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# Oocyte Factors Suppress Mitochondrial Polynucleotide Phosphorylase to Remodel the Metabolome and Enhance Reprogramming

## Highlights
- Oocyte-enriched Tcl1 enhances the efficiency and quality of somatic reprogramming
- Tcl1b1 promotes Akt phosphorylation to promote somatic reprogramming
- Tcl1 suppresses mitochondrial biogenesis via PnPase to promote somatic reprogramming
- The Tcl1-PnPase switch remodels the metabolome to enhance somatic reprogramming

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## In Brief
Khaw et al. identify the oocyte Tcl1-PnPase pathway as a critical mitochondrial switch during reprogramming into iPSCs. They find that Tcl1 suppresses the mitochondrial localization of PNPase, thus inhibiting mitochondrial biogenesis and oxidation phosphorylation.
Oocyte Factors Suppress Mitochondrial Polynucleotide Phosphorylase to Remodel the Metabolome and Enhance Reprogramming

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SUMMARY

Oocyte factors not only drive somatic cell nuclear transfer reprogramming but also augment the efficiency and quality of induced pluripotent stem cell (iPSC) reprogramming. Here, we show that the oocyte-enriched factors Tcl1 and Tcl1b1 significantly enhance reprogramming efficiency. Clonal analysis of pluripotency biomarkers further show that the Tcl1 oocyte factors improve the quality of reprogramming. Mechanistically, we find that the enhancement effect of Tcl1b1 depends on Akt, one of its putative targets. In contrast, Tcl1 suppresses the mitochondrial polynucleotide phosphorylase (PnPase) to promote reprogramming. Knockdown of PnPase rescues the inhibitory effect from Tcl1 knockdown during reprogramming, whereas PnPase overexpression abrogates the enhancement from Tcl1 overexpression. We further demonstrate that Tcl1 suppresses PnPase’s mitochondrial localization to inhibit mitochondrial biogenesis and oxidation phosphorylation, thus remodeling the metabolome. Hence, we identified the Tcl1-PnPase pathway as a critical mitochondrial switch during reprogramming.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) was the first nuclear reprogramming method to be developed. In this method, a somatic nucleus is rapidly reprogrammed by oocyte cytosolic factors to gain pluripotency in a deterministic manner (Brambrink et al., 2006). The cells generated from SCNT are bona fide pluripotent stem cells (iPSCs) (Figures S1A and S1B). The mammalian oocyte-enriched T-cell leukemia (Tcl1) protein family consists of cytosolic, non-enzymatic proteins that are known to bind the Akt kinase, among other targets (Laine et al., 2000). Here, we show that Tcl1 suppresses PnPase’s mitochondrial localization to inhibit mitochondrial biogenesis and oxidation phosphorylation, thus remodeling the metabolome. Hence, we identified the Tcl1-PnPase pathway as a critical mitochondrial switch during reprogramming.

RESULTS

Oocyte Tcl1 and Tcl1b1 Enhance Reprogramming of Fibroblasts

We screened 20 oocyte-enriched genes, selected based on their abundance in ESCs and oocytes (Wang et al., 2010b; Zhang et al., 2009), by testing their effects on iPSC reprogramming of mouse fibroblasts using retroviral Oct4, Sox2, and Klf4 (OSK; Figures S1A and S1B). Out of the 20 oocyte-enriched genes, only Tcl1 and Tcl1b1 significantly increased the number of alkaline phosphatase (AP)-positive iPSC colonies (Figure 1A). Endogenous Tcl1 expression is similar to Nanog in that it is highly upregulated only at the late stage of iPSC reprogramming, when cells begin to acquire pluripotency (Figure 1B), whereas endogenous Tcl1b1 is undetectable throughout iPSC reprogramming.
Figure 1. Overexpression of Tcl1 and Tcl1b1 Enhance Somatic Reprogramming

(A) Fold change in AP-positive colonies after OSK-mediated reprogramming of WT MEFs in a mini-screen against oocyte-enriched genes, relative to empty vector (EV) control.

(B) Gene expression of endogenous Nanog and Tcl1 during the time course of iPSC reprogramming of MEFs.

(C and D) Gene expression of (C) Tcl1 and (D) Tcl1b1 in the ovary, two ESCs, and two MEF lines.

(E) Fold change in Oct4-GFP+ colonies after Tcl1 and Tcl1b1 overexpression during OSK reprogramming of WT MEFs.

