) in the treatment with Rosiglitazone. This group was not or downregulated in the TNFA treatment compared to the control and downregulated in the Rosiglitazone/TNFA treatment compared to Rosiglitazone indicating their positive correlation with insulin sensitivity (Figure 3A, B, Tables S3, S4). For instance, peptides from INSR were changing from 0.84 to 2.61 fmol/µg in insulin sensitive cells (Rosiglitazone), were not changing in the TNFA treatment 0.61 fmol/µg and displayed slightly lower level in the double treatment. Compared to this group, a second group of 17 proteins including for example proteins such as PP1G, KAP0, SHC1, and CRK were significantly downregulated in the Rosiglitazone treatment and in the double treatment compared to the control indicating their negative correlation with insulin sensitivity (Figure 3A,B, Table S3).

In contrast, hexokinase 2 (HK2) was upregulated under all three treatment conditions. Hexokinase catalyzes the phosphorylation of glucose, the rate-limiting first step of glycolysis. If the levels are compared to control (dimethyl sulfoxide [DMSO]), HK2 levels are increasing from 0.45 fmol/µg base line to 3.23 fmol/µg in the Rosiglitazone, 0.88 fmol/µg in the TNFA, and 4.23 fmol/µg in the combined treatment (Figure 3B). HK2 is the predominant isoform in insulin-sensitive tissues such as heart, skeletal muscle, and adipose tissues [28], where HK2 is constantly adapting its concentration level as highly insulin-responsive element to the sensitivity of the ISP (simulated through different treatments). The slight upregulation in case of the TNFA treatment may be interpreted as a compensatory mechanism to cope with the induced insulin resistance. Note that the adenosine triphosphate (ATP) from oxidative phosphorylation coupling of HK2 to the insulin signaling cascade is leading to an increased rate of glycolysis [29] based on the cascades hyper activation [30] this can also be observed in rapidly growing cancer cells such glioblastoma multiforme. However, to interpret the concentration dependent regulation of the entire insulin signaling network, more detailed investigations are mandatory and the here reported changes can be regarded as a proof of concept to demonstrate the usability of the chosen quantification approach and developed PRM assay.

3 | DISCUSSION

3.1 | Absolute quantification strategy

The insulin signaling pathway is an important signaling pathway that stimulates muscle and adipose tissue to exert hypoglycemic effects through the progressive uptake of glucose and inhibition of gluconeogenesis, which is involved in many diseases such as type 2 diabetes, neurodegenerative disorders, or cancer.

Since this pathway is affected in the whole system during diseases, absolute quantification of the insulin signaling pathway at the protein level is of utmost importance to study the fine tuning of insulin signaling and adaptation. However, the challenge remains that absolute results often differ caused by the selection of different peptides, modification of molecules, or different types of sample preparation. Nevertheless, the way the absolute values are calculated also impacts the quantitative results making comparisons unnecessarily difficult. Therefore, in this work, we summarize multiple calculation methods and try to determine the most accurate and reproducible absolute quantification workflow for our insulin signaling pathway mapping. This will help to develop more robust assays that can be used to investigate the regulation of the insulin signaling pathway across different cell and tissue types.

There are basically two different ways to approach absolute quantification of proteins: by targeted LC-MS/MS, the one-point calibration, or the use of a calibration curve. Although the linear range of type 1–3 calibration curves showed no difference, the LOQ from different calibration methods varied due to their different relative standard deviation and accuracy.

By analyzing different parameters of calibration curves type 1–3, we observed that the ratio-to-ratio method (type 3), in comparison to the other calibration curve designs obtains the best accuracy and reproducibility.

There are several reasons for this result. Looking at the calibration curve of type 1 (amount to area), it can be seen that only the heavy peptide is considered here in the external calibration curve with the matrix. Since only the intensity of the respective peptide amount is read out directly, there is no correction for signal variations introduced by pipetting errors, sample preparation, and LC/MS system variations. In contrast, in type 2 and 3 due to the use of an additional internal standard, these systematic errors can be corrected and thereby reduced, which is clearly reflected in the calculation of the accuracy, CV, and LLOQ. Where in the type 2 calibration curve only the Y-axis contains this information in the type 3 calibration curve both axis presenting ratios that consider internal standards and therefore the type 3 displays best accuracy.

