JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v2i2.108

Normalization of protein at different stages in SILAC subcellular proteomics affects functional analysis

Kerry L. Inder[1], Dorothy Loo[1], Yu Zi Zheng[2], Robert G. Parton[3], Leonard J. Foster[2], Michelle M. Hill[1]*

[1]The University of Queensland Diamantina Institute, Brisbane, Queensland, 4102, Australia; [2]Centre for High-Throughput Biology and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4; [3]Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia.

Received: 30 August 2012 **Accepted:** 12 November 2012 **Available Online**: 16 November 2012

ABSTRACT

Quantitative subcellular proteomics is a powerful method to interrogate spatial dynamics of cells or tissues. Stable isotope labeling by amino acids in cell culture (SILAC) is a popular quantitative approach that is ideally suited to subcellular proteomics because samples can be combined very early to reduce technical variability in the subcellular fractionation and downstream processing. However, validation of results using orthogonal methods such as immunoblotting do not allow mixing of samples prior to fractionation, leading to potentially different outcomes. Here we have investigated the impact protein normalization before or after subcellular fractionation has on the functional analysis and experimental conclusions. As a model system, we compared the detergent-resistant membrane (DRM) fraction of mouse embryonic fibroblasts (MEF) from caveolin-1-null mice with wildtype controls. Caveolin-1 is cholesterol-binding protein which is essential for formation of plasma membrane caveolae, a subtype of lipid raft membrane microdomains. Surprisingly, we found that the relative protein content of DRM as a percentage of total protein content is 1.6 fold higher for Cav1-/- MEF compared to wild type MEF, leading to different SILAC ratios in pre fractionation mix and post fractionation mix experiments. Most of the observed differences were replicated by mathematical modeling of the normalization effect, with the striking exception for mitochondrial DRM proteins. Interestingly, caveolin-1 affected DRM proteins in the post fractionation mix data showed a significant enrichment of the mitochondrial oxidative phosphorylation pathway, which was not observed in the pre fractionation mix experiment. The observed quantitative changes in mitochondrial DRM proteins using different analyses suggest a caveolin-1 induced change rather than simple contamination, and may support recent reports of caveolin-1-dependent mitochondrial cholesterol changes. Based on these results, we recommend a thorough understanding of how experimental conditions impact relative subcellular fraction in order to make an informed decision on the most appropriate point to combine SILAC samples for quantitative subcellular proteomic analysis.

Keywords: Quantitative subcellular proteomics; Organellar proteomics; Caveolin-1; Caveolae; Lipid raft; Detergent resistant membranes.

Abbreviations:

SILAC, Stable isotope labeling by amino acids in cell culture; **DRM,** Detergent resistant membrane; **MS,** Mass spectrometry; **MEFs,** Mouse embryonic fibroblasts.

1. Introduction

The functional organization of the cell is central to its biological activity. Trafficking to specific subcellular compartments facilitates the functional activity of proteins. While some proteins predominantly localize to a particular organelle, many proteins traffic to multiple locations where they exert distinct functions [1,2]. Understanding the spatial con-

***Corresponding author:** Michelle M. Hill, Level4, R-wing, Princess Alexandra Hospital Ipswich Rd, Wooloongabba, QLD, 4102; E -mail adress: m.hill2@uq.edu.au; Phone number: +61 7 3176 7774; Fax number: +61 7 3176 5946.

figuration of proteins and how this influences cell processes has therefore been an important question since the advent of cell biology. Over the last decade, proteomics has emerged as a powerful method to study the total protein complement of cells or tissue. More recently, proteomics has proven to be a successful approach to characterizing the repertoire of proteins within subcellular compartments [3,4]. This technology presents researchers with a high-throughput method to assign protein localizations. Furthermore, a particular organelle of interest can be focused upon or instead changes in protein distribution after perturbations can be assessed.

In general, subcellular proteomics is achieved through subcellular fractionation to enrich for organelle/s of interest. Well established protocols are available for enrichment of most subcellular compartments. Organelles such as the nucleus and mitochondria are easily purified based on their density and size and as such the protein complement of these organelles has been well documented using proteomics [5-7]. However, enrichment of some organelles such as the endoplasmic reticulum (ER) and Golgi apparatus are less straight-forward resulting in considerable levels of contaminating proteins [8]. Often, validation using techniques such as western blotting and electron microscopy is needed to distinguish bona fide organelle components from contaminating proteins. Subcellular fractionation also reduces sample complexity and increases detection of low abundant proteins. Whole cell analyses contain large amounts of protein which generates an overwhelming number of peptides. This is further complicated by large deviation in protein expression levels, ranging up to as much as 10 orders of magnitude [9]. As a result, low abundance proteins, which are often important regulatory proteins such as kinases, are concealed by highly abundant proteins. To date, organellar proteomics has been performed on the phagosome, lysosome, lipid rafts, exosomes, plasma membrane, clathrin-coated vesicles, spliceosome, nuclear pore, nucleolus, ER-Golgi intermediate compartment(ERGIC) and peroxisome, highlighting the depth and scope of this technique [3,8].

