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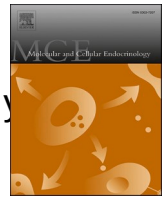

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ABSTRACT

Insuilaininmportreagnutloafwthoorle-gbloudcyhoosmeeostaisnisss.leinnsitisvseueasmuscle and dipoise,uiInidnutchetesrans lodgalutictonsaensp4(GtLeUiT4t)hceemhembratmhe,reby incregalsuicuogpsteaHkoew.evienrs,uallisnigniatlissstheastneogtenerasIloycwiatghedcose homeostatshiesmarneprodenodiovaexinsy,perinsuipperemeaseecsreotfgiocomado-tropfirnogmonadotcedopftshaentepiotuitthaerryle,bnykiinnsgudlyisnregutloautbiompt imal reprodbeaIlvtnhehmeousgeo,nadotexpoprtehssiesnsurleicrewyhiobnlatshceanonsiicganlaling responRsEeAK,Ta,nndTOBactivkadwivevntehfeunctiuontcaobmfienssualcitnogmonadotrope aruencleaarveed,demonsthnactuegdefanoptimcizetdlactipnratiobnathsusItiimmu-latiodtnhBT2gonadotcreolplimceesuiIntsheenxpetcrtaends lodGaLtUiTo1nthpelasma membraUnseinughrighurftyactipnratiwcedur,tdheemronsthratheuAgkhsignaiIning responmisesuilsinntaicnts,ulin-tirnadhusdeodGaLtUiTo1ncuinslependfekatdyiviantion LβT2cells.

1. Introduction

The hypothalamic-Pituitary-Gonadal axis regulates reproductive function in all sexually reproducing vertebrates. Gonadotropin releasing hormone (GnRH) neurons in the hypothalamus project to the pituitary gland where they stimulate the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These pituitary glycoprotein hormones are released into the general circulation and act on receptors expressed in the gonads to regulate gametogenesis and steroidogenesis [1]. Gonadotropin secretion is controlled by the pulsatile release of GnRH from the hypothalamus, which is regulated by a complex network of neuronal and hormonal inputs that convey information about the internal and external environment [2]. Gonadotropes express receptors for GnRH, gonadal steroids, and peptide hormones, including activins and inhibins, which modulate the synthesis and release of gonadotropins [3]. Gonadotropes also express receptors for neurotransmitters and neuropeptides, which can directly or indirectly regulate GnRH secretion [4]. The integration of these diverse inputs by gonadotropes is critical for the precise control of reproductive function.

Insulin is a peptide hormone produced by pancreatic β cells that plays a central role in glucose homeostasis [5]. Insulin binds to the insulin receptor (IR), a receptor tyrosine kinase that is widely expressed in many tissues, including the brain, liver, muscle, and adipose tissue [6]. Upon insulin binding, the IR undergoes autophosphorylation and activates downstream signaling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway [7]. These pathways regulate a variety of cellular processes, including glucose uptake, glycogen synthesis, protein synthesis, and cell growth [8]. In addition to its metabolic effects, insulin has been shown to modulate the activity of the hypothalamic-pituitary-gonadal axis in both males and females [9]. Insulin receptors are expressed in GnRH neurons, pituitary gonadotropes, and gonadal tissues, suggesting that insulin can directly regulate reproductive function at multiple levels [10]. Insulin has been shown to stimulate GnRH release from hypothalamic explants and GnRH neuronal cell lines [11]. Insulin also stimulates the synthesis and release of LH and FSH from pituitary gonadotropes [12]. In addition, insulin has been shown to modulate steroidogenesis in the gonads [13]. These findings suggest that insulin can act as a metabolic signal that links energy status to reproductive function.

Insulin signaling is initiated by the binding of insulin to the IR, which is a heterotetrameric protein composed of two α subunits and two β subunits [14]. The α subunits are extracellular and contain the insulin-binding domain, while the β subunits are transmembrane and contain the tyrosine kinase domain [15]. Upon insulin binding, the IR undergoes autophosphorylation on multiple tyrosine residues in the β subunits [16]. This autophosphorylation activates the kinase domain and creates docking sites for downstream signaling molecules [17]. The IR can also be internalized into endosomes, where it can continue to signal or be recycled back to the plasma membrane [18]. The duration and intensity of insulin signaling are regulated by a variety of mechanisms, including receptor internalization, degradation, and dephosphorylation [19]. Dysregulation of insulin signaling can lead to insulin resistance, a condition in which cells become less responsive to insulin [20]. Insulin resistance is a hallmark of type 2 diabetes mellitus and is also associated with obesity, polycystic ovary syndrome, and other metabolic disorders [21]. In addition to its role in glucose homeostasis, insulin resistance can also affect reproductive function [22]. For example, insulin resistance has been shown to impair ovulation and fertility in women with polycystic ovary syndrome [23]. In men, insulin resistance has been associated with decreased testosterone levels and impaired spermatogenesis [24]. These findings suggest that insulin signaling is important for maintaining normal reproductive function in both sexes [25].

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Dysregulation of the HPG axis is characteristic of various reproductive disorders, such as lack of reproductive development or delayed puberty, polycystic ovary syndrome (PCOS), hyperandrogenism or amenorrhea, or hypogonadism. Many of these reproductive disorders are linked to derangements in energy balance and inflammation, and changes in systemic metabolism are common disruptors of the HPG axis. Insulin is a prime example. Complex relationships between insulin and gonadotropins have been observed in humans (Burt Solorzano et al., 2018; Distiller et al., 1997; Navratil et al., 2009; Stepto et al., 2013; Rojas et al., 2014; Santos et al., 2021). In healthy women, infusion of insulin increased basal and GnRH-stimulated LH (Santos et al., 2021), while in both normally cycling women and women with PCOS, it is apparent that insulin negatively correlates with LH levels (Lawson et al., 2008). Given that hyperinsulinemia commonly associates with conditions that impair reproductive health, including PCOS, it is important to understand the molecular mechanisms that may be responsible for the observed regulation of LH by insulin (Burt Solorzano et al., 2018; Stepto et al., 2013; Chosich et al., 2017).

The primary action of insulin is to regulate glucose homeostasis by facilitating the uptake of glucose from the circulation into target tissues, largely through the insulin receptor (IR), a transmembrane receptor tyrosine kinase. Upon binding, insulin induces a conformational change that activates autophosphorylation. This event triggers signaling cascades that result in the phosphorylation and activation of protein kinase B (Akt). Akt then phosphorylates the Rab GTPase-activating protein Akt substrate of 160 kDa (AS160), phosphorylated and inactivated AS160, which negatively regulates the trafficking of glucose transporter 4 (GLUT4)-containing vesicles. This deactivation allows the fusion of GLUT4-containing vesicles to the cell membrane, where GLUT4 then facilitates glucose uptake. Recently, we and others have demonstrated that glucose metabolism and homeostasis is integral to the regulation of gonadotrope function in the pituitary (Ntiachy et al., 2022; Harris et al., 2012; Buggs-Saxton, 2014). Furthermore, we have also demonstrated that GnRH can potentiate their signaling responses in the context (Navratil et al., 2009; Buggs et al., 2006). Given that GnRH-induces localization of GLUT1 to the membrane of gonadotropes, and as insulin can regulate glucose uptake as well, we surmise that regulation of GLUT1 could be occurring in gonadotropes and be a function of insulin stimulation.

Using the gonadotrope cell line LβT2, we show insulin-stimulated GLUT1 and phospho-Akt are redistributed with the use of an optimized subcellular fractionation protocol. We show that insulin induces GLUT1 membrane localization and Akt independent of phospho-Akt distribution in cytosolic fraction (Manning and Toker, 2017). Regulation of GLUT1 by insulin may provide the mechanism basis for the context-dependent signaling integration between insulin and GnRH for the physiological observations of insulin-GnRH regulation of gonadotropin secretion.

2. Materials and methods

2.1. Tissue culture

The female mouse-derived LβT2 gonadotrope cell line (Nguyen et al., 2004; Windle et al., 1990; Ruf-Zamojski et al., 2019) was maintained in high-glucose (4.5 g/L) HEPES-buffered DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS; FB-11, Omega Scientific) at 37 °C in a humidified atmosphere of 5% CO_2. EA.hy926 (ATCC, CRL-2922) were grown at 37 °C, 8% CO_2 in 10% FBS-DMEM (Gibco, #10313-021, #CV0437-028) supplemented with fresh 20% pre-conditioned medium every 2 days and cultured to passage 8. HeLa cells were grown in DMEM containing 10% (v/v) FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a humidified incubator at 5% (v/v) CO_2. PC12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% donor horse serum (DHS) at 37 °C with 5% CO_2.

2.2. Insulin treatment and Akt inhibitor treatment

To test the effect of insulin, LβT2 cells were seeded at 2×10^5 cells per cm², cultured for 24 h, and pretreated with serum-free DMEM for 12–16 h prior to incubation with 10 nM insulin (1 pmol/L; I9278 Sigma) or 1 μM MK-2206 (Selleck #S1078) treatment. MK-2206 was dissolved in DMSO and DMSO was used as the vehicle control.

2.3. Subcellular fractionation established protocol

The Abcam fractionation protocol is available online at https://www.abcam.com/protocols/subcellular-fractionation-protocol and is widely used for separating cellular components. We have optimized this protocol that we refer to as the Established Protocol (EP) from here on.