(F) Percentage of AP+ colonies that are Oct4-GFP+ after Tcl1 and Tcl1b1 overexpression during OSK reprogramming of WT MEFs.

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Furthermore, Tcl1 is expressed highly in both mouse oocytes and ESCs (Figure 1C), whereas Tcl1b1 is expressed only in oocytes (Figure 1D), consistent with previous findings (Hallas et al., 1999). These data support their candidacy as oocyte reprogramming factors.

To validate our initial screen results, we repeated Tcl1 and Tcl1b1 overexpression during reprogramming of Oct4-GFP mouse embryonic fibroblasts (MEFs) and found that both Tcl1 factors could significantly increase Oct4-GFP+ colonies (Figure 1E). Tcl1 and Tcl1b1 overexpression also significantly increased the percentage of Oct4-GFP+ colonies within AP+ colonies (Figure 1F). This enhancement was independent of c-Myc overexpression (Figure 1G) and p53 deletion (Figure S1C).

Conversely, somatic reprogramming efficiency was significantly impaired by the knockdown of endogenous Tcl1 (Figure 1D) during iPSC reprogramming of either wild-type (WT) or p53 deficient (p53−/−) MEFs (Figures 1H–1J), thus highlighting its necessity in somatic reprogramming. Taken together, these observations suggest that the Tcl1 oocyte factors represent a distinct pathway critical to reprogramming.

Oocyte Tcl1 and Tcl1b1 Enhance Pluripotency Biomarkers after iPSC Derivation

Recent studies have suggested that other biomarkers, besides Oct4-GFP fluorescence, can support the assessment of bona fide iPSC reprogramming efficiency (Buganim et al., 2012; Golipour et al., 2012). Therefore, we used a single-clone profiling assay with nanofluidic chips to profile Tbx3, Nanog, and the imprinted Dlk1-Dio3 gene cluster as biomarkers for iPSC quality (Figure S2A), as these biomarkers had been shown to predict bona fide pluripotency and differentiation potency (Han et al., 2010; Niwa et al., 2009; Silva et al., 2009; Stadtfeld and Hochedlinger, 2010). Our analysis revealed that Nanog, Tbx3, Gli2, and Rian were upregulated to pluripotent ESC-like levels in Tcl1- and Tcl1b1-expressing clones, in contrast to OSK-only or partially reprogrammed iPSCs (Figures S2B–S2E), whereas Dlk-1, which is imprinted and anti-correlated with pluripotency, was downregulated to ESC-like levels in Tcl1- and Tcl1b1-expressing clones (Figure S2F). In total, either Tcl1 or Tcl1b1 overexpression could enhance reprogramming to the ESC-like state (Figure S2G) with ~50% efficiency among Oct4-GFP+ colonies, whereas OSK-only could produce ESC-like colonies with only 10% efficiency (Figure S2H). This further supports our findings that Tcl1 and Tcl1b1 enhance iPSC reprogramming.

Tcl1b1 Enhances Somatic Reprogramming by Increasing Akt Activity

Next, we investigated the molecular mechanism for Tcl1- and Tcl1b1-mediated enhancement of somatic reprogramming. Since both Tcl1 and Tcl1b1 are known Akt1/2 cofactors (Laine et al., 2000), we explored the role of Akt during reprogramming. Although Akt1 can stimulate heterokaryon and iPSC reprogramming, Akt’s role in SCNT reprogramming had remained unclear (Nakamura et al., 2008; Zhu et al., 2010). We observed a gradual increase in phospho-Akt during iPSC reprogramming by OSK factors (Figure 2A), and Akt was predominantly phosphorylated in ESCs relative to MEFs (Figure 2B). The pro-reprogramming role of Akt was confirmed when ectopic Akt1 increased reprogramming efficiency (Figure S3A), whereas inhibition of endogenous Akt activity suppressed iPSC reprogramming (Figure S3B). When we compared reprogramming using OSK factors alone, or OSK factors with either Tcl1 or Tcl1b1 overexpression, we found that only ectopic Tcl1b1 increased Akt phosphorylation far above the level achieved by OSK factors alone (Figure 2C). This suggests that only Tcl1b1, but not Tcl1, significantly enhances Akt activation.