Recently, quantitative approaches have been applied to subcellular proteomics enabling the comparison of organelles under different experimental conditions. Stable isotope labeling by amino acids in cell culture (SILAC) is particularly well suited for subcellular proteomics. This method involves metabolic labeling of cells in culture with different isotopic labeled amino acids that can be distinguished by the mass spectrometer [10]. One advantage of SILAC is that samples can be mixed prior to the subcellular fractionation processes, eliminating variation between sample handling. The reduction in sample number simplifies and speeds up the otherwise time consuming and often cumbersome subcellular fractionation protocols. In addition, the disparity between mass spectrometry runs is also circumvented, significantly reducing the error rate.

Combining quantitative methods with subcellular proteomics has allowed the spatial dynamics within cells to be interrogated on a large scale. Emmott et. al. [11] used SILAC to study the host subcellular proteome in response to infection with Coronavirus Infectious Bronchitis Virus. Work by Dhungana and colleagues [12] focused on the detergent resistant membrane (DRM), a fraction enriched in lipid rafts, of macrophages in response to lipopolysaccharide treatment. They found that compartmentalization and activation of the 26S proteasome in DRM mediates activation of the MAPK pathway. More recently, we used quantitative SILAC and subcellular proteomics to investigate the role of caveolin-1, an integral membrane protein, in the aggressive prostate cancer cell line PC-3 [13]. We analyzed total membrane, DRM, prostasome, and secreted fractions. Our results suggested a role for caveolin-1 in modulating the lipid raft environment that accentuates secretion pathways possibly via ER sorting.

In the current study, we have investigated the widely accepted practice of mixing SILAC samples prior to subcellular fractionation and importantly, the impact of this on the functional analysis and experimental conclusions. In our model we have studied the protein caveolin-1 which is a major structural protein of caveolae. Caveolae are specialized lipid raft microdomains on the plasma membrane that are characterized by their flask shaped invaginations [14]. Caveolae are involved in many cellular processes including endocytosis, lipid regulation, and signal transduction [14]. Caveolin-1 deficient mouse embryonic fibroblasts (MEFs) have been widely used to ascertain the functions of caveolin-1 and caveolae [15, 16]. Here we have employed SILAC and subcellular proteomics to compare wild type MEFs and caveolin -1 deficient MEFs. We focused on DRM that includes caveolae and non caveolae lipid rafts and investigated the consequence of mixing SILAC samples before or after fractionation. Interestingly our results clearly demonstrate dramatic differences in the functional analysis between the different mixing steps. Our results caution the general practice of mixing SILAC samples prior to fractionation and instead recommend a thorough understanding of the changes in biology caused by experimental treatments in order to make an informed decision on the most appropriate point to combine SILAC samples for quantitative subcellular proteomic analysis.

2. Material and Methods

2.1 *Cell culture and SILAC*

Immortalized wild type (WT) and Caveolin-1-null (Cav1-/-) MEFs were generated as previously described [16]. MEFs were grown and maintained in DMEM lacking Lysine and Arginine (Sigma) with 10% dialyzed FBS (Bovogen) and supplemented with the following amino acids: '0/0' for the normal isotopic Lys and Arg (Sigma) and '8/10' for $^{13}C_6{}^{15}N_2$-Lys and $^{13}C_6{}^{15}N_4$-Arg (Silantes). Cell populations were amplified 200-fold in the labeling media to achieve > 99% incorporation as confirmed by LC-MS/MS. For each analysis, two 15 cm plates of cells were used for detergent-resistant mem-

brane extractions.

2.2 *Detergent-resistant membrane (DRM) preparation*

Detergent resistant membranes (DRMs) were prepared as previously described [13].

2.3 *LC-MS/MS, database searching and SILAC quantitation*

Protein samples (30ug) were separated on a 10% SDS-PAGE to 8-9 mm and stained with colloidal coomassie. Protein gel slices (1 mm) were excised and de-stained with a solution of 50% acetonitrile in 25mM ammonium bicarbonate before reduction with 20mM DTT (Sigma) and alkylation with 50mM IAA (Sigma). The gel pieces were subsequently pH adjusted with 50mM ammonium bicarbonate and dehydrated before overnight digest at 37°C with 0.01mM Trypsin (Promega) in a buffer of 10% acetonitrile and 50mM ammonium bicarbonate. Samples were extracted with 60% acetanitrile, 1% formic acid, dried in a speed-vac and resuspended in 10µl of 5% v/v formic acid for LC-MS/MS.

Peptides were analyzed using a 1200 Series nano HPLC and Chip-Cube Q-TOF 6510 (Agilent Technologies). Peptides were resolved using the Agilent large capacity HPLC chip (G4240-62010) 150 mm 300 Å C18 chip with 160nL trapping column. A 45 minute gradient from 10% to 45% solvent B was used. Solvent A was 0.1% formic acid and solvent B was 0.1% formic acid and 90% acetonitrile. HPLC loading pump was set to 3% B, flow rate of 4uL/min while analytical pump was set to 10%B and flow rate of 0.3uL/min. Q-TOF mass spectrometer was programmed to acquire 8 MS spectra/sec and 4MS/MS spectra/sec with dynamic exclusion after 2 MS/MS and released after 0.2min.