3.2 | Insulin signaling pathway regulation in fat cells

Both TNFA and Rosiglitazone can regulate insulin sensitivity [11, 14]. Our results demonstrated clearly that proteins in the insulin signaling pathway are systematically regulated under different conditions. From the results, all proteins of our targeted assay can be classified into three groups: proteins that were not regulated across all conditions, proteins which are positively or proteins which are negatively correlated with insulin sensitivity. In summary, these results indicate (besides a fast posttranslational response mainly driven by phosphorylation of Tyr at the insulin receptor) that there is also a much slower response adapting the insulin signaling pathway at the protein concentration level. At this point, we can only speculate why this is of importance but one rational would be that with this changing protein concentration the system has an additional parameter to regulate the response to insulin, which allows for a long-term adaptation of the signaling pathway in fat cells. However, this hypothesis has still to be proven and carefully investigated.
FIGURE 3  The insulin signaling pathway. (A) Expression profiles of all 58 proteins across the insulin signaling pathway under four different conditions (DMSO, Rosiglitazone, TNFA, Rosiglitazone followed by TNFA) visualized in the STAMPS pathway tool. The gradient color scale was generated based on absolute amount log2 transformed quantities. Here, n = 3 independent biological replicates. (B) Selected pathway proteins which positively, negatively or not correlated across the different treatments. An ANOVA with Bonferroni post hoc test was the method used to calculate the adjusted p-value (*p ≤ 0.05). ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; TNFA, tumor necrosis factor-A.
CONCLUSION

In conclusion, we evaluated different approaches for the calculation of the absolute amount of proteins in targeted proteomics. Based on our evaluation, we applied the ratio-to-ratio method (type 3) to monitor and determine the absolute amount of key proteins such as INSR, INPPL1, and MTOR in insulin signaling. Our analysis clearly indicated that regulation of insulin signaling takes place on the protein level. Therefore, our results pinpoint which branches of the insulin signaling pathway are active or less active in concentration adaptation during insulin sensitive or resistance cellular stages, which indicates a potential long-term adaptation of this insulin signaling branches.

MATERIALS AND METHODS

5.1 | Cell culture

The mouse bone marrow stromal cell line (OP9, CRL-2749) was cultured at 37°C in Mem-alpha medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin streptomycin glutamine (PSG) in the presence of 5% CO2 until 80% confluence was reached. The differentiation process is usually induced by adding an adipogenic mixture to the growth medium containing dexamethasone, insulin, 3-isobutyl-1-methylxanthine (IBMX), and 10% FBS and takes approximately 72 h [11]. This process can be jump-started by direct activation of PPARG using agonists, such as thiazolidinediones such as Rosiglitazone [11] which reduces the time to 48 h. This is based on the fact that the early differentiation process that starts with a feedback between CEBPβ and CEBPA is skipped and PPARG is directly activated. To create insulin resistant and sensitive cell the following protocol was applied: (1) TNFA treatment with 10 ng/mL TNFA in 10% FBS and 1% PSG followed by 0.01% DMSO for 24 h; (2) Rosiglitazone group: Rosiglitazone treatment for 24 h (1 μM Rosiglitazone in 10% FBS and 1% PSG) followed by 0.01% DMSO for 24 h; (3) TNFA/Rosiglitazone group: first with 1 μM Rosiglitazone for 24 h followed by 10 ng/mL TNFA for 24 h. As control, cells were incubated with 0.01% DMSO for the same time period. The cells were harvested by trypsin-EDTA treatment followed by centrifugation at 200 g for 5 min. Harvested samples were washed with phosphate-buffered saline (PBS) and then centrifuged again to obtain the cell pellet. For each condition 3 biological replicates were generated.

5.2 | Proteomics sample preparation

Cells were lysed using 1% SDS buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.8) and Rosche complete™ Protease Inhibitor Cocktail tablets (Merck, Darmstadt, Germany). Benzonase was added into samples to degrade all forms of DNA and RNA for 30 min at 37°C. After a centrifugation for 30 min at 4°C and 18,000 g the resulting pellet was dissolved in 6 M urea, 10 mM NH4HCO3 buffer. Samples were diluted 1:4 with 10 mM NH4HCO3 buffer (pH 7.8) and protein concentration was measured using bicinchoninic acid assay according to the manufacturer’s instructions (BCA, Thermo Fisher Scientific, Bremen, Germany). Proteins were reduced and alkylated using TCEP (Tris-(2-carboxyethyl)-phosphin, final concentration 10 mM) for 30 min at 37°C followed with IAA (iodoacetamide, final concentration 40 mM) in the dark for 30 min at 20°C. After dilution to 1 M urea, the proteins were digested using a trypsin to protein ratio of 1:40 w/w at 37°C in a ThermoMixer (Eppendorf) at 650 rpm, overnight. Protein digestion was stopped by adding formic acid and the samples were centrifuged at 15,000 g for 10 min. The supernatant was desalted using C18 cartridges (Sep-Pak, 50 mg, Waters). Samples were diluted using a vacuum concentrator and dissolved in 0.1% TFA. Peptides were quantified by amino acid analysis (AAA) to get the absolute amount and then 1 μg of peptides of each replicate was used for later measurements. The digested and desalted peptides were checked for digestion efficiency on an Ultimate 3000 HPLC (Dionex, Germany) with a monolithic column (PepSwift monolithic PS-DVB PL-CAP200-PM, Dionex) as described previously [31].