To test whether Tcl1 and Tcl1b1 act through Akt activation to promote iPSC reprogramming, we sought to verify whether pharmacological inhibition of Akt1/2 might specifically abrogate Tcl1’s and Tcl1b1’s enhancement of iPSC reprogramming. Although Akt1/2 inhibition abrogated Tcl1b1’s enhancement of iPSC reprogramming (Figure 2D), Akt1/2 inhibition failed to abrogate the 3-fold enhancement promoted by Tcl1 (Figure 2E). A different Akt1/2 inhibitor yielded similar results (Figure S3C). On the other hand, co-overexpression of both Tcl1 and Tcl1b1 together synergistically increased reprogramming efficiency more than expected (Figure 2F), implying that Tcl1’s enhancement mechanism does not completely overlap with that of Tcl1b1 and that another mechanistic target besides Akt exists downstream of Tcl1 for reprogramming (Figure 2G).

Tcl1 Promotes Reprogramming by Suppressing Mitochondrial PnPase

To identify this other mechanistic target of Tcl1 in reprogramming, we performed co-immunoprecipitation (coIP) experiments in MEFs expressing hemagglutinin (HA)-tagged Tcl1 (HA-Tcl1), with and without the OSK reprogramming factors. We readily identified endogenous Pnpt1 as Tcl1’s main binding partner (Figure 3A). Pnpt1 encodes mitochondrial polynucleotide phosphatase (PnPase), an RNA-binding protein that plays an important role in RNA import and processing in mitochondria and, thus, mitochondrial homeostasis (Chen et al., 2006; Wang et al., 2010a). When we overexpressed Tcl1, the mitochondrial fraction of endogenous PnPase was significantly reduced but not the total pool of PnPase (Figures 3B and 3C). This suggested that Tcl1 suppressed the mitochondrial localization—and, thus, the mitochondrial activity—of PnPase.

Furthermore, we discovered that overexpression of Pnpt1 (Figure S4A) significantly suppressed somatic reprogramming by 60% (Figure 3D), whereas knockdown of the Pnpt1 gene with two independent small hairpin RNAs (shRNAs) (Figure S4B)
Figure 2. Tcl1b1 Promotes Akt Phosphorylation to Enhance iPSC Reprogramming

(A) Abundance of phospho-Akt (Ser473) protein during the time course of iPSC reprogramming of WT MEFs. GAPDH served as the total protein loading control.

(B) Abundance of phospho-Akt (Ser473) protein and densitometric quantification of phospho-Akt relative to total Akt (pAkt/tAkt) in mouse ESCs and MEFs. GAPDH served as the total protein loading control.

(C) Abundance of phospho-Akt (Ser473) protein after Tcl1 and Tcl1b1 overexpression during OSK-mediated reprogramming of WT MEFs.

(D and E) Fold change in Oct4-GFP+ colonies after (D) Tcl1b1 and (E) Tcl1 overexpression during OSK-mediated reprogramming of WT MEFs, with or without Akt1/2 inhibitor (Akt1/2i).

(F) Fold change in Oct4-GFP+ colonies after double co-overexpression of Tcl1 and Tcl1b1 during OSK-mediated reprogramming of WT MEFs.

(G) Diagram model depicting how Tcl1 and Tcl1b1-Akt regulate somatic reprogramming.

All data are shown as the mean ± SEM of at least three biological replicates. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figure S3.
Figure 3. Tcl1 Suppresses Mitochondrial PnPase to Enhance iPSC Reprogramming

(A) Western blots of co-IPs (IP) between ectopic HA-Tcl1 and endogenous PnPase, immunoblotted (IB) with anti-HA or anti-PnPase. Tim13a/b is a mitochondrial inner membrane protein and served as a negative control. Three experiments were performed with similar results. IgG, immunoglobulin G.

(B) PnPase protein in mitochondrial fraction (Mito) and total protein (Total) pools. COX IV served as the mitochondrial loading control, while GAPDH served as the total protein loading control. EV, empty vector.

(C) Densitometric quantification of mitochondrial PnPase protein, relative to mitochondrial COX IV loading control. Three biological replicates were quantified.

(D) Fold change in Oct4-GFP+ colonies after Pnpt1 overexpression during OSK-mediated reprogramming of WT MEFs.

(E) Fold change in Oct4-GFP+ colonies after Pnpt1 shRNA knockdown during OSK reprogramming of WT MEFs.

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significantly enhanced iPSC reprogramming by ~4-fold (Figures 3E and 3F). This enhancement was similar in magnitude to pS3 knockdown (Figure S4C), but it was even further enhanced to ~12-fold by pS3 deletion (Figure S4D), suggesting that Pnpt1 depletion also works independently of pS3 deletion. Thus, mitochondrial PnPase does inhibit iPSC reprogramming, independently of the pS3 pathway and its effects on cellular proliferation and apoptosis.