Mass spectrometry data was analyzed using Spectrum Mill (Agilent, B.04.00.127) search engine. Data was extracted with carbamidomethylation cysteine and SILAC amino acids N-Lys, $^{13}C_6{}^{15}N_2$-Lys, N-Arg and $^{13}C_6{}^{15}N_4$-Arg as a fixed/mix modifications. Extracted data were searched against SwissProt (release-2010_03 containing 23,000 entries) mouse database with carbamidomethylation cysteine and SILAC amino acids N-Lys, $^{13}C_6{}^{15}N_2$-Lys, N-Arg and $^{13}C_6{}^{15}N_4$-Arg as a fixed/mix modifications as appropriate, and oxidized methionine as variable modifications. Precursor and product mass tolerance was set to +/- 20ppm and ±=/- 50ppm respectively. Reverse database scores were calculated with Spectrum Mill search engine. All peptides identified had a global false discovery rate of less than 0.9%. Protein identification cut-offs were set to protein score > 11, peptide score > 10 and scored peak intensity > 60%.

Single peptide identifications were excluded from further analysis. Mean SILAC ratio (L/H) and standard deviation was calculated using all the peptide ratios matched to a protein, and p-values were calculated using the peptide SILAC ratios.

2.4 *Assignment of gene ontology and functional enrichment analysis*

Proteins with a SILAC p-value < 0.05 were submitted to GeneGo for identification of Gene Ontology (GO) terms over represented in each list. Correction for multiple hypothesis testing was performed by controlling for the False Discovery Rate at p = 0.05. Subcellular localization was assigned using UniProt.

2.5 *Mathematical modeling*

Representative SILAC fold changes (20 values in total) ranging from -10, -8, -6, -4, and -2 fold decrease to a 2, 4, 6, 8, and 10 fold increase were used in the model. Half of the SILAC fold changes (10 values) were a SILAC fold change of 1 since the majority of proteins remain unchanged. The pre fractionation values were adjusted by 1.6 fold to account for the increase contribution from Cav1-/- MEFs. The post fractionation values were left unchanged. The predicted outcomes were plotted as a line graph.

3. Results and Discussion

3.1 *Sample preparation and mass spectrometry analysis*

Using SILAC subcellular proteomics, we investigated if normalization of protein amount before and after subcellular fractionation would impact upon the functional analysis. To this end, we compared the subcellular proteomes of WT MEFs and Cav1-/- MEFs. Given that caveolin-1 is necessary for caveolae formation, we analyzed DRM, a fraction enriched in all cholesterol dependent lipid raft domains. Typical of SILAC subcellular proteomics, we combined equal amount of protein after cell lysis but prior to fractionation from SILAC-labeled WT MEFs and Cav1-/- MEFs, then iso-

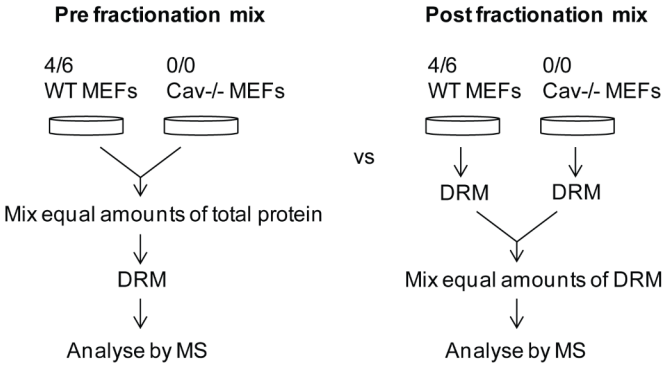

Figure 1. Work flow comparing mixing SILAC samples before or after subcellular fractionation. Typical of most SILAC subcellular proteomics experiments, SILAC labeled cells were lysed and equal levels of total protein were combined pre fractionation. DRM were purified from the combined sample and then analyzed by mass spectrometry (MS). In comparison, DRM was isolated from each SILAC condition (4/6 and 0/0) and then equal amount of DRM was combined (post fractionation) and then analyzed by mass spectrometry (MS).

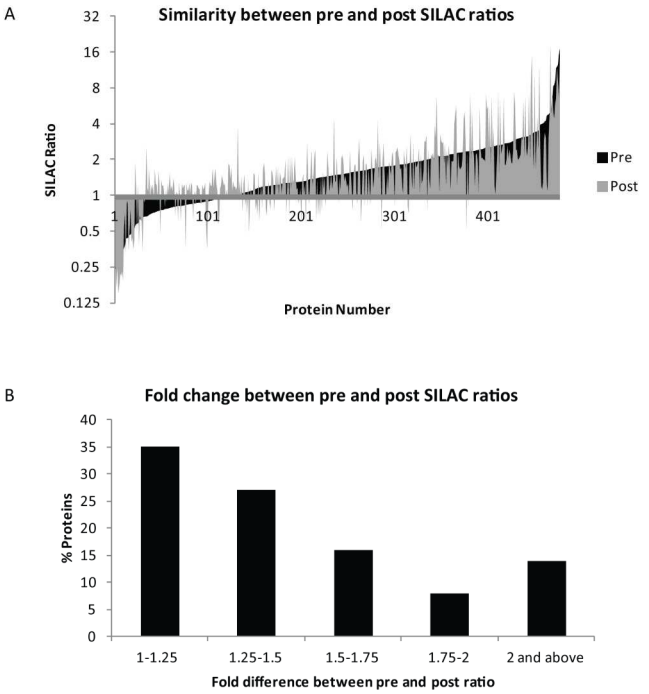

Figure 2. Similarity of SILAC ratios between mixing methods. (A) Pre fractionation mix SILAC ratios were sorted in ascending order and plotted (black). The corresponding post fractionation mix SILAC ratio was overlayed in grey. (B) Bar graph representing the fold difference in SILAC ratios from pre fractionation and post fractionation mix methods.