Established Protocol isolated separate mitochondrial, nuclear, membrane, cytoplasmic fractions as summarized in Fig. 1A. Cells were grown to confluence in 10 cm² plates. Cell monolayers were washed twice with cold PBS. Next, 500 μL of ice-cold subcellular fraction buffer (20 mM HEPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Halt)) was added and cells were harvested by scraping. Cells were lysed by incubating on ice for 15 min and then by passing through a 27 G needle 10 times. Lysates were incubated on ice for 20 min. The nuclei were pelleted by centrifugation at 720×g for 5 min at 4 °C and the supernatant, which contains the mitochondria, membrane, cytosol, was saved for further processing. The nuclear pellet was washed and resuspended in 500 μL of fresh subcellular fractionation buffer, passed through a 25 G syringe 10 times and clarified by centrifugation at 720×g for 10 min at 4 °C. In the Established Protocol, the supernatant at this step (S6) is discarded. However, we analyzed this fraction (W6) for subcellular fraction contamination markers by immunoblot (Supplem. Fig. 1A). The cleared nuclear pellet was resuspended in 500 μL TBS 0.1% SDS and the clear nuclear suspension was sonicated for 3 seconds at a power setting of 2 in non-continuous shearing of DNA and homogenize the suspension. The supernatant containing the cytoplasm, mitochondria and membrane, was centrifuged for 5 min at 10,000 g to pellet the mitochondria. The supernatant was transferred to a new Eppendorf tube and the mitochondrial pellet was processed exactly like the nuclear pellet. The fraction containing the membrane and cytoplasm was ultracentrifuged at 100,000×g for 1 h at 4 °C. The supernatant was saved as cytoplasmic fraction 1. The membrane pellet was washed, resuspended in 500 μL of subcellular fractionation buffer, passed through a 25 G needle, re-centrifuged for 45 min at the same settings as the cytoplasmic fraction. The wash supernatant was saved as cytoplasmic fraction 2 and the membrane fraction was processed the same way as the mitochondrial fraction. Samples were mixed with 2x Laemmli Sample Buffer (Bio-Rad) containing 200 mM dithiothreitol, resolved by SDS-PAGE and immunoblotted.

2.4. Subcellular fractionation OM protocol

Cells were washed twice with cold 1x PBS and then harvested by scraping in 1x PBS. Subsequently, cells were centrifuged at 12,655 ×g at 4 °C, resuspended in 250 μL of 1x hypotonic buffer (20 mM HEPES pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, complete protease and phosphatase inhibitor cocktail, 1 mM PMSF), and incubated on ice for 30 min. Cells were passed through a 27½ G needle 10 times to maximize lysis and spun down at 2,655 ×g to pellet the nuclear membrane. After BCA quantification of protein concentration, concentrations were normalized to the sample with the lowest protein concentration (~2.5 mg/mL). Lysates were then combined

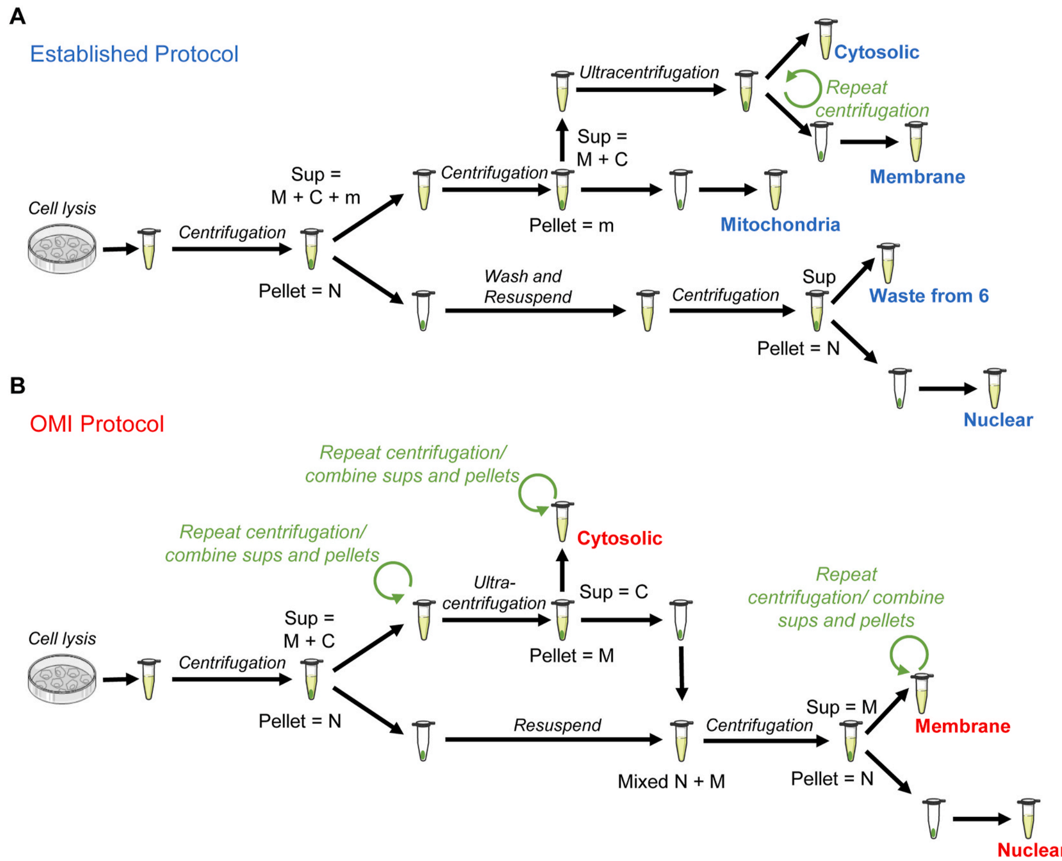


Fig. 1. Schematic of the Established and OMI subcellular fractionation protocols. (A) In the Established protocol, samples followed a centrifugation procedure to produce a pellet containing the nuclear fraction and a supernatant containing cytosolic, mitochondrial and membrane fractions. The nuclear pellet was then washed with fraction buffer and recentrifuged (Waste from 6) and the nuclear pellet resuspended. The supernatant from the initial centrifugation then underwent two centrifugation steps to pellet the mitochondria followed by the membrane fraction. The remaining supernatant constituted the cytosolic fraction. (B) In the OMI protocol, samples underwent centrifugation to produce a supernatant containing membrane and cytosolic proteins as well as the nuclear pellet. The supernatant was then processed by ultracentrifugation to obtain a cytosolic fraction and a pellet containing membrane protein. The membrane and nuclear fractions were combined, followed by centrifugation to obtain the nuclear and membrane fractions. M = membrane, C = cytosol, N = nuclear, m = mitochondria.

in 250 µL of hypotonic buffer. The supernatant was then ultracentrifuged (Rotor TLA 120.2, Beckman Coulter, cat# 343778) at 100,000×g for 1 h at 4 °C. The supernatant was the cytoplasmic fraction. The membrane pellet was resuspended in 250 µL of hypotonic buffer, and the sample was passed through a 27½ G syringe 10 times, and centrifuged for 5 min at 9600 g at 4 °C. The supernatant was combined with the cytoplasmic fraction to a total of 500 µL. The membrane pellet was resuspended in 250 µL of hypotonic buffer, combined with the nuclear fraction and solubilized in NP-40 at a final concentration of 0.5%. The combined membrane and nuclear fractions were centrifuged twice at 2655×g to pellet the nuclear portion. The supernatant then became the membrane fraction. The nuclear pellet was resuspended in 500 µL of hypotonic buffer and sonicated to solubilize. This procedure is summarized in Fig. 1B and can be found in detail in Appendix A.

2.5. Flow cytometry

LβT2 cells were grown in complete medium (4.5 g/L glucose, sodium pyruvate) supplemented with 10% fetal bovine serum. After serum starvation and additional MK-2206 treatment, the cells were washed with PBS, harvested with 0.25% trypsin. Cells were washed in PBS and then resuspended in PBS containing Zombie NIR Live/Dead stain (BioLegend, San Diego, CA, cat# 423105) at 1:400. Samples were incubated for 20 min at 4 °C protected from light, washed with FACS buffer (0.1% BSA, 2 mM EDTA in 1x PBS). Following washing with FACS buffer, cells were blocked with TruStain FcX (anti-mouse CD16/32 antibody, BioLegend, RRID: AB_1574975) in FACS buffer for 20 min at 4 °C protected from light with rabbit anti-GLUT1 antibody (Abcam, ab195020, RRID: AB_2783877) in 50 µL per 1 × 10^6 cells. Cells were fixed according to (Fixation Buffer, BioLegend 420801). Single stain UltraComp eBeads and ArC Amine Reactive Compensation Beads (Thermo Fisher) for compensation. Samples were acquired in duplicate on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA), and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

2.6. Imaging flow cytometry

1 × 10^6 LβT2 cells in 2 mL of medium were seeded for each treatment in a 6 well plate. The following day, cells were serum starved, replacing with serum-free medium. After 16 h serum starvation, GnRH group were treated with 10 nM GnRH, and the remaining groups received PBS as the control. Cells were then trypsinized, and live-dead staining was performed using 1:1600 Zombie Green (BioLegend, cat# 423111) in PBS. Cells were then blocked specifically for the GLUT1 antibody with 1:50 TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (BioLegend cat# 156603, RRID: AB_2783137) in FACS buffer (PBS + 0.1% BSA + 2 mM EDTA) followed by surface staining with 1:400 rabbit anti-GLUT1 antibody (Abcam, ab195020, RRID: AB_2783877) in FACS buffer. The cells were then fixed with 4% paraformaldehyde and with 1x perm/wash

buffer according to the manufacturer (Biolegend, cat# 421002). For intracellular staining, anti-GLUT1 at 1:800 dilution. The cells were then washed with perm/wash buffer and resuspended in PBS for imaging using the Amnis ImageStream Mark II imaging cytometry was repeated three independent times. Data was analyzed with the IDEAS 6.0 software.

