

2015

GPS2 nuclear localization and TBL1-mediated stabilization are important in regulating nuclear encoded mitochondrial gene expression

<https://hdl.handle.net/2144/16013>

"Downloaded from OpenBU. Boston University's institutional repository."

BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**GPS2 NUCLEAR LOCALIZATION AND TBL1-MEDIATED STABILIZATION
ARE IMPORTANT IN REGULATING NUCLEAR ENCODED
MITOCHONDRIAL GENE EXPRESSION**

by

JIAWEN HUANG

B.S., University of California–Berkeley, 2012

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2015

© 2015 by
Jiawen Huang
All rights reserved

Approved by

First Reader

Valentina Perissi, Ph.D.
Assistant Professor of Biochemistry

Second Reader

Susan K. Fried, Ph.D.
Professor of Nutrition and Metabolism

ACKNOWLEDGMENTS

Foremost, I would like to express my heartfelt appreciation and gratitude to my thesis advisor, Dr. Valentina Perissi, and my co-mentor, Dr. Dafne M. Cardamone, for their support, guidance, and immense knowledge throughout my master's thesis research.

I would like to acknowledge my Nutrition and Metabolism program advisors, Dr. Susan Fried and Dr. Lynn Moore, for challenging me to think critically and for imparting their passion for science as well as vast knowledge.

I would also like to thank Dr. Romy Chakraborty for all the opportunities to nurture my interest in scientific research since undergraduate.

I'm appreciative of my mother, Huixin Li, for always showing me the strength needed to overcome challenges and supporting me unconditionally. Lastly, I would like to thank my boyfriend, Daniel Nguyen, for his love, understanding, and encouragement throughout all my master's studies.

**GPS2 NUCLEAR LOCALIZATION AND TBL1-MEDIATED STABILIZATION
ARE IMPORTANT IN REGULATING NUCLEAR ENCODED
MITOCHONDRIAL GENE EXPRESSION**

JIAWEN HUANG

ABSTRACT

G-protein pathway suppressor 2 (GPS2) is a 36kD protein involved in a number of regulatory functions in key metabolic organs. First discovered as a suppressor of the RAS- and MAPK- signaling pathways, GPS2 is subsequently identified as part of the NCoR/SMRT corepressor complex that play an important regulatory role in gene transcription, and GPS2 is also involved in meiotic recombination in the nucleus. Recently, we identified a non-transcriptional role of GPS2 as an inhibitor of the pro-inflammatory JNK pathway activation in response to tumor necrosis factor alpha (TNF- α) in the cytosol. This suggests that GPS2 function may be dependent on its cellular localization. However, an understanding of how GPS2 differentially target cellular compartments is still lacking. In this study, we show that a tightly controlled balance between GPS2 protein stabilization and degradation regulates the function of nuclear GPS2. Our results reveal that methylation by arginine methyltransferase PRMT6 and interaction with exchange factor TBL1 cooperate to protect GPS2 from Siah2-dependent proteasomal degradation, thus promoting GPS2 nuclear localization. In addition, our results link GPS2 protein instability to decreased nuclear-encoded mitochondrial gene expression, suggesting that GPS2 may play an important role in regulating mitochondrial oxidative capacity, whose imbalance has been linked to chronic inflammation and insulin

resistance. In conclusion, our findings illustrate post-transcriptional modification is important in the regulation of GPS2 cellular function. Understanding such molecular regulation of GPS2 is critical in furthering future efforts to investigate its roles in cellular homeostasis and inflammatory responses.

TABLE OF CONTENTS

TITLE.....	i
COPYRIGHT PAGE.....	ii
READER APPROVAL PAGE.....	iii
ACKNOWLEDGMENTS	iv
ABSTRACT.....	v
TABLE OF CONTENTS.....	vii
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS.....	x
INTRODUCTION	1
MATERIALS AND METHODS.....	4
RESULTS	9
DISCUSSION.....	27
REFERENCES.....	30
VITA.....	36

LIST OF TABLES

Table	Title	Page
1	Nuclear-encoded mitochondrial genes of interest	20

LIST OF FIGURES

Figure	Title	Page
1	Interaction with TBL1 via the N-terminal domain of GPS2 is required for nuclear localization and protein stabilization	10
2	Ubiquitination of specific lysines in the GPS2 C-terminal domain is required for its proteasomal degradation	12
3	GPS2 ubiquitination and degradation by Siah2	13
4	PRMT6-mediated methylation of arginine323 prevents proteasomal-dependent degradation of GPS2	16
5	Proposed model mechanism of GPS2 regulation	18
6	GPS2 is required for transcriptional regulation of nuclear encoded mitochondrial gene in response to cellular stress	21–26