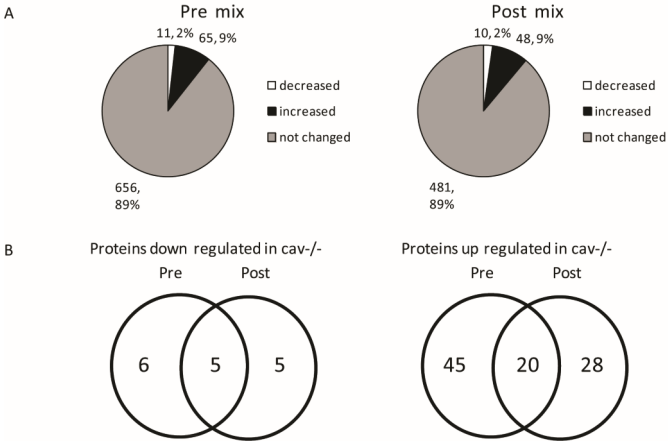

Figure 3. Proteins regulated by caveolin-1 in pre fractionation and post fractionation methods. (A) Pie chart indicating proteins not changed, increased or decreased (mean±2SD) in the pre fractionation and post fractionation mix methods. (B) Overlapping up regulated and down regulated proteins (mean±2SD) from pre fractionation and post fractionation methods is displayed in Venn diagrams.

lated the DRM for LC-MS/MS analysis (referred to as pre fractionation mix) (Figure 1). To compare combining SILAC samples downstream of fractionation, we separately isolated DRM from SILAC labeled WT MEFs and Cav1 -/- MEFs then combined equal amounts of DRM prior to LC-MS/MS (referred to as post fractionation mix) (Figure 1).

SILAC ratios were determined by calculating an overall mean for each protein using SILAC ratios for peptides from 3 independent biological replicate experiments (Supplementary Table 1). We examined the similarity of the SILAC ratios produced from the pre fractionation and post fractionation mixing methods for all overlapping protein (Figure 2A). We found that 62% of proteins had ratios within 1.5 fold and 86% displayed less than 2 fold variation (Figure 2B). These data indicate that normalization of protein amount pre fractionation or post fractionation generates similar SILAC ratios for the majority of proteins. However, a small subset of proteins (14%) showed a greater than 2 fold difference in SILAC ratios between mixing methods.

3.2 *Analysis of proteins regulated by caveolin-1*

To discover proteins affected by the absence of caveolin-1, we analyzed proteins that were significantly different between WT MEFs and Cav1-/- MEFs. Proteins that had a SILAC ratio two or more standard deviations from the mean and a p-value less than 0.05 were considered significantly different (Figure 3A). The pre fractionation method identified 11 (2%) down regulated proteins and 65 (9%) proteins upregulated from a total of 732 proteins (Figure 3A, left panel). The post fractionation mix method produced very similar percentages with 10 (2%) proteins that were down regulated and 48 (9%) upregulated proteins from the 539 proteins quantified (Figure 3A, right panel). Despite the comparable percentages, only 5 down regulated proteins were common to both mixing methods from the 11 and 10 quantified proteins respectively, and 20 up regulated proteins common from 65 and 45 identified (Figure 3B). Analysis of the overlapping proteins significantly altered by caveolin-1 (Table 1) revealed extensive variation between pre and post fractionation SILAC ratios. This was unexpected since we had shown considerable similarity between SILAC ratios when assessing all quantified proteins. It is likely that the small percentage of proteins that have a greater than 2 fold variation between SILAC ratios are biologically responsive to loss of caveolin-1.

Since the pre fractionation mixing is normalized by total protein amount, one potential explanation of the observed difference is that Cav1-/- MEFs contain a different proportion of DRM compared to WT MEF. To address this possibility, we measured DRM protein amounts from the two cell types and expressed it as a percentage of the starting total cellular protein amount. This experiment determined that the DRM constitutes 0.51 ± 0.06 SEM % of total protein in WT MEFs and 0.84 ± 0.09 SEM % in Cav1-/- MEFs (Figure 4A). This equates to a 1.6 fold increase in protein recruitment to DRM. While biologically interesting, this result has serious implications on the SILAC pre fractionation mixing method since the Cav1-/- MEFs will contribute more protein to the mix than WT MEFs. It is probable the SILAC ratios will be skewed making the data difficult to interpret. To understand how this result could potentially influence our data, we mathematically modeled the effect on the pre and post fractionation mix methods. Since equal levels of DRM protein was combined in the post fractionation method, the