2.7. Signaling assays

LβT2 cells were seeded in 12-well plates and allowed to adhere overnight before serum starvation. Serum-starved cells were treated with 1 nM insulin (Sigma I9278) for 30 min at 37°C. For Akt inhibition, prior to insulin stimulation, serum-starved cells were incubated at 37°C with 1 µM MK-2206 (Selleck #S1078) for 1 h and subsequently treated for 30 min with 1 nM insulin. Cells were washed with ice cold PBS, then lysed in 2× LSB containing 200 mM dithiothreitol and boiled. Equivalent volumes of cell lysates were resolved by SDS-PAGE and immunoblotted.

2.8. Immunoblotting

Samples were prepared with LSB containing dithiothreitol and resolved by SDS-PAGE (9% gel). Proteins were transferred to PVDF membranes, which were blocked in 4% BSA and probed for rabbit anti-Na^+/K^+-ATPase (Cell Signaling, Cat# 3010S, RRID:AB_2060983), mouse anti-p84 (GeneTex, 1:2,000, Cat# GTX70220, RRID:AB_372637), mouse anti-GAPDH (GeneTex, 1:5,000, Cat# GTX627408, RRID:AB_11174761), and rabbit anti-GLUT1 (Abcam, 1:2,000, Cat# ab15309, RRID:AB_301844) overnight at 4°C. Rabbit anti-GLUT1 (Abcam, Cat# ab15309, RRID:AB_301844) was previously validated using LβT2 cells and stably transfected LβT2 cells expressing shRNA targeting GLUT1 mRNA (Slc2a1). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, goat anti-rabbit IgG-HRP: sc-2004, RRID:AB_631746 and goat anti-mouse IgG-HRP: sc-2005, RRID:AB_631736, both at 1:15,000) were used at room temperature for 1 h. Blots were visualized by chemiluminescence and imaged using a Pxi system (Syngene). After imaging, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Cat# 21059) for 15 min and reblotted with subsequent primary antibody at 4°C overnight. Quantitative densitometry was performed using ImageJ software 1.52 (https://imagej.nih.gov/ij/download.html). To ensure accurate results, exposures of blots were developed lower than peak. Quantification of bands was done within maximal signal without saturation. To determine the relative expression of proteins by immunoblots, the signal of each marker in a single exposure was transformed to percentage of total signal of that protein. The values were then divided by the sample with the highest expression to derive relative expression. The relative expression of experimental markers was normalized to relative expression of organelle markers to represent enrichment in each fraction to control for fold change. For example, GLUT1 is normalized to the concentration of Na^+/K^+-ATPase enrichment. The percentage of signal each sample was calculated for each target and expressed as the percent signal per target in each subcellular fraction.

2.9. Early endosome antigen 1 (EEA1) immunoprecipitation

Early endosomes from cytoplasmic fractions derived from the MI fractionation were enriched by adapting the approach described (Gosney et al., 2018) (Gosney et al., 2018). Protein concentration was determined by BCA and normalized. Approximately 0.4 µg of EEA1 antibody was added to 400 µL of cytoplasmic fraction and allowed to incubate at 4°C. Protein G beads were washed 3 times in PBS and resuspended. Next, 10 µL of beads were added to each sample and rotated at 4°C for 1 h. The endosomes associated with the anti-EEA1 antibody/G protein complexes were pelleted by centrifugation and the supernatant was saved for analysis by immunoblotting. Samples were then washed 3 times by adding wash buffer followed by centrifugation. Finally, affinity-purified endosomes were eluted by resuspending beads in 30 µL of 2× LSB containing dithiothreitol. For immunoblotting, 15 µL of eluted endosome-associated proteins were used for analysis.

2.10. Extracellular flux analysis

For mitochondrial stress test, LβT2 cells were seeded on poly-L-lysine coated XFe96 plates at 6×10^4 cells per well in HEPES-buffered DMEM supplemented with 10% FBS and antibiotics as described above. Cells were cultured for 24 h then serum starved overnight. Prior to XF analysis, LβT2 cells were washed with extracellular flux (XF) assay media (5 mM HEPES buffered DMEM, 10 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate). The media was changed to XF assay media with or without insulin and equilibrated 1 h before XF analysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using modified mitochondrial stress test procedures as follows: OCR followed after sequential addition of 10 nM GnRH (Inject 1c), followed by 2.5 µM oligomycin (Inject 2), 150 µM 2,4-dinitrophenol (DNP) (Sigma-Aldrich) (Inject 3), and 1 µM rotenone + 1 µM antimycin A (Inject 4) with XFe96 Extracellular Flux Analyzer (Agilent). Data were extracted by Wave software 2.6.1 (https://www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis) and manually analyzed using GraphPad Prism 10.

2.11. Immunofluorescence

Mouse experiments were performed according to approved protocol of the UC Institutional Animal Care and Use Committee. Whole pituitaries were collected from wild-type C57BL/6 male mice at 9–10 weeks of age. Individually, pituitaries were dissected in ice-cold PBS and dispersed individually by incubation with 0.25% collagenase and 0.25% trypsin-EDTA (Life Technologies) as previously described (Nicholes et al., 2020). Primary pituitary cells were seeded at a density of 5×10^6 cells per cm^2 on poly-lysine (Sigma) coated 8-chamber slides (Lab-Tek). The cells were cultured at 37°C and 5% CO_2 in high-glucose HEPES-buffered DMEM with 10% FBS. Next, cells were serum starved for 2 h before treatment with GnRH for 30 min. Cells were washed in ice cold PBS, then fixed for 15 min with 4% formaldehyde in PBS followed by PBS wash. Cells were stained with guinea pig anti-rat LH antiserum from the National Hormone and Peptide Program (NHPP) (Parlow, 2004) at a 1:200 dilution and rabbit anti-GLUT1 (see above). Secondary staining for LH was performed with goat anti-guinea pig Alexa 568 (1:800 dilution, Molecular Probes Cat# A-11075, RRID:AB_141954). Slides were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI). Wide-field images were captured at 60× with a Nikon TE2000-U microscope (Nikon, Melville, NY) equipped with an X-Cite 120 PC collimated light source (Lumen Dynamics Group, Mississauga, ON, Canada), DAPI-1160A-000, FITC-5050A-000, and mCherry filter sets (Semrock, Rochester, NY), using a CoolSNAP monochrome camera (Photometrics, Tucson, AZ) using NIS-Elements software (Nikon America).

2.12. Statistics

All experiments were repeated at least three times in independent experiments and data are presented as the mean ± standard error of the mean. JMP software (SAS Institute, https://www.jmp.com/en_us/home.html) was used to perform statistical analysis

was performed on normalized, or Box-Cox transformed data, to correct for heteroscedasticity. Data were evaluated by t-test or ANOVA and appropriate post-hoc tests when there was a significant interaction. Specifically, data analysis utilized a mixed model with GLUT1 expression as the dependent variable. Both treatment (with insulin or without insulin) and passage were included as independent variables. This mixed model allowed us to account for the response of the cells to insulin being independent of the experiment. This approach adjusts for the effects of passage of cells between experiments. With this adjustment, a t-test was performed with three independent variables. A p-value < 0.05 was considered significant. Asterisks indicate a significant difference from the control. Data sharing a letter are not significantly different from each other, while data marked by letters exclusive to another data point are significantly different from each other. Multivariate regression demonstrated that individual experiments had significant variability. Therefore, statistical tests included adjusted ANOVAs to account for this variability.

3. Results

3.1. An optimized subcellular fractionation method

To determine the distribution of GLUT1 in the gonadotrope, we chose a subcellular fractionation approach with the Established Protocol (Fig. 2A). Subcellular fractionation is achieved via chemical lysis and mechanical disruption and homogenization, generating fragments of organelles and membranes (high-frequency pressure, and/or osmotic shock), followed by a separation of cell compartments based on their sedimentation coefficient during ultracentrifugation. While subcellular fractionation allows quantitative analysis, most fractionation methods for purifying specific subcellular compartments are intended for comparison of subcellular compartments, and we found that many methods of fractionation gave high cross-contamination with LβT2 cells (Windle et al., 1990; Ruf-Zamojski et al., 2018), a mouse gonadotrope cell line. We optimized a fractionation protocol and demonstrate an improvement in the enrichment of fraction marker proteins in LβT2 cells. As a benchmark, we compared a commercially available subcellular fractionation kit (Established protocol, Fig. 1A) to our optimized OMI protocol (Fig. 1B). The OMI protocol contains steps that include intermediate washes and a series of combination centrifugation to improve subcellular fractionation (Supplementary Fig. 1). Fig. 2A shows immunoblot analysis of organelle marker proteins for the nuclear (N), cytosolic (C), and membrane (M) fractions. In immunoblots of the cytosolic and membrane fractions from the Established protocol, nuclear matrix protein p84 (Durfee et al., 1994), though mostly in the nuclear fraction, also appears as a band in the cytosolic and membrane fractions. GAPDH, a cytosolic marker, is present primarily in the cytosolic fraction, but also in the nuclear and membrane fractions. Na+/K+ ATPase, a plasma membrane marker, appears in the nuclear fraction instead of the membrane fraction in the Established protocol. In contrast, the OMI protocol revealed distinct bands for p84 at ~75 kDa in the nuclear fraction, GAPDH at ~37 kDa in the cytosolic fraction, and a band at ~100 kDa for Na^+/K^+ ATPase in the membrane fraction (Fig. 2A). Although GAPDH is present in the membrane fraction with the OMI protocol, the high density of appropriate organelle marker presence in each fraction at equivalent estimated molecular weights and not in the other fractions indicates less cross-contamination.