To validate whether Pnpt1 functions downstream of Tcl1 during reprogramming, we performed genetic epistasis experiments. We found that Pnpt1 knockdown fully rescued the inhibitory effect of Tcl1 knockdown during reprogramming (Figure 3G). On the other hand, Pnpt1 overexpression fully abrogated the enhancement effect of Tcl1 during reprogramming (Figure 3H). These data prove that mitochondrial Pnpt1 functions downstream of Tcl1 in reprogramming (Figure 3I).

**Tcl1-PnPase Pathway Reprograms the Cellular Metabolome**

While somatic cells depend mostly on mitochondrial oxidative phosphorylation (OxPhos), pluripotent stem cells possess immature mitochondria and preferentially use glycolysis as their major source of energy (Prigione et al., 2010; Folmes et al., 2011). Although this preference has been well characterized in ESCs, the pluripluripotency or oocyte factors responsible for this metabolic switch during somatic reprogramming had remained incompletely resolved. To test whether the Tcl1-Pnpt1 switch regulates somatic reprogramming by regulating mitochondrial homeostasis, we first examined the effects of Tcl1-Pnpt1 on mtDNA levels as an indicator of mitochondrial replication and biogenesis. Pnpt1-mediated import of nucleus-encoded RNAs—including MRP and RNase P RNAs, tRNAs, and SS rRNA—is thought to be essential for mtDNA replication and gene expression (Wang et al., 2010a). Interestingly, we found that Tcl1 suppressed mtDNA by 30%, whereas Pnpt1 increased it by 50% (Figure 4A). Moreover, Pnpt1 overexpression overrode the effects of Tcl1 overexpression (Figure 4A), proving that it lies downstream of Tcl1 in regulating mitochondrial biogenesis.

To understand the roles of mitochondrial Pnpt1 and Tcl1 in regulating cellular metabolism, we profiled the metabolomes of WT MEFs overexpressing either Tcl1 or Pnpt1, using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform. We observed that Tcl1 and Pnpt1 overexpression transformed significant segments of the metabolome in opposite directions, relative to the empty vector control (Figure 4B). In the glycolysis pathway, Tcl1 significantly increased fructose-1,6-bisphosphate (F-1,6-BP), suggesting higher phosphofructokinase (PFK) flux than the control, whereas Pnpt1 significantly decreased F-1,6-BP, suggesting lower PFK flux than the control (Figure 4C). PFK is the major rate-limiting irreversible step in glycolysis. Thus, Tcl1 increased input flux into glycolysis, whereas Pnpt1 decreased input flux into glycolysis, which is also evident from the significantly lower levels of glycolytic intermediates from G3P to PEP (Figure 4C). At the final irreversible step of pyruvate kinase (PK) in glycolysis, which controls the glycolytic outflows, Tcl1 and Pnpt1 again show opposite trends. Tcl1 significantly increased phosphoenolpyruvate (PEP) and decreased pyruvate, whereas Pnpt1 caused opposite changes (Figure 4C), suggesting that Tcl1 decreased PK flux to promote the accumulation of glycolytic intermediates for growth, whereas Pnpt1 increased PK flux to feed pyruvate into mitochondrial OxPhos. These are supported by observations that Tcl1 increased the glycolysis-derived D-glucono-δ-lactone-6-phosphate (Figure 4D), which shunts into the pentose phosphate pathway for nucleotide synthesis, whereas Pnpt1 increased the ATP/AMP ratio (Figure 4E) and decreased the glutathione GSH/GSSG ratio (Figure 4F), indicating that Pnpt1 promoted mitochondrial OxPhos and ROS production.

To prove that these metabolic effects of Tcl1 and Pnpt1 are relevant to reprogramming and pluripotency, we imaged the bioenergetics profiles of MEFs by measuring the mitochondrial membrane potential (ΔΨm) of ESCs and MEFs undergoing OSK reprogramming (Figure 4G). Previous studies had shown that a high ΔΨm can result from a preference for glycolysis and a lower rate of respiration, leading to a slower dissipation of the mitochondrial membrane potential (Fantin et al., 2006; Yoshida et al., 2009). Using a live ΔΨm probe, we performed flow cytometry analysis on live cells and found that Tcl1 significantly increased the proportion of ESC-like ΔΨmHIGH cells during OSK reprogramming (Figure 4H). Similarly, Pnpt1 knockdown significantly increased the proportion of ESC-like ΔΨmHIGH cells during reprogramming (Figure 4I).