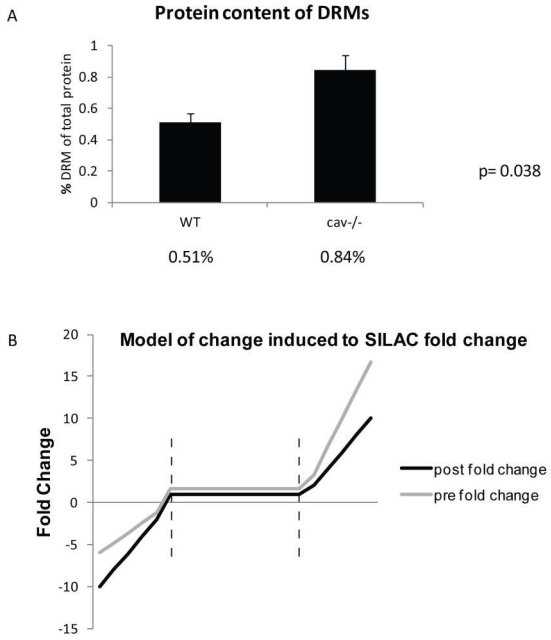

Figure 4. Loss of caveolin-1 increases protein recruitment to DRM. (A) Protein content of DRM and whole cell lysate was determined using Bradford assay. The graph shows the percentage of DRM protein represents from total cellular protein (WT MEFs = 0.51 ± 0.06 SEM % and Cav1-/- MEFs = 0.84 ± 0.09 SEM % of total protein). Using student T-Test, there is a significant difference between WT MEFs and Cav1-/- MEFs, p = 0.038. (B) Model of the affect that increased DRM protein in Cav1-/- MEFs is predicted to have on pre fractionation mixing and post fractionation mixing. The pre fractionation mix method fold changes were adjusted to reflect the predicted 1.6 fold increase contributed by Cav1-/- DRM protein.

Table 1. Overlapping proteins altered by caveolin-1

ID	Protein Name	Pre ratio	Post ratio
Q9JM51	Prostaglandin E synthase	0.27	0.15
P01831	Thy-1 membrane glycoprotein	0.30	0.18
Q9JLI3	Membrane metallo-endopeptidase-like 1	0.32	0.21
P21995	Embigin	0.27	0.25
P97449	Aminopeptidase N	0.24	0.26
P25911	Tyrosine-protein kinase Lyn	8.52	4.39
Q9CYL5	Golgi-associated plant pathogenesis-related protein 1	3.47	4.54
P62071	Ras-related protein R-Ras2	4.93	4.57
Q3UMR5	Coiled-coil domain-containing protein 109A	5.35	4.66
Q9D379	Epoxide hydrolase 1	8.36	4.92
P21956	Lactadherin	9.26	5.86
Q9DCJ5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	3.09	6.03
Q9CU24	Protein THEMIS3	11.76	6.48
Q8VHL0	Urea transporter 1	15.27	6.62
Q9WV54	Acid ceramidase	3.12	6.64
Q6X893	Choline transporter-like protein 1	6.59	6.85
P19783	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	3.14	6.91
P12787	Cytochrome c oxidase subunit 5A, mitochondrial	3.25	8.49
Q6ZQM8	UDP-glucuronosyltransferase 1-7C	12.56	8.80
Q9CPQ1	Cytochrome c oxidase subunit 6C	3.42	9.77
Q80Z24	Neuronal growth regulator 1	12.17	11.58
P56391	Cytochrome c oxidase subunit 6B1	3.73	11.67
Q61468	Mesothelin	17.13	13.69
P48771	Cytochrome c oxidase subunit 7A2, mitochondrial	3.25	14.56
Q7SIG6	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2	4.95	17.29

SILAC fold change was left unchanged. This was compared to the pre fractionation mix method, which the SILAC fold change was adjusted by 1.6 fold to account for the predicted increased contribution by Cav1-/- MEFs (Figure 4B). The model calculated a minor increase in the SILAC fold change for pre fractionation mixing compared with post fractionation mixing for proteins that were not altered by caveolin-1 (fold change between the broken lines). However, in stark contrast, proteins that were altered by caveolin-1 (fold change outside of the broken lines) were predicted to show variation between mixing methods that radically increased as the fold change increased. More specifically, our model predicted that protein down regulated in Cav1-/- MEFs (a negative fold change) in the post fractionation mix exhibited an increased SILAC fold change in the pre fractionation mix thereby making it less significant. The opposite trend was predicted for up regulated proteins with an increase in the SILAC fold change with pre fractionation mix compared to post fractionation, making it appear more significant. Consistent with this model, we found the majority of overlapping proteins showed relatively similar SILAC ratios between mix methods, but large differences in proteins altered by caveolin-1 expression. This would have a major impact on whether a protein is considered significantly altered or not. This may also explain the lower percentage of overlap of altered protein we observed between mix methods.

We next examined our list of overlapping altered proteins to see if they behaved as the model predicted. Table 2 shows that 11 of the 25 of the proteins did behave as predicted, a small subset of proteins (5) showed no change in SILAC ratios between mix methods, while 9 proteins showed opposite trend to the model. Given that DRM is derived from membrane from all cellular compartments including plasma membrane, Golgi, and ER, we also assigned the subcellular location using Uniprot to our list of altered proteins (Table 2). Interestingly, the altered proteins that behaved as the model predicted were found to be predominately membrane/cell membrane localized. However, those that behaved in the opposite manner to the model were mainly mitochondria and Golgi localized. These data suggest that there is