Quantification of organelle marker presence in each fraction was performed across three independent experiments. The analysis indicated that the OMI protocol exhibited less cross-contamination for nuclear, cytosolic, and membrane fractions. The OMI protocol was significantly improved over the Established protocol with a very high > 97%, 92%, and 95%, respectively, for Na^+/K^+ ATPase (Fig. 2B). Relative to the Established protocol, the OMI protocol minimized cross-contamination of the markers enriched in the cytosolic and membrane fractions. Further, we found that this protocol was applicable to other cell lines, PC12, an epithelial cell line, HeLa, and a human umbilical vein-derived endothelial cell line, EA.hy926, in addition to LβT2 cells. The Established and OMI protocols were used to process LβT2, HeLa and PC12 cells (Supplementary Fig. 2A). Using the Established protocol, GAPDH was observed in PC12 cell cytosolic fractions, while Na^+/K^+ ATPase appeared in the nuclear fraction in HeLa cells. The Established protocol resulted in the presence of GAPDH in both the cytosolic and membrane fractions with Na^+/K^+ ATPase being completely absent from the cytosolic fraction (Supplementary Fig. 2A). This cross-contamination was eliminated by the OMI protocol (Supplementary Fig. 2B), where Na^+/K^+ ATPase, p84 and GAPDH were detected predominantly in their proper cellular fractions. The Established protocol for EA.hy926 cells showed a pattern of contamination like LβT2 and PC12 and HeLa cells (Supplementary Fig. 2C). In contrast, the OMI protocol resulted in fractions enriched in markers for EA.hy926 cells. Overall, when compared to the Established protocol, our optimized OMI fractionation method reduces cross-contamination of nuclear, cytosolic and membrane fractions, while maintaining consistency across a variety of cell types. With the improvement over the Established protocol, the OMI protocol will allow us to accurately quantify the effects of insulin on GLUT1 compartmentalization in LβT2 cells.

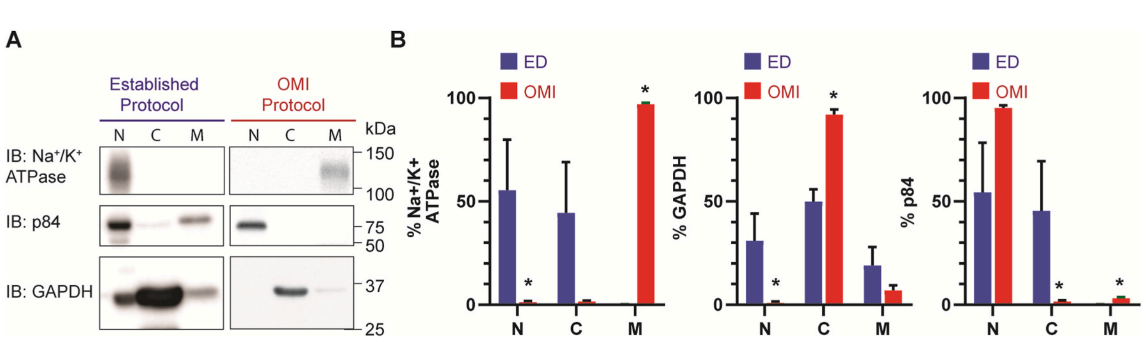


Fig. 2. Optimized fractionation method (OMI Protocol) yields higher enrichment of subcellular markers than the Established subcellular fractionation protocol. (A) Representative immunoblot of LβT2 cell fractions using the Established protocol (n = 4) and the OMI Protocol (n = 3). Na^+/K^+ ATPase is the marker for the membrane fraction (M), p84 for nuclear (N), and GAPDH for the cytosol (C). (B) The percent of subcellular fraction marker in each fraction, quantified from immunoblots, as quantified for the Established protocol (ED) and red bars from the OMI Protocol. The percent of the designated marker in each fraction is the mean ± SEM. Data was analyzed by t-test after transforming with the mixed model with independent variables treatment and passage. Asterisks indicate significance between ED protocol compared to OMI protocol at $p < 0.05$.

3.2. Insulin regulates subcellular localization of GLUT1 in LβT2 cells

Using our optimized OMM protocol, we examined the distribution of GLUT1 protein and Akt in LβT2 cells. GnRH from the hypothalamus stimulates gonadotropes in the pituitary to synthesize and secrete LH, a key regulator of reproduction. We have demonstrated that in response to GnRH, gonadotropes increase expression of glucose transporter 1 (GLUT1) in the cell surface and increase glycolysis (Nicholas et al., 2020). This translocation of GLUT1 to the membrane is required for maximal LH secretion. Furthermore, insulin regulates the translocation of GLUT4 to the cell surface in muscle and adipose tissue, and in addition to GnRH signaling, insulin plays a role in LH production in gonadotropes depending on context (Navratil et al., 2009; Bugeja et al., 2006). In addition, in some cell types, insulin regulates GLUT1 (Cifuentes et al., 2011), which is typically insulin-independent. Therefore, we hypothesized that insulin may increase the translocation of GLUT1 to the surface of gonadotropes as a mechanism to increase glycolysis, which would support LH production.

To partially test our hypothesis, LβT2 cells were stimulated with insulin and the OMM protocol was used to assess the distribution of GLUT1. Since GLUT1 supports LH secretion and insulin augments LH secretion from gonadotropes (Weiss et al., 2003) (Fig. 3). Immunoblotting of GLUT1 in fractions isolated from insulin-treated LβT2 cells after 30 min showed that GLUT1 was localized to the cytosol and membrane as expected in each condition (Fig. 3A). We found that insulin caused a statistically significant increase in membrane GLUT1 (Fig. 3B), as quantified by densitometry. The magnitude of this response is similar to our published quantification of GnRH-induced GLUT1 translocation (Nicholas et al., 2020). Standard GnRH-induced redistribution of GLUT1 was similarly confirmed in LβT2 cells and primary gonadotropes (Supplemental Fig. 3).

If insulin induced an increase in translocation of glucose transporter to the membrane, we expected that it would result in a functional increase in glycolysis. To test whether insulin increases glycolysis in LβT2 cells, we performed extracellular flux analysis as previously published (Nicholas et al., 2020). GnRH stimulation mediated an increase in extracellular acidification rate (ECAR, proxy for glycolysis) in these cells and had minimal impact on oxygen consumption rate (OCR, proxy for oxidative phosphorylation in mitochondria) (Fig. 4A and B). The switch to increased glycolysis is demonstrated by a significant increase in the OCR:ECAR ratio (Fig. 4C). We found that pretreatment with insulin increased basal glycolysis but the OCR:ECAR ratio is similar to GnRH. In addition, we observed that insulin did not inhibit, but further increased GnRH-induced glycolysis (Fig. 4A–C).

Using flow cytometry and surface staining, we confirmed the increase of surface GLUT1 in response to insulin that was observed with the OMM fractionation method (Fig. 4D). Insulin is known to induce phosphorylation of Akt downstream of the insulin receptor in adipose and muscle. This is also true in gonadotropes (Navratil et al., 2009). In insulin-sensitive tissues, phosphorylated Akt mediates the translocation of GLUT4 to the cell membrane. We therefore tested whether the insulin-induced GLUT1 translocation to the membrane in LβT2 cells were dependent on Akt. We used the Akt allosteric inhibitor MK-2206. We have previously demonstrated that it inhibits phosphorylation and activation of Akt in these cells (Molina-Inglis et al., 2021). Surprisingly, we found evidence that the insulin-induced increase in membrane GLUT1 were independent of Akt, as seen with treatment with MK-2206 (Fig. 4D). In fact, MK-2206 caused increased GLUT1 surface expression when used alone in LβT2 cells. We conclude that GLUT1 is significantly linked to increase the surface of glycolysis in gonadotropes through non-conventional signaling.

3.3. Optimized fractionation method reveals localization of pAkt in the cytoplasm of LβT2 cells

Although Akt is known to regulate docking and fusion of GLUT4 vesicles to the plasma membrane, we found that insulin-induced GLUT1 translocation was not impacted in the presence of the Akt inhibitor MK-2206, which targets the closed Akt conformation preventing T308 and S473 phosphorylation (Barnett et al., 2005). Given these findings, we examined insulin-induced phosphorylation of Akt in LβT2 cells using anti-phospho-Akt-S473 antibody (Fig. 5A and B). We found that Akt is phosphorylated in response to insulin in LβT2 cells as early as 2 min post stimulation, with phosphorylation persisting to 1 h. We next used the OMM protocol to determine whether we could assess the subcellular localization of insulin-stimulated phosphorylated Akt in LβT2 cells. LβT2 cells were treated with insulin for 30 min prior to fractionation using our OMM protocol and the fractions were immunoblotted for phosphorylated Akt in the various fractions. We found that total Akt was localized to the cytosol and the plasma membrane. Remarkably, phosphorylated Akt was primarily associated with the cytosolic fraction (Fig. 5C and D) and insulin caused a marked significant 5-fold increase in phosphorylated Akt.

Several groups have shown that phosphorylated Akt is localized to the cytosol in association with endosomal membranes (Siess and Leonard, 2019; Ebner et al., 2017; Živić et al., 2018). To determine whether the phosphorylated Akt we observed is associated with endosomes, we probed the cytosolic fraction of LβT2 cells for endosomal specific markers Rab5, member of the Ras-like small GTPase superfamily important for endosome targeting, and early endosome antigen 1

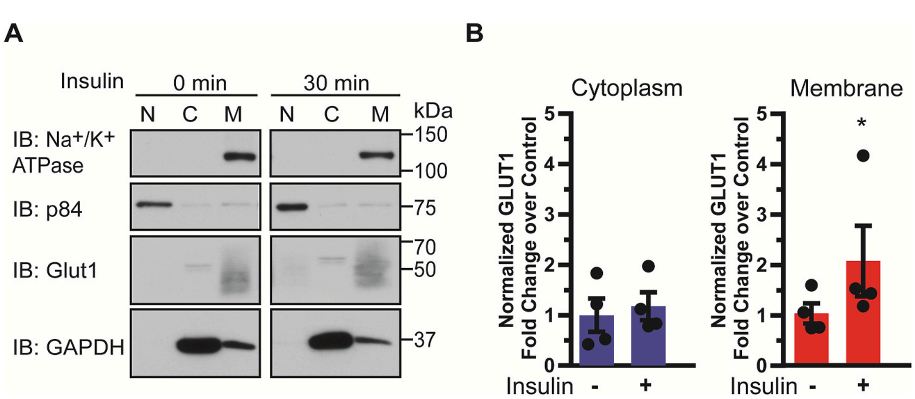


Fig. 3. In response to insulin, GLUT1 traffics to the membrane in LβT2 cells. (A) Representative immunoblot of subcellular fractionation of LβT2 cells probed for GLUT1 in response to 30 min insulin treatment. (B) Protein expression of GLUT1 in immunoblots was normalized to organelle markers (specific normalization: p84 for nuclear, Na+/K+ ATPase for membrane, and GAPDH for cytosolic). Data are presented as mean ± SEM. Data were analyzed by student's t-test comparing insulin treated with untreated control separately in each fraction, *$P < 0.05$. N = 3–4.