To confirm these metabolic observations, we examined the bioenergetics profiles of MEFs by measuring the mitochondrial oxygen consumption rate (OCR) to assay respiration rates and by measuring the extracellular acidification rate (ECAR) to assay glycolytic rates. Relative to the scrambled control, we observed that Pnpt1 was required for normal basal and maximal respiration after Pnpt1 knockdown (Figure 4J). On the other hand, Pnpt1 knockdown increased the basal glycolysis rate, suggesting that Pnpt1 suppressed glycolysis (Figure 4J). Similarly, Tcl1 overexpression decreased basal respiration and maximal respiration by 40% (Figure 4K). Furthermore Tcl1 overexpression increased the glycolytic rate by 40%–60% (Figure 4K), similar to Pnpt1 knockdown. Most importantly, we found that Pnpt1 overexpression could rescue not only Tcl1’s inhibition of respiration (Figure 4L) but also Tcl1’s enhancement of glycolytic flux (Figure 4M), confirming that Pnpt1 acts downstream of Tcl1 in metabolic reprogramming.

**DISCUSSION**

Our results contribute to solving a long-standing mystery of how oocyte-enriched cytosolic factors facilitate reprogramming (Gordon and Melton, 2008). In this study, we demonstrated that...
Figure 4. Tcl1-PnPtase Pathway Remodels the Cellular Metabolome during Reprogramming

(A) Relative mtDNA copy number after Tcl1 and Pnpt1 overexpression in MEFs, relative to the empty vector (EV) control.

(B) Heatmap of metabolite levels, as measured by LC-MS/MS metabolomics in MEFs after overexpression of Tcl1 and Pnpt1, relative to the EV control.

(C) Percent change in glycolysis intermediate levels in MEFs after overexpression of Tcl1 and Pnpt1, relative to the EV control.

(D) Percent change in D-glucono-δ-lactone-6-phosphate levels in MEFs after overexpression of Tcl1 and Pnpt1, relative to the EV control.

(E) Percent change in the ATP/AMP ratio in MEFs after overexpression of Tcl1 and Pnpt1, relative to the EV control.

(F) Percent change in the GSH/GSSG ratio in MEFs after overexpression of Tcl1 and Pnpt1, relative to the EV control.

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the oocyte-enriched Tcl1 and Tcl1b1 proteins enhance reprogramming of iPSCs. Furthermore, we established that Tcl1b1 promotes Akt activation, whereas Tcl1 suppresses mitochondrial PnPase localization in order to promote reprogramming. As shown previously, mitochondrial PnPase serves to promote mitochondrial biogenesis and homeostasis (Chen et al., 2006; Wang et al., 2010a). This supports our observations that the Tcl1-PnPase mechanism operates as a metabolic switch to remodel the cellular metabolome during reprogramming. Furthermore, our genetic experiments with Tcl1 and Pnpt1 perturbation in WT versus p53−/− backgrounds suggest that the Tcl1-Pnpt1 switch regulates metabolic reprogramming independently of effects on cellular proliferation and senescence. In fact, Tcl1 is directly activated by Oct4 and Stat3 (Matoba et al., 2006).

Given that we explored the mechanistic basis for the iPSC reprogramming enhancement by the Tcl1 oocyte factors, we initially focused on Akt since it is a well-established binding target of Tcl1 (Laine et al., 2000). We found that Akt phosphorylation increased during iPSC reprogramming and that Akt signaling was both necessary and sufficient for iPSC reprogramming. While we found that both Tcl1 and Tcl1b1 enhanced Akt activity, Tcl1b1 was a significantly more potent Akt activator. Consistently, we found that Tcl1b1-overexpressing cells were much more sensitive to Akt inhibition than Tcl1-overexpressing cells during reprogramming. Previous studies had also shown that Tcl1 only modestly increases Akt activation in lymphoma cells (Hoyer et al., 2002). Therefore, it seems that Tcl1b1 is a more potent Akt coactivator, activating the proliferative and metabolic pathways downstream of Akt to boost reprogramming. In fact, a previous study had shown that a small molecule agonist of the PI3K-PDK1-Akt pathway can also boost reprogramming by upregulating glycolysis (Zhu et al., 2010).