5.3 | Synthesis of synthetic isotopic labeled (SIS) peptides

The SIS peptides with C-terminal 15N- and 13C-labeled arginine and lysine residues were in-house synthesized by a Fomc-chemistry-based solid-phase peptide synthesis system (Syro, MultiSynTech, Germany). The concentration of SIS peptides was determined by AAA.

5.4 | Calibration curves and sample measurements

To establish the calibration curves, different amounts of SIS peptides range from 0.1 to 50 fmol were spiked into 1 μg of tryptic digest control which was used as the matrix for the calibration curves in the experiment. The amount of SIS peptides in the assay for high abundant peptides contains: 0.25, 0.5, 1, 2, 3.5, 6, 10, 15, 20, 30, and 50 fmol. For low abundant peptides, the following amounts of SIS peptides were used: 0.1, 0.25, 0.78, 3.14, 12.52, 25.04, and 50.08 fmol. In order to measure the endogenous peptide amount, different amount of SIS peptides (6 fmol for high abundant peptides and 0.3 fmol for low abundant peptides) were spiked into 1 μg of all tryptic digest samples, respectively.

5.5 | LC-MS/MS analysis

PRM measurements were performed on a QExactive HF mass spectrometer (Thermo Fisher Scientific) in line with a nanoliquid chromatography (nanoLC) system Ultimate 3000 Rapid Separation Liquid Chromatography (RSLC) system (Thermo Fisher Scientific). The mobile phase consisted of solvent A (0.1% formic acid) and solvent B (84%...
acetonitrile with 0.1% formic acid). The peptides were separated at a flow rate of 250 nL/min on a reversed phase main-column (Acclaim C18 PepMap100, 75 μm 50 cm, Thermo Scientific, Waltham, MA, USA) with a gradient of 3%–35% solvent B in 60 min followed by a 10 min wash period with 90% solvent B. The mass spectrometer was operated in PRM mode with normalized collision energy set to 27% and AGC target value at 3 × 10⁶. The targeted assay was split into two parts: abundant peptides were measured at a resolution of 15,000 with an isolation width of 0.6 m/z and an injection time of 80 ms. Low abundant peptides measured at a resolution of 240,000 and an injection time of 500 ms, using the same isolation width. In order to monitor the performance of the MS platform during measurements, seven in-house synthesized peptides were measured every 24 h for quality control. Retention times as well as peak intensities and peak widths were monitored and compared to ensure a stable performance. For each biological replicate, three technical replicates were generated.

5.6 | Targeted assay establishment and optimization

Three peptides per protein of insulin signaling pathway were chosen from the assay database STAMPS [22] according to the following criteria: (i) the peptides had to be unique in their protein of the mouse proteome, (ii) the peptide length was between eight and 20 amino acids, (iii) tryptic peptides had no missed cleavages, (iv) the methionine amino acid was not included in the peptide, (v) the peptide was validated by its corresponding SIS peptide based on the same retention time and transition pattern, and (vi) the peptides were detected by PRM under all experimental conditions. In order to evaluate the reproducibility, three technical replicates were used to establish each calibration curve.

The initial targeted assay covered high and middle abundant proteins. According to a recently published paper [21], increasing the resolution, AGC values and injection time of the mass spectrometer can lower the LOQ of analytes, which is helpful for the detection low abundant proteins. Therefore, we extended the initial experiment to include low abundant proteins as well by using the optimized MS parameters. In the final targeted analysis, abundant peptides were measured at a resolution of 15 k and an injection time of 80 ms and low abundant peptides measured at high resolution 240 k and injection time 500 ms. In comparison, to previously published targeted assays [24], 58 proteins of insulin signaling pathways were covered in our targeted analysis and absolutely quantified. After an initial screening some of the chosen proteins were left with only one peptide which was still included into the assay for completeness of the monitored insulin signaling pathway (Table S1).

After established targeted assays, four different conditions were analyzed – the control (0.1% DMSO, Rosiglitazone a PPARG agonist activating also insulin signaling, TNFA to induce insulin resistance, and Rosiglitazone followed with TNFA (RT) representing dropping insulin sensitivity (Figure 3). To do so, the endogenous peptide digest was spiked with a mix of the standard peptides and each peptide was targeted with a time window of 5 min using a 60 min revers phase gradient.

5.7 | Data processing

The acquired raw files were imported to Skyline version 4.2.0. Each endogenous peptide was identified with its corresponding SIS peptide based on the same retention time and the occurrence of the same fragment pattern. The mass deviation compared to its corresponding synthetic peptide had to be less than 10 ppm. All interference and tracked fragments were excluded for the later calculation. The summed-up area under the curve (AUC) of all validated and optimized transitions was then used for all subsequent calculations. An ANOVA with Bonferroni post hoc was the method used to calculate the p-value.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available from Panorama Public: https://panoramaweb.org/tObdcX.url. User: panorama+reviewer31@proteinsms.net. Password: njYBvzT. ProteomeXchange: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD026204.

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SUPPORTING INFORMATION
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