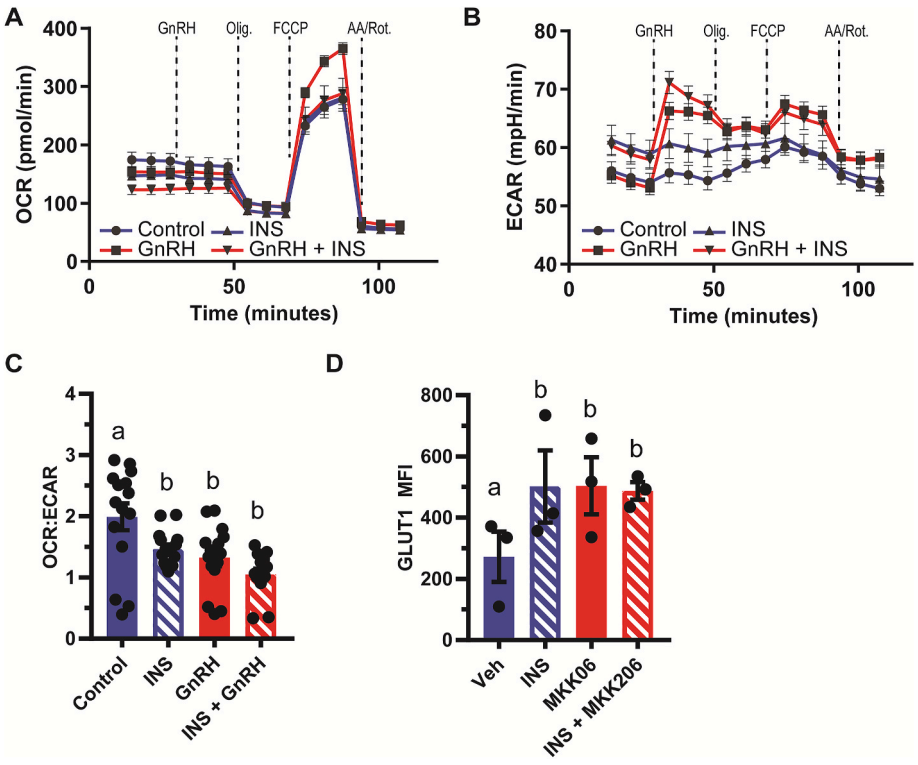


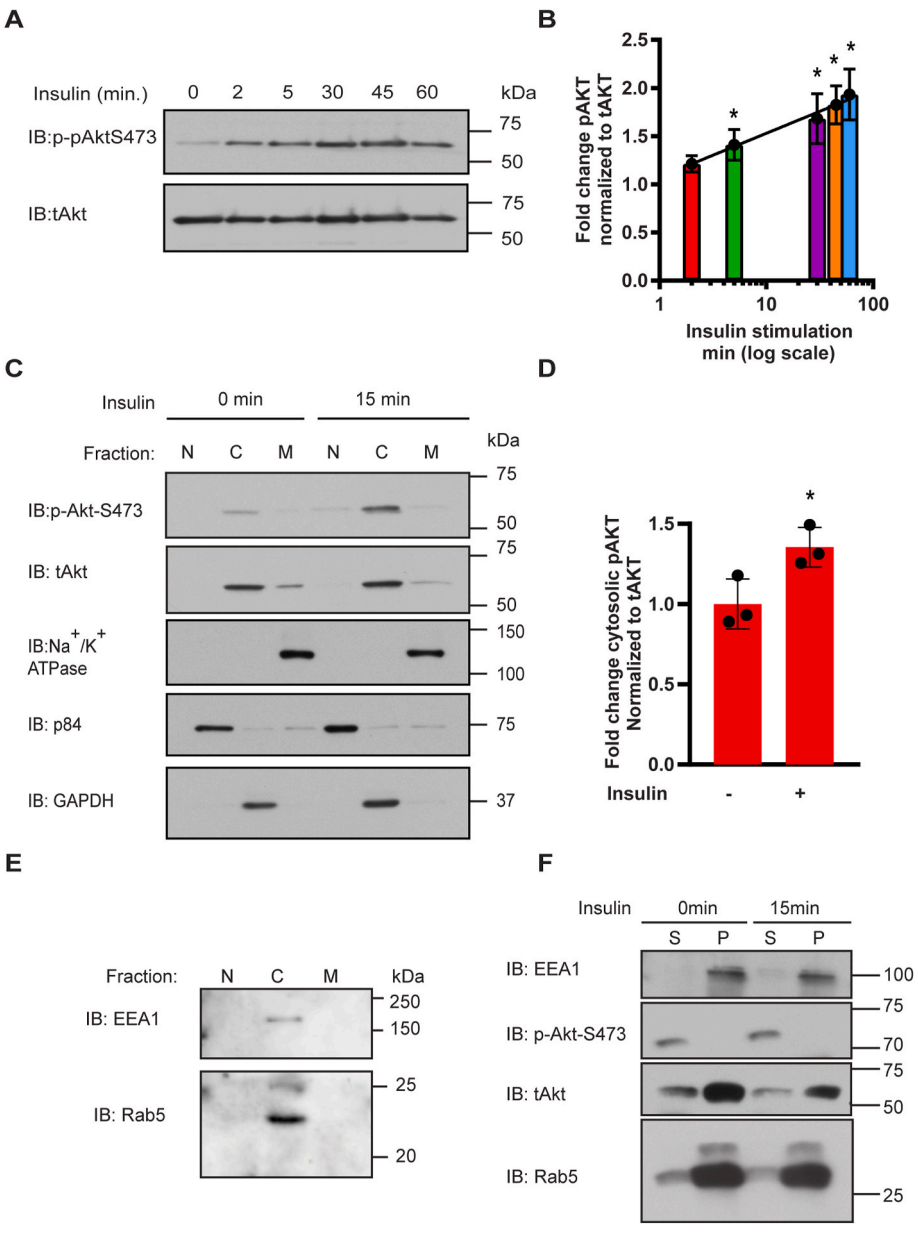
Fig. 4. Insulin induces glycolysis and mitochondrial respiration in GnRH neurons. Mitochondrial respiration (A) and glycolysis (B) from mitochondrial stress test assay in GT1-7 cells treated with vehicle (Control), insulin (INS), GnRH, or GnRH + INS. Cells were treated with oligomycin (Olig.), FCCP, and antimycin A/rotenone (AA/Rot.) at the indicated time points. Data are presented as mean ± SEM. (C) OCR:ECAR ratio from mitochondrial stress test assay in GT1-7 cells treated with vehicle (Control), INS, GnRH, or INS + GnRH. Data are presented as mean ± SEM. (D) Surface GLUT1 expression in GT1-7 cells treated with vehicle (Veh), INS, MKK06, or INS + MKK206. Data are presented as mean ± SEM. Different letters indicate significant differences between groups ($p < 0.05$).

(EEA1), membrane receptor tyrosine kinase 5 (ephrin type-A receptor 5), and insulin-sensitive endosomal vesicles (GLUT4) (Fig. 5E). We then immunoprecipitated insulin receptor substrate 1 (IRS1) and 2 (IRS2) and probed for GLUT1 to determine if GLUT1 localizes to the insulin receptor signaling complex. We found that GLUT1 co-immunoprecipitated with IRS1 and IRS2 (Fig. 5F). Together, these data suggest that GLUT1 localizes to insulin-sensitive endosomes in GnRH neurons.

Using immunofluorescence and confocal microscopy, we found that insulin increased the number of GLUT1 puncta in the perinuclear region of GnRH neurons (Fig. 6A–C). Using a biotinylation assay, we found that insulin increased the amount of GLUT1 in the plasma membrane fraction of GT1-7 cells (Fig. 6D–F). We then performed a real-time glucose uptake assay in GT1-7 cells and found that insulin increased glucose uptake (Fig. 6G–I). Together, these data suggest that insulin increases GLUT1 trafficking to the plasma membrane in GnRH neurons.

4. Discussion

We have utilized GnRH neuron-specific deletion of the insulin receptor to demonstrate that insulin signaling in GnRH neurons is required for normal fertility in female mice. Specifically, we found that deletion of the insulin receptor in GnRH neurons impairs the preovulatory LH surge, ovulation, and fertility in female mice. Moreover, we found that insulin increases glycolysis and mitochondrial respiration in GT1-7 cells, a model of GnRH neurons. Finally, we found that insulin increases GLUT1 trafficking to the plasma membrane in GT1-7 cells. Together, these data suggest that insulin signaling in GnRH neurons is required for normal fertility in female mice and that insulin increases glucose uptake in GnRH neurons by increasing GLUT1 trafficking to the plasma membrane.