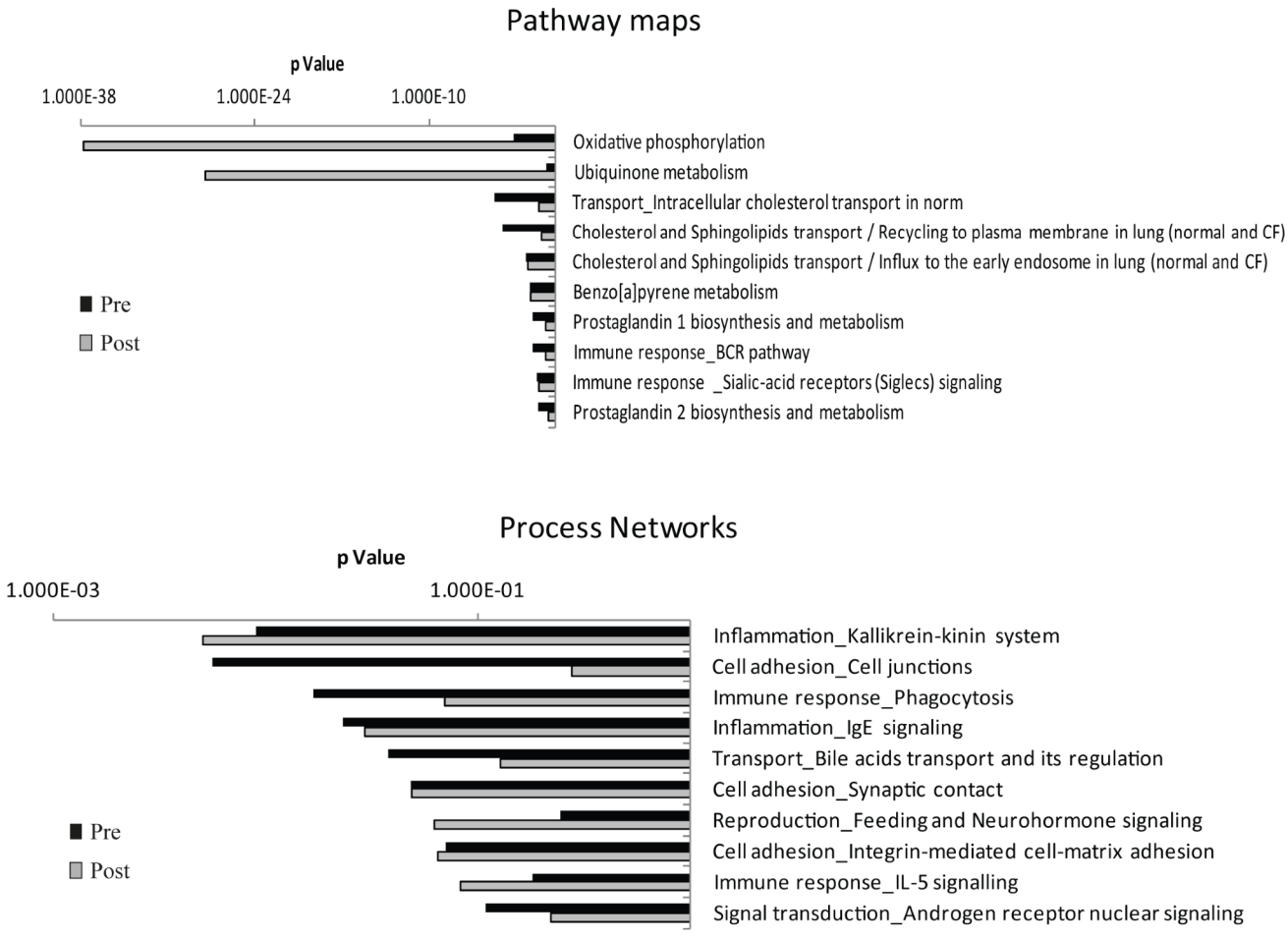

Figure 5. Mixing method impacts on the functional pathways enriched in the significantly altered protein list. Bar chart of GeneGo analysis displaying the most significantly enriched pathways and biological processes for pre fractionation mixing and post fractionation mixing.

Table 2. Proteins altered by caveolin-1, compliance with model and the subcellular localisation.

ID	Protein Name	Behave as modeled	Subcellular Location
P25911	Tyrosine-protein kinase Lyn	Yes	Cell membrane. Nucleus. Cytoplasm. Cytoplasm > perinuclear region. Golgi apparatus.
P01831	Thy-1 membrane glycoprotein	Yes	Cell membrane; Lipid-anchor > GPI-anchor.
Q61468	Mesothelin	Yes	Cell membrane; Lipid-anchor > GPI-anchor. Golgi apparatus. Secreted.
Q8VHL0	Urea transporter 1	Yes	Cell membrane; Multi-pass membrane protein. Basolateral cell membrane.
Q9JM51	Prostaglandin E synthase	Yes	Membrane; Multi-pass membrane protein.
P21956	Lactadherin	Yes	Membrane; Peripheral membrane protein. Secreted.
Q9JLI3	Membrane metallo-endopeptidase-like 1	Yes	Membrane; Single-pass type II membrane protein. Secreted.
Q9D379	Epoxide hydrolase 1	Yes	Microsome membrane; Single-pass type II membrane protein. Endoplasmic reticulum membrane; Single-pass type II membrane protein.
Q6ZQM8	UDP-glucuronosyltransferase 1-7C	Yes	Microsome. Endoplasmic reticulum membrane; Single-pass membrane protein.
Q3UMR5	Coiled-coil domain-containing protein 109A	Yes	Mitochondrion inner membrane; Multi-pass membrane protein.
Q9CU24	Protein THEMIS3	Yes	unknown
Q80Z24	Neuronal growth regulator 1	No change	Cell membrane; Lipid-anchor > GPI-anchor.
P62071	Ras-related protein R-Ras2	No change	Cell membrane; Lipid-anchor; Cytoplasmic side.
Q6X893	Choline transporter-like protein 1	No change	Cell membrane; Multi-pass membrane protein. Mitochondrion outer membrane; Multi-pass membrane protein.
P21995	Embigin	No change	Membrane; Single-pass type I membrane protein.
P97449	Aminopeptidase N	No change	Membrane; Single-pass type II membrane protein.
Q7SIG6	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2	No	Golgi apparatus > Golgi stack membrane; Peripheral membrane protein. Cell membrane; Peripheral membrane protein. Cytoplasm
Q9CYL5	Golgi-associated plant pathogenesis-related protein 1	No	Golgi apparatus membrane; Lipid-anchor.
Q9WV54	Acid ceramidase	No	Lysosome.
P19783	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	No	Mitochondrion inner membrane.
P12787	Cytochrome c oxidase subunit 5A, mitochondrial	No	Mitochondrion inner membrane.
Q9CPQ1	Cytochrome c oxidase subunit 6C	No	Mitochondrion inner membrane.
P48771	Cytochrome c oxidase subunit 7A2, mitochondrial	No	Mitochondrion inner membrane.
P56391	Cytochrome c oxidase subunit 6B1	No	Mitochondrion intermembrane space.
Q9DCJ5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	No	Mitochondrion. Mitochondrion intermembrane space.