For Tcl1, which did not seem as dependent on PI3K-Akt signaling as Tcl1b1, we found instead that the mitochondrial RNA-binding PnPase is the relevant binding target of Tcl1 during reprogramming. While total PnPase levels were unaffected by Tcl1, the mitochondrial fraction of PnPase was significantly reduced by overexpression of Tcl1, indicating that Tcl1 suppresses PnPase’s mitochondrial localization. Suppression of mitochondrial PnPase by RNAi also significantly enhanced iPSC reprogramming. Through genetic epistasis experiments, we established that PnPase is the downstream target of Tcl1 in remodeling the metabolome during reprogramming.

PnPase regulates RNA import and processing in the mitochondria, which are complex processes crucial for mitochondrial biogenesis and homeostasis (Wang et al., 2010a). Although the multitude of RNAs that are transiently processed by PnPase remains difficult to elucidate, PnPase-mediated import of the MRP and RNase P RNAs, 5S RNA, and tRNAs is known to be essential for mtDNA replication and biogenesis (Wang et al., 2010a). Indeed, cells depleted of Pnpt1 are known to have less mitochondrial biogenesis and activity (Wang et al., 2010a), consistent with our observations. This is also consistent with others’ observations of lower mitochondrial biogenesis and activity in pluripotent stem cells (Prigione et al., 2010; Folmes et al., 2011; Liu et al., 2013). As a regulator of mitochondrial homeostasis, we found that Tcl1-PnPase regulates mitochondrial OxPhos, the ATP/AMP ratio, the redox balance, and, thus, the glycolytic flux. In particular, Tcl1-PnPase also regulates the increased PFK flux and decreased PK flux observed during the glycolytic switch in iPSC reprogramming (Zhu et al., 2010; Shyh-Chang et al., 2013a; Prigione et al., 2010). Thus, the Tcl1-PnPase mechanism serves as a metabolic switch that is capable of remodeling the metabolome during reprogramming.

Given this important role in regulating mitochondrial homeostasis, it is unsurprising that PnPase is required for mouse embryogeneration, muscle, brain, and inner ear development (Wang et al., 2010a; Vedrenne et al., 2012; von Ameln et al., 2012). Since mitochondrial numbers and functions are also tightly regulated in pre-fertilization oocytes and early embryonic development (Shyh-Chang et al., 2013b), the Tcl1-PnPase switch might be relevant not only for regulating mitochondria in iPSC reprogramming but also for mitochondrial replacement in aged oocytes and during in vitro fertilization.

**EXPERIMENTAL PROCEDURES**

**Murine iPSC Reprogramming Assay and Drug Treatment**

Reprogramming of primary MEFs was performed according to previously published protocols (Takahashi and Yamanaka, 2006).

**Protein Extraction and Western Blotting**

Cells were harvested and processed according to previously published protocols (Shyh-Chang et al., 2013a). Immunoblot membranes were probed with specific antibodies: GAPDH, (Santa Cruz Biotechnology, SC137179), PnPase (Santa Cruz, SC365049), Akt (Cell Signaling Technology, #9272), phosphoAkt1 (Ser473) (Cell Signaling Technology, #4060), Tim13A/B (Santa Cruz, SC17065), and HA (Covance MMS-101P). Antibody-protein complexes were detected by horseradish peroxidase (HRP)-conjugated antibodies and ECL-Plus (Amersham Biosciences).

**Oxygen Consumption and Extracellular Flux Measurement**

Infected MEFs were seeded onto Seahorse assay plates (pre-coated with 0.1% gelatin) at 15,000 cells per well 1 day before the assay. We replaced culture media with assay media (Seahorse Bioscience) 1 hr before data collection using the XF96 Seahorse analyzer. Glucose (Sigma-Aldrich; final concentration, 25 mM), oligomycin (Sigma-Aldrich; final concentration, 1 μM), FCCP (Sigma-Aldrich; final concentration, 1 μM), and a mixture of antimycin and rotenone (Sigma-Aldrich; final concentration, 1 μM) were injected sequentially in log2 scale to capture both the efficiency gains and losses adequately.

All data are shown as the mean ± SEM of at least three biological replicates. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. See also Figure S5 and Table S1.
during measurement for bioenergetics profile analysis. Measurements were taken according to manufacturer’s instructions.

**Targeted LC-MS/MS**

LC-MS/MS was performed according to previously published protocols (Shyh-Chang et al., 2013a).

For detailed materials and procedures, please see the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.crep.2015.07.032.

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