F i g 5 . P h o s p h o A y k l t a c t a d i t z h e e s y t o p h a a t p m l a s m m e a m b r a n i e e n d o s o m a c t i n h β T n 2 c e l (A) R e p r e s e i n m a t u r i v o d e f l o s t u l i n - s t i m u p A k t e i d n c e o u i s h β T 2 c e l (b) Q u a n t i f c a p A k i d m m u n o b l e p r e s i e n (B) t D e a d t i a p r e s e a s m e a n S . E (M = 4) S . i g n i f w c a s n c e d e t e r m b i y A n N e O d V a A n p l o s t o a c n a l w y s i D h s n n s e c t o t m p a r t i c s o c o m m t t r e o s h B o x - C o a n s f v o a r h m u A e s l s t . e r i n s k i a c s a i t g e n i d i c t a h e (P e n c e < 0 . 0 5 r) o t m i n 0 e n i n (C) R e p r e s e i n m a t u r i v o d e f c e t e f l u r l a c r o i L β T n 2 c e l p l r s o b f e o A r k a n d A k a t f t 1 e 5 m i n s t i m u l w a i t t i h o s u (D) i n . Q u a n t i f c a p A k i d m m u n o b l f r o t t h s e y t o p l a r s a m c i r t c e i p o n e s i e n (C) t D e a d t r a e p r e s e a r t o s p o o f t i o o t p e A l k f t o u i n d t h G e f r a c a f i b e n r n o r m a l i t z d a o t A a k a t h n d i p r e s e a n s m e a n S . E a M W a a n a l y z S e t d u d s e t - n t t e (s = t 6) P , < 0 . 0 (5) R e p r e s e i n m a t u r i v o d e e l m o t o n s t r a t i n e n d o s o m a a k E E A 1 a n R a b a 5 r a e s s o c w i a t t h e e y t o s y s o u l b c e I f h a c t a r i (n = a t) (F) D R e p r e s e i n m a t u r i v o d e f l u o l i c e l a s s u b a c i o a f t i o n p A K T β T 2 c e l w l e s r s e t i m u w a i t t i e n d s u f l o l r 5 m i n T . h e β T 2 c e l w l e s r t e h e f n r a c t i a s i a (C) e a c h d l h e y t o s f o r l a i c t f i r o u p h T 2 c e l w l e s r e i m m u n o p r e c v i i p t a i m t t a i t - e E d E A 1 b o T h S e s p e r n (a S t) a e b f e t h i a n f d t i e m m u n o p r e c a i n p t d i h P e e t l (R) t h e e n d o s o m a c t w i e o p e r , o b e d b y i m m u n o l (n = 3 t) , .

A c l e p a o r t e m n t e i c a h l a r o i f i s m s u i l m i p r a o o n l β T 2 c e G L U T 1 t r a n s l o c o a u t o i e t o h m r o T X N l a P t , r a n s c f i a p c t t h o a i n t s g l u c s o e s n e s i l t n r i e v s e p . d o n s i e g g h l u c T X N e l P o s l e e p e n d e n p t h l o y s p h o A y k w i a t e l a n d l o s o m y s e e s p a r e a n t d o n s g f o m e n t s h e i n c r e (C a h s o e i s E l a r 2 k 0 , 2) 3 l b l o p k s d u c t G L U T 1 R N A w h i s i e m u l t a p r e o o n u s G L U T e 1 n d o c y (W o s i a s 2 0 , 1) 3 . K n o c k d a o n v o h v e r e x p e e x p s e i r o i e n e e g a s c h e t m l o y n s t r a p t h e o d r y l A a k t t h e e d a y , t h e p l a s m e a m b r a n a n d a l c o s c y t o s a o n b l i c t h a t a o f T X N l i P n c r e g a l s u e c u o p s t e a (F k a r i e k a h 2 0 , 0) G i v e n p o t e n t h u a c l l s e y a b r s t R a c t e s s t t u d c i h e a s l I t e h n p i g a e r a d i g m , i n s u i l n β T n 2 c e l c l o s u b l e d t h A e k t - i n d e p p a e t n h d w e a n y t m o t i p l y G L U T 1 t r a n s l o c a t i o n . s u g g e s t h a A r k g u e e d o m a i n t i a n i t n e r a v c i t t i h r o m s p i h t o s l - i n g o l i a p t i h d e s l a s h m y a s , o s e m o d l s , c o m p a t h e e n r d o m e m b r a n e (S i e a s n d L e o n a 2 r 0 d 1 ; E b n e r e t a l 2 0 , 1 ; Z ú ñ i g a e t a l 2 0 , 1 ; 8 S u g i y a m a a l 2 0 , 1) 9 . I n d e s t u d s i h e o s w h a A t k t i s r a p i d l y

dephosphorylated in lipid membrane rafts. In contrast, our results support a different model in which Akt in βT2 cells is activated in lipid rafts, and phosphorylated Akt appears in the cytoplasm (Fig 5C), and Akt that is associated with the endomembrane fraction in cells is not phosphorylated (Fig 5F). The observation that total phosphorylated Akt is found in the cytoplasm 15 min after insulin treatment, combined with our observation that Akt remains phosphorylated at 1 h after insulin treatment in βT2 cells, suggests that in this system, Akt may not significantly associate with the endomembrane. While this is in apparent contrast to previous reports that experimentally demonstrate Akt at the endomembrane, it is possible that Akt is continuously being phosphorylated. Additionally, it is possible that phosphorylated Akt is rapidly dephosphorylated after being released, as has been suggested by others (Manning and Toker 2017). While our observations suggest that Akt could be phosphorylated when associated with cell membrane fractions, further investigations are required to identify these individual Akt isoforms at these observations, as they may explain why the canonical insulin signaling pathway in gonadotropes is intact.

In conclusion, our observations in βT2 cells were made possible by the optimized OMI fractionation. The findings in this cell line, using GLUT1 protein localization and that phospho-Akt may not be associated with the endosomal membrane, form the mechanisms in gonadotropes that provide a basis to understand the observed clinical impact of hyperinsulinemia on reproduction.

CRediT authorship contribution statement

Olivia Molina: Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Waiting: Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. Anjali Varma: Writing – review & editing, Visualization, Investigation, Formal analysis. Zenia Desmund: Writing – review & editing, Visualization, Methodology, Investigation. Jonas Medina: Writing – review & editing, Investigation, Formal analysis, Data curation. Alyson Cozzi: Writing – review & editing, Investigation, Formal analysis, Data curation. Oscar Muñoz: Writing – review & editing, Investigation, Formal analysis, Data curation. Lu Yeom: Writing – review & editing, Investigation, Formal analysis, Data curation. Visualization, Methodology, Investigation, Formal analysis, Data curation. Trin: Writing – review & editing, Visualization, Methodology, Investigation, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. Zhang: Writing – review & editing, Supervision, Resources, Funding acquisition. Nicholas: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

None.

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Appendix

OMI Subcellular Fractionation Protocol.

A. Reagents

Name	Company	Catalog Number
Phosphate Buffered Saline (PBS)	Gibco	14190-144
Pierce Protein Kit	Thermo Scientific	23225
Bovine Serum Albumin	Thermo Scientific	23210
Nonidet™ P-40 Substitute solution	Sigma Millipore	98379
Protease Inhibitor	Roche	11697498001
Phenylmethylsulfonyl Fluoride (PMSF)	Calbiochem	52332

B. Recipes: Hypotonic Buffer*

* Buffers are to be kept at 4 °C. For each experiment, make fresh hypotonic buffer.

S t o H c y k p o t B u n i f c e r			
R e a g e n t	S t o C c k n c e n t r a t i o n	V o l u m e	F i n C a o l n c e n t r a t i o n
T r i s - p H H C . l 4	1 M	1 m L	2 0 m M
N a C l	5 M	0 . n 5 L	1 0 m M
M g C l 2	1 M	1 5 µ 0 L	3 m M
d d H 2 O	N / A	4 8 . n 8 L 5	
	F i n \ a o l l u = m e	5 0 m L	1 3 : 2 "

W o r k H y g g o t B u n i f * c e r
* O n t h y v e o r k h i y g g o t b u n f i v c e h b e u s e d d u r i e n x g p e r i m e n t s t a t t o h c y k p o t b u n f i c e r .

W o r k H y g g o t B u n i f c e r			
R e a g e n t		V o l u m e	F i n C a o l n c e n t r a t i o n
H y p o t S o n l u c t i o n		9 . n 9 L	N / A
P r o t e a s e i b R i o t c o h r e s		1 t a b l e t	N / A
P M S F		1 0 µ 0 L	1 0 n 0 M
	F i n \ a o l l u = m e	1 0 m L	

C . P r e p a r a t i o n

- C o o d l o w n e n t r i t f 4 ü C g . e s
- M a k 1 e 0 m L w o r k h i y g g o t b u n f i c e r .
- K e e w p o r k h i y g g o t b u n f i c e e o h i c e .
  - M a k 1 e 0 % N P - 4 0 l u t i o n .

D . P r o t o c o l
G e n e N r o a t l e s .

- 5 t d 1 0 m i l l a i d o h n e r c e e n (t t 1 s 0 m 2 l i s a h r) e e q u f o s e u d f f c p i r e o n t y t e i i e n l d .
- K e e c p e l o h i s c t e h r o u g t h e e n t i o r f e h y r o t o c o l .
  - R e a t d h r o t u g e h n t p i r r e t b e o f o b e g i n n i n g .

C e H a r v æ n s d t y s i n g .