probably not a global up regulation of all DRM proteins in Cav1-/- MEFs. Rather, caveolin-1 has distinct effects on DRMs from different subcellular compartments. Although caveolin-1 is critical for plasma membrane caveolae, it has also been identified at multiple other locations with no morphological caveolae, including the Golgi [17], endosomes [18] and the mitochondria [19]. However the role of non-caveolar caveolin remains unclear.

3.3 *Functional analysis of caveolin-1 regulated proteins*

To discover how the pre and post fractionation mix methods impact upon functional analysis of proteomics data, we performed pathways enrichment analysis using GeneGo software on the proteins significantly altered by caveolin-1 from both mixing methods (Figure 5). Post fractionation mixing data suggested that caveolin-1 regulated DRM proteins involved in oxidative phosphorylation and ubiquitone

metabolism (Figure 5, top panel) however, these pathways were far less significant with the pre fractionation mix method. In addition, pre and post fractionation mix methods revealed caveolin-1 regulated inflammation but cell adhesion was dramatically over represented in the pre fractionation mix method (Figure 5, bottom panel). Therefore, these results clearly demonstrate that different conclusions are derived when mixing pre or post fractionation.

Specific quantitative differences in the mitochondria DRM proteins were observed between pre and post fractionation mixing set up (Table 2). The post fractionation mix method highlighted a role for caveolin-1 in regulation of mitochondrial proteins and oxidative phosphorylation. Identification of proteins annotated as mitochondrial proteins in the DRM have been reported in several proteomics studies, and a number of different methods have been used to determine if these proteins were contaminants during biochemical fractionation [21-23]. Foster et al. differentiated true DRM proteins from contaminants using sensitivity to cholesterol disruption. In this scenario, only *bona fide* DRM proteins are responsive to changes in cholesterol [20]. Follow up studies with this method found that the major mitochondrial proteins reported in proteomics studies such as ATP synthase subunits and voltage-dependent anion selective channels (VDACs) were DRM contaminants [21]. In stark contrast, a recent study demonstrated the opposite result with certain mitochondrial proteins sensitive to cholesterol disruption [22]. Elegant studies using affinity purification of cell surface lipid rafts, a method optimized to exclude mitochondrial contamination, reported the presence of mitochondrial proteins ATP synthase and cytochrome c oxidase [23]. Therefore it remains highly controversial if mitochondrial proteins are true DRM proteins, a topic reviewed extensively elsewhere [23, 24]. In our study, the observed consistent quantitative differences in mitochondrial proteins points to a true caveolin-1 effect. Further support of this comes from recent studies showing that caveolin-1 participates in mitochondrial cholesterol regulation [25, 26]. In the absence of caveolin-1, cholesterol was found to congregate in the membrane of mitochondria, affecting the function of mitochondria [26]. Importantly, this finding would not have been revealed without comparison of pre and post fractionation methods, and examination of relative DRM amounts in the cell types.

4. Concluding Remarks

Finally, our results indicate that careful consideration is needed when deciding on what step is best to combine SILAC samples. This will vary with each experimental model and we therefore recommend a preliminary examination of how experimental conditions affect the organelle/s of interest before proceeding with SILAC subcellular proteomics in order to make sure correct interpretation can be made.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/108/0.

Supplementary Material includes Supplementary Table 1. SILAC ratios and p values for all proteins identified.

Acknowledgements

We thank Eunju Choi for technical assistance and useful discussions. MMH is supported by a National Health and Medical Research Council of Australia (NHMRC) Career Development Fellowship (App ID 569512). Access to GeneGo was provided by the Queensland Facility for Advanced Bioinformatics (QFAB) through LIEF Grant LE098933.

References

1. K. Inder, A. Harding, S. J. Plowman, M. R. Philips, R. G. Parton, and J. F. Hancock, Mol Biol Cell 19 (2008) 4776-4784.
2. K. L. Inder, C. Lau, D. Loo, N. Chaudhary, A. Goodall, S. Martin, A. Jones, D. van der Hoeven, R. G. Parton, M. M. Hill, and J. F. Hancock, J Biol Chem 284 (2009) 28410-28419.
3. S. Brunet, P. Thibault, E. Gagnon, P. Kearney, J. J. Bergeron, and M. Desjardins, Trends in cell biology 13 (2003) 629-638.
4. J. R. Yates, 3rd, A. Gilchrist, K. E. Howell, and J. J. Bergeron, Nature reviews. Molecular cell biology 6 (2005) 702-714.
5. J. S. Andersen, Y. W. Lam, A. K. Leung, S. E. Ong, C. E. Lyon, A. I. Lamond, and M. Mann, Nature 433 (2005) 77-83.
6. V. K. Mootha, J. Bunkenborg, J. V. Olsen, M. Hjerrild, J. R. Wisniewski, E. Stahl, M. S. Bolouri, H. N. Ray, S. Sihag, M. Kamal, N. Patterson, E. S. Lander, and M. Mann, Cell 115 (2003) 629-640.
7. S. W. Taylor, E. Fahy, B. Zhang, G. M. Glenn, D. E. Warnock, S. Wiley, A. N. Murphy, S. P. Gaucher, R. A. Capaldi, B. W. Gibson, and S. S. Ghosh, Nature biotechnology 21 (2003) 281-286.
8. C. E. Au, A. W. Bell, A. Gilchrist, J. Hiding, T. Nilsson, and J. J. Bergeron, Curr Opin Cell Biol 19 (2007) 376-385.
9. S. D. Patterson and R. H. Aebersold, Nat Genet 33 Suppl (2003) 311-323.
10. S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey, and M. Mann, Mol Cell Proteomics 1 (2002) 376-386.
11. E. Emmott, M. A. Rodgers, A. Macdonald, S. McCrory, P. Ajuh, and J. A. Hiscox, Mol Cell Proteomics 9 (2010) 1920-1936.
12. S. Dhungana, B. A. Merrick, K. B. Tomer, and M. B. Fessler, Molecular & cellular proteomics : MCP 8 (2009) 201-213.
13. K. L. Inder, Y. Z. Zheng, M. J. Davis, H. Moon, D. Loo, H. Nguyen, J. A. Clements, R. G. Parton, L. J. Foster, and M. M. Hill, Molecular & cellular proteomics : MCP 11 (2012) M111 012245.
14. R. G. Parton and K. Simons, Nat Rev Mol Cell Biol 8 (2007) 185-194.
15. M. M. Hill, M. Bastiani, R. Luetterforst, M. Kirkham, A. Kirkham, S. J. Nixon, P. Walser, D. Abankwa, V. M. Oorschot, S. Martin, J. F. Hancock, and R. G. Parton, Cell 132 (2008) 113-124.
16. M. Kirkham, A. Fujita, R. Chadda, S. J. Nixon, T. V. Kurzchalia, D. K. Sharma, R. E. Pagano, J. F. Hancock, S. Mayor, and R. G. Parton, J Cell Biol 168 (2005) 465-476.

17. T. V. Kurzchalia and R. G. Parton, Current opinion in cell biology 11 (1999) 424-431.
18. A. Pol, M. Calvo, A. Lu, and C. Enrich, Hepatology 29 (1999) 1848-1857.
19. W. P. Li, P. Liu, B. K. Pilcher, and R. G. Anderson, Journal of cell science 114 (2001) 1397-1408.
20. M. Bosch, M. Mari, S. P. Gross, J. C. Fernandez-Checa, and A. Pol, Traffic 12 (2011) 1483-1489.
21. M. Bosch, M. Mari, A. Herms, A. Fernandez, A. Fajardo, A. Kassan, A. Giralt, A. Colell, D. Balgoma, E. Barbero, E. Gonzalez-Moreno, N. Matias, F. Tebar, J. Balsinde, M. Camps, C. Enrich, S. P. Gross, C. Garcia-Ruiz, E. Perez-Navarro, J. C. Fernandez-Checa, and A. Pol, Current biology : CB 21 (2011) 681-686.
22. K. B. Kim, J. W. Lee, C. S. Lee, B. W. Kim, H. J. Choo, S. Y. Jung, S. G. Chi, Y. S. Yoon, G. Yoon, and Y. G. Ko, Proteomics 6 (2006) 2444-2453.
23. B. W. Kim, C. S. Lee, J. S. Yi, J. H. Lee, J. W. Lee, H. J. Choo, S. Y. Jung, M. S. Kim, S. W. Lee, M. S. Lee, G. Yoon, and Y. G. Ko, Expert review of proteomics 7 (2010) 849-866.
24. L. J. Foster and Q. W. Chan, Sub-cellular biochemistry 43 (2007) 35-47.
25. M. Bosch, M. Mari, S. P. Gross, J. C. Fernandez-Checa, and A. Pol, Traffic 12 (2011) 1483-1489.
26. M. Bosch, M. Mari, A. Herms, A. Fernandez, A. Fajardo, A. Kassan, A. Giralt, A. Colell, D. Balgoma, E. Barbero, E. Gonzalez-Moreno, N. Matias, F. Tebar, J. Balsinde, M. Camps, C. Enrich, S. P. Gross, C. Garcia-Ruiz, E. Perez-Navarro, J. C. Fernandez-Checa, and A. Pol, Current biology : CB 21 (2011) 681-686.