1 . G e n t w l a y s c h e l w l i s t 5 h m L o f c o P B S 2 t i m a s p i r P a B t S i f n t g e a r o w h a s h .
2 . A f t s e e r c o P n B o W a s h a d 2 1 0 µ 0 L o f P B S .
3 . S c r a c p e e w l i s t c h e s l d r a a p n e d r o l l i e p o c r t e - c l a i n p l f r e e l l a E h p e p l e e n d l u b r e f .
4 . A d d a l n o t 2 h 0 e µ 0 L o f P B S f o t h p e l a s t c e r , a c p e e l c o s l , l a e n c d t o , m b i n e t o s e a m E e p p e n d l u b r e f .
5 . S p i d r o w n e l a l t s C a t 1 0 × 5 g f o 3 r m i n .
6 . A s p i P B S a n d e s u s p h e p e n d l i l 2 5 µ 0 L o f w o r k h i y g g o t b u n f i c e r .
7 . V o r t f e 1 r 0 s a n d i n c u b a i t c f e e o 3 r 0 m i n .
8 . H o m o g e u n s i z a t 2 g 7 C S y r i a n t g e e a 1 s 0 t t i m t e o s n s u t h e e l a l r k e y s e d .

B C A a n d N o r m a l i z a t i o n .

1 . P e r f a B r C n A a s s u a s y i y n c g u p r r e f e m r e r t e h d o b p d l r o t q e u i a n n t i f W c e a u t t i i o t n h P e i e e B C A P r o t A e s i s n a y .
2 . U t i l a i p z l a r t e a c t e d r e t e b d e o l o r i c n h e a t n o g i f t e h s e a m p C e a s l c u c l o a n t c e e n t f r r a o t n h B e C n A a s s b a y c a l c u l t a h p e i r o g e i n c o n c e n t t r h a r t o i a B g S h A s t a n d c a u r n d v æ n u a o l v l i y t a h p e r o t c e o i n n c e n t c r a a l t c i u o s n a t f i t o w n a r e .
3 . N o r m a l i s l a e n p i l r e s r e a n g 2 . n 0 g / m t L 2 . n 5 g / m b L y a d d i e n q u a m l o u n t p s r o t t e o n i e n E p p e n d l u b r i e f a s f n a v l o l u o m f e 2 5 µ 0 L o f h y p o t b u n f i c e r .
4 . O p t i C h n a i l s p o s s i t b o l p e p o i n S t a t m p c l a b s e s t o a r t e C l u n t n i e l x d t a y .

S e p a r a t C i y c t r o s F o r l a i c c f i r d V m e m b r a n e l u c l C e a m p o n e n t s .

1 . C e n t r i f i n e u g r e m a h i o z n e o d g e f n o 1 r 0 n e i a 2 6 5 × g a t 4 ° C .
2 . S a v t e h p e e l a n e s t u p e r n i a n t d i a n f f e t r u e b n E t h p e e l l c e o n t s t a h i n e u c l f e r a a r c a t i n o d h n s e u p e r n c a o t n a t n a t i i n t s c h o n d r i m e m b r a n e l y t o s p o r b i t e e i n s .
3 . C e n t r i f i n s e u g p e e r n f a r t o a m n 2 p o 1 r 0 n i a 2 6 5 × g a t 4 ° C .
4 . S a v t e h p e e l a n e s t a v s e u p e r n i a n t d i a n f f t e r u e b n A s i . n s t 2 p t h p e e l l c e o n t s t a h i n e u c l f e r a a r c a t i n o d h n s e u p e r n c a o t n a t n a t i n s m i t o c h o n n e d m r b i r a a , n e l y t o s p o r b i t e e i n s .
5 . T r a n s f i n s e u p e r n f a r t o a m n 4 p i n h o g s h p e e d - c e n t t r u i b f e u s g . a t i o n
6 . W e i g t h h e i g h - s u p l e t e r e d a c e n t t r u i b f a e u n s g d a n t e c i i c o m m t e U n s t e s h v e e i g t h p t r e p b a a r l e a t n u c b e e s .
7 . C e n t r i f i n s e u g p e e r n f a 0 1 a h n a t 9 s 6 0 × g 0 a 4 ° C i n a n u l t r a c e n t r i f u g e .

8 W i t h d o i u s t r u p h t p i e n l g g y e n t w a y s t h h p e e l l f e r t o p s m r e v i s t o u e 2 p a s n 4 l t w i w c i e t 2 l 5 µ 0 L o f h y p o t w o n r i k c t i o n g f A e r t t e h r e s e c o v r a d s a n d , 2 l 5 µ 0 L t o n p e e l f e s t u , s a p r e t h h d e t r r a n t s h f r e e r s u s p p e e n l d t e d e d h t s e e c o t n u d c t e e n t a i p n e i l n R g e s u s p p e e n d c o m b i t h p e e l l f e r t o s m t e 2 p a s n 4 l i n a t o t v a o l l u o m f 2 5 µ 0 L *. T h e s s t e c c p a s h n e c o m p l d e u t r e t d h 1 g h o f u l t r a c e n t r i f u g a t r e s u s p p e e n l d d e e d t s n a u i c n l p e r a o r t s e d s i a n v s e i c f e o f r u r p h r e c e s s i n g .

9 .T r a n s t i p e r n f a t a t m h t e s t r a c e t h a n r e i v E p g e n t l o b T e h . i s s u p e r n i a y t o a u n r t e a c r y e t d o s f o r l a i c t i o n .

1 0 R e s u s p l e n e d l m a i m e i m l g r p a e e i l 2 5 µ 0 L o f h y p o t b u n f i a c e n p l a s t s h r o 2 l 7 G i y r i 1 n 0 g i e m t e d s o m o g e n T i h z i s e s t . e p m a x i m t i h z y e i s o l f n d e m b r p a r n o c e t a e n i d h n s c r e a s e v e y y o p s o d t i e c i n s .

1 1 W i t h h e o m o g e n p i e z l e r d e p S a t e 6 p a s n 7 l .

1 2 A d d l h s e u p e r n f a r t o t a n h r e t e p e a s t e r p d t h E e p p e n d l o w f t t h c e y t o s f o r l a i c t i o n s m 0 f p o a r t o t v a o l l u o m 5 e 0 µ 0 L T h i i s t h f e n a c l y t o s f o r l a i c t U s i e h r e e m a i m e i m l g r p a e e i l e h r e e x s t e c t i o n .

S e p a r a M i e o m b r E a n a c t i o n V n u c l F e r a a r c t i o n .

1 R e s u s p l e n e d l m b r p a e e i l 2 µ 5 L o f h y p o t b u n f i a c e n d o m b w i n e t h r e e s u s p r e u n d l e p e d e a l r f l o a r t t o t o a 4 l 7 µ 5 L .

2 A d 2 5 µ L o f 1 0 % N P 4 0 .

3 .V o r t f e 1 r O s .

4 .I n c u l s a t h e f l o 3 s 0 m i o n i c e .

5 .C e n t r f 1 u 0 g i e a 2 6 5 x E g a 4 ° C .

6 .T r a n s f i s e u r p e r n t a a t n a e n E p p e n d l o a e f n s l a v t e h p e e l T h e p e i l y e c t u n r u c l f e r a a r c t i o n .

7 .C e n t r f i f i s e u g p e e r n a a s t e a c n o t t n i d h f e o 1 r 0 m i a 2 6 5 x E g a 4 ° C .

8 .C o l l t h e s e t u p e r n a a n t s l a a m a t e s f n a m l e m b r á n a c S á v a t e h p e e l T h e l i s y o u 2 m d u c l p e e a l r f l e n t t h . t i u s b t e h p e e l w l i e b l e t e r y s m a s l d h a , a n d l h s e a m p c l a e r e f u l l y .

9 .G e n t w a y s t h h r e e m a i p n e i h g e t i s m v e i s t h h y p o t b u n f i d e i r s , c a r l d b e i u n f g f e a c t h i m e .

1 0 C o m b i t h p e e l l e n a t t s o t o a 5 l 0 µ 0 L o f h y p o t b u n f i a c e n s l a v a e s t h f e n a n l u c l f e r a a r c t i o n .

1 1 S o n i c t a n t e e m b r a n e d u c l f e r a a r c t a 1 L o f 1 % s m p l i f t o 1 L D s e .

A p p e n B L i S x u p p l e m e d n a t t a a r y

S u p p l e m e d a r a t t a t m h y a i s t i c a l h e f o u o n d l a i n e t h t p s : / / d o i . o r g / 1 0 . 1 0 1 6 / j . m c e . 2 0 2 4 . 1 1 2 4 0 5

D a t a a v a i l a b i l i t y

D a t w a i l b l e m a d æ v a i l o a n b l e q u e s t .

R e f e r e n c e s

A n d r a J d Q e , u , i n J n B , e c k R e . r Z S . h , u p M i . k A 2 . 0 , 1 A 8 M P - a c t p i v c a t k e i d h n a s e i a k e i y n t e r m e d G i n a R H y - s t i t n d H g l a e n t e r d a n s c M i o p l E t . n i c b o n c . r i n g , B a l t M i c h 2 . 7 8 , 2 – 8 8 3 . 9

B a r - T L e M e , t a , l 2 . 0 , 1 F 5 o l o e P l 4 a K n l E l 3 K - i A E K R T K 1 a / c 2 t i v b a y G i n E R H n t h p e i t u g b a n a y d o t M r o o k P . e l E l n . d o c r 4 i 1 n 5 b , 2 1 2 . 3

B a r n S t . R e . t a , l 2 . 0 , 0 5 d e n t i f a c n a d t h i a c r n a c t e o r p i z e a c t k i s o t n r i n - h o m o l d o m a i n - d e a p n e d n s d o b e e m n t z y m e A - k s i t p n e h c i o b E d t o c r h s E 8 n 8 . 5 3 9 – 9 4 0 . 8

B u g g C s . e , t a l 2 . 0 , 0 6 n s u a l u i g m n e G n n t R s H - s t i t n d l b e g t e a n d e x p r e l s y s i o n E g r M d C . e l E l n . d o c r 2 i 4 n 9 9 , 9 1 1 . 0 . 6

B u g g s - S C a . 2 , 0 d A e , g u l a n d G i l o u n c T o s a e n s p 1 o (r S t l e c r 2 a t 1 n P e i t u i t a r y G o n a d o t o M o i p æ e f t P e u r b e v t o) ,

B u S t o l o r C 2 a M e o a , l 2 . 0 , 1 B n s u r l e i s n i s h y a p e e e , i n s u a l n d h e r e a l a t i v e o r o l i e p s e r i p u d o l e e r s t i a t l y - a h s y s p o c e r i a a n t d e r d o J g C e l n i E m m . d l a c r i n o l . M e t a 1 b 0 . 3 2 , 5 7 2 1 5 8 . 2

C h o E i . , - P l a r , S k . , - 2 J 0 . 2 , B X N l a P k : e y r o t i e n t i h m e e l s u t l r a v e r e s s p o n s e p a t h v a r a y p o t e n t h i e a r l a p t e a u r t g E i e x c p M . o M . e d 5 . 5 1 , 3 4 1 8 3 5 . 6

C h o s J C e h a , l 2 . 0 , 1 A C u n t e c a p i t o u t l h a h e t y i p o e n r i n s u h d n e m i a h y p e r l i p h a l e m a c a d o e f m r e i t s a t b i s o y l n i d c r s o u m p e p r g e s s a e d o t r o p i n s O b e S . i . l S p e r M r d @ 5 5 , 5 – 5 6 . 0

C i f u e M n . t e , e t a s l 2 . 0 , 1 1 n s u r l e i g n u G a L U e T s 1 - m e g l i u a c t o r s h a e n s i p M G r - t 6 3 h u m a o n s t e o s a c r e c l d M G s e a . P l h . y s 2 2 0 d L , 4 2 5 4 3 . 2

D i s t L L A S e a r , g , e M , o r J e . y E S e , f t H e C 1 , 9 , 7 F 3 i . t u i r t e a s p y p o n s t i o v e n e s s l u t e i h o i z m o g e - r h e o l r e m a o s m i e m g u l i n - d b l e p a e b m e d e d e n t s t i t u s . D i a b e 2 4 2 8 7 5 – 8 8 . 0

D u r f T e M , a n c M n A J . o , n E S E , l l e S l g J e , e V , H 1 9 , 9 7 4 h a m i n o - t e r m i n a l r e g i o d t h r e e t i n o b g l e a s p e r o o n d l a u i c n t a d n s o v r e u l c l m e a a t r p i r x o t t e h i a n t c o - l o c t a c l e i n z t e f e s R N s A p r o c e s J S C i e n B g l . c l 1 2 . 7 6 , 0 – 9 6 2 . 2

E l b n M r . L , i c í | L e o n A T r . e V , u , d u s h K 2 i 0 n 1 F l . (3 , 4 e , n 5 g) a R g 3 e m r e e s n t t r i c t A k a t c t i v o i e t l y l n u e l m a b r r a M h o e l S . e 6 5 4 , 1 – 6 4 3 1 . e 6

G o s n J e . y A V . i , l k D e . y W V M e , r c h M u n L C . e , r e B s . a P 2 , 0 , 1 R 8 r . o t e o m e i v e n s o l v s e l p r o t a e s i s n o c i v a i t t e h o n e l s n y d o s o i m a e n e s p i d e g r m o a v l f t a h c t o r - d e p e n d e n t m a n n e d B i o C l h . e n 2 9 3 5 , 8 9 5 9 0 . 8

H a r M i . s M e . t a , l 2 . 0 , 1 C 2 n R H l n c r e g a l s u e c t o r s a e n s p e r e x p e r e a s t i o n s t i m u g l a u t e u s p s t e a i k n t e h g e o n a d o t U r E o m p d h o . c r 2 i 1 n 2 o , 3 1 – 9 1 4 . 7

L a w s M d n , A e . t a , l 2 . 0 , 0 B v i d e f r o c n e s u s l u i p r e o s f s a i s o e n l u i t e i n i z i n g h o r m o i n m v e o m e v n i t p h o l y c o y v s a t r i s i c y a m d r a o m n d e o r m v a o l m e J h C l i n . E n d o c r M i e n t o c a 0 b 3 2 , 0 8 2 9 0 9 . 6

L i c c i J . e , t a l 2 . 0 , 1 C o n f o r m a s t a i m o p n l o a i n f n e g m b r a b n y A e l s c t o n t r o b s i s a c t i v a n t d i n o a n c t i R A a d N d a i a o t A l c . a S l c U . S A . 1 1 5 E , 3 9 – 4 E 0 3 9 4 9

M a n n i B n . g D T . o , k e A r . 2 , , 0 1 A K T / P s K i B g n a n l a i v n i g g : a t h n e e n g w C r e k L . 6 9 , 3 8 – 1 4 0 5

M o l i n a r O . l e n a g l 2 . 0 , 2 d 1 P . C / P A B 1 f e e n r d s o t h a e n l t i i a - l a p a o c p t t i o v t i a t c y a d i s c p r - e a t r e r , e s t i n - S 2 p - h n K e 1 d - i S a 1 t P s R d g - n A a k x t i P h s r g . o N c a . t A l c . a d . S c U . S A . 1 1 8 e , 2 1 0 6 6 2 3 1 1 8

N a v r a A t M e l . a , l 2 . 0 , 0 9 n s u a l u i g m n e g n o t n s a d o t r o p i h n o - r m e c i l m e e d a u s c i n i g o n o f t r a n s l a t o b i e c t m a E 2 M l o s I C . e l E l n . d o c r 3 i 1 n 1 0 , 7 5 . 4

N i c h c D l . a A e . t a , l 2 . 0 , 2 C G L U T 1 - m e g l i y a c t e s t b u y p s p i G s n R l s l - i n d u c e d s e 9 C y r o t f l i u o t n e i h o i z m o f g r e o f m e m a g l o e n a d o t S r c o R p e e p l s . 0 1 , 3 0 6 3

P a r i H k . l e , t a , l 2 . 0 , 0 7 X N l r E g u l æ t r e i s p g h l e u r a m d e s t e a b o i l h i u s m m a P l s . o S M e d 4 . e 1 5 8

P a r l A o w F 2 . 0 , 0 M a t i e l h o a r m h o a n n e E e p t P i r d c e g (A l H P P e , v r e c o m b i n a n t h o r m o h y e p o t h a p l e a p m t i i n d c a t e s u h r o a r l m o n e v a n t i a e n d a x p a n d e d h o r m o a n s e s s a e y r v a i v c a e i s l J a C o l l i E m n . d o c r M i e n t o c a E b 9 3 , 6 1 3 8 6 2 . 0

P r o j a l s e , t a , l 2 . 0 , 1 P 4 o l y c o y v s a t s r i y c n d r i o m n e u r l e i s n i s i a n d o b e s , i t y : n a v i g a t h p e a n g h o p h y s i a l o l y o l g m i J t C h e . p r M o e l d 2 . 0 1 7 4 1 , 9 0 . 5 0

R u f - Z a m F o . e , t a k 2 1 0 , 1 9 y . t o g e g n e r t o i m a i n d o f , u n c t i o n a l a c t e o r f i z a t i o p i t u i g b a n a y d o c t e r l o b j n e J e E s . n . d o S c r C 3 . 9 0 – 2 9 2 2 . 0

S a n t N l r e o a , l 2 . 0 , 2 C l o n a d o t r r e o s p p i d m o i s n e s u a l n i t h r i p i n d f u s e i p o n o d u c e s t h r e e p r o m e s y s t o b d f o o f o b e e s i n e t u y m e n o r l r e h a v e o i m c e a r : a n d o m i z e d - c r o s s t o r v i E a e l r . S f i e 1 . 1 6 5 , 6 – 6 5 7 . 4

S i e k s . s M L e , o n a T r . o A 2 , . 0 , 1 9 i . p i d - d e a p k e t n - d i e v M n i e t w y c h : , e a n d l o w . B i o c h S e o m T . r a r 4 3 8 , 9 – 7 9 0 . 8

S t e p N t . d K e . t a , l 2 . 0 , 1 W 8 o m e v n i t p h o l y c o y v s a t s r i y c n d r h o a m v e e n t r i n s u i t c i n r e s i s o t n e r o u g e l y c a e m i c - h y c p l e a m H p u n n s R u e l p a r e 0 m d i E c n g 2 l 8 . , 7 7 – 7 8 . 4

S u g i y M l m G F , a , i G n D A . n , t o n e C s . M 2 0 , 1 A 9 k t - u i p j g a s h o e v t e r y w h e r e : d m p a r t m e n A k a s o p t e i c v i a f n t o f l u o m c t i r m e n c e p t y o o k s l i m a s e s i g n a F l r i o n G g e . D e v B . i o 7 l 7 . 0

W e i s i s M P . o , l a S c . B , i , e d r K i . C , h t , m a C n . 2 , 0 0 E 3 f . f e o d f t n s s u o l n l i u n t e i n i z i n g h o r m o a m e d r o l a s c e t c i r e a t n i d o a n l c s i u g n n a i l r f i e m g a r l a p t e i t u i c t e a l r l y s . A r c G h y . n e C o l s . t 2 e 6 t 9 4 , 5 5 0

W i n d J u e W e , i n R e . r M e , l I P o . n L 1 , 9 , 9 0 E l l n o e f t s h p e i t u i g b a n a y d o t r o p e i n t e d a e g r e i b v y e a l r g e n t e d g e i n n t e r s a i n s s r g i e c M i o c E n . d o c r B i a n l o t l i m . M d 4 5 , 9 – 7 6 0 . 3

W u N . e , t a l 2 . 0 , 1 A 8 M P . K - d e p e d e n g l e a d o a f f t X N o u n P o e n n e r s g t y r l e e s a s t d o s e n h a n g c l e u d c u o p s t e a v k i G a L U T M 1 o . I C . e 4 9 1 , 1 6 7 1 7 . 5