LIST OF ABBREVIATIONS

3T3-L1	Mouse Embryonic Fibroblasts
BCS	Bovine Calf Serum
ChIP	Chromatin Immunoprecipitation
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide 4-(trifluoromethoxy)phenylhydrazone
GPS2	G-protein Pathway Suppressor 2
HEK293T	Human Embryonic Kidney Cells
Hela	Human Cervical Cancer Cells
kD	Kilodalton
LB	Lysogeny Broth
NCoR	Nuclear Receptor Co-Repressor
PMSF	Phenylmethanesulfonylfluoride
PRMT6	Protein Arginine Methyltransferase 6
PVDF	Polyvinylidene Fluoride
SIAH2	Drosophila Seven-in-Absentia Protein 2
SMRT	Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor
TBL1	Transducin β -Like Protein 1
TNF- α	Tumor Necrosis Factor Alpha

INTRODUCTION

Metabolic diseases such as obesity, Type II diabetes mellitus, and insulin resistance have been linked to chronically uncontrolled cellular inflammation (Wellen et al., 2005; Hotamisligil, 2006). Inflammatory markers such as, TNF- α , are increased in obesity and are associated with diminish insulin sensitivity (Hotamisligil, 1993; Uysal et al., 1997). G-protein suppressor 2 (GPS2) is a small protein that was originally discovered to be involved in the suppression of the RAS- and MAPK- signaling (Spain et al., 1996). GPS2 is indicated to have transcriptional regulatory roles through its interaction with several transcriptional regulators (Peng et al., 2000, 2001; Lee et al., 2006; Cheng et al., 2009; Sanyal et al., 2007; Venteclef et al., 2010) and through its participation to the NCoR/HDAC3 corepressor complex (Zhang et al., 2002, 2008). In addition, our lab recently reported a non-transcriptional role of GPS2 in the cytoplasm via the inhibition of JNK activation in response to TNF- α (Cardamone et al., 2012). Our findings indicate that both GPS2's transcriptional and non-transcriptional functions are dependent on the inhibition of ubiquitin ligases (Cardamone 2012, 2014). These results, together with evidences of the dysregulation of SMRT- and GPS2-mediated inhibition of pro-inflammatory genes in human obese adipose tissue (Toubal et al., 2013), define GPS2 as a key regulator in the suppression of inflammatory responses in metabolically relevant organs. Presently, our lab generated adipose tissue-specific GPS2 KO mice and observed obesity-induced upregulation of pro-inflammatory genes and altered lipid metabolism *in vivo* (unpublished data). Despite this emerging important role of GPS2, little is known about the endogenous mechanism regulating GPS2 protein stability or

degradation and its intracellular localization. Understanding the pathways and mechanisms governing the expression of GPS2 protein will be important in characterizing the GPS2 KO mice.

Preliminary results in the lab show that GPS2 is highly unstable unless it is complexed with Transducin β -like protein 1 (TBL1), which contains a F-box/WD-40 repeat, and it is associated with GPS2 in the NCoR/HDAC nuclear receptor corepressor complex (Zhang et al., 2002). However, the mechanism by which TBL1 stabilize GPS2 is unknown. It has been reported that phosphorylation, sumoylation, and ubiquitination are important in regulating cellular localization and functions for components of the NCoR/SMRT complex (Zhang et al., 2005; Choi et al., 2011, 2013; Perissi et al., 2008; Huang et al., 2011), therefore we hypothesize post-translational modification could similarly regulate the function and expression of GPS2 level (Bi et al., 2014; Jarmalavicius et al., 2010; Guo et al., 2014). Also, our previous work has shown the mechanism for the dismissal of the NCoR/SMRT corepressor complex depends on TBL1/TBLR1-dependent recruitment of Siah2/UbcH5 ubiquitin machinery (Perissi et al., 2004; Huang et al., 2009; Pascual et al., 2005). Here, we hypothesize that GPS2 protein level would be similarly regulated. Our results reveal the existence of a similar but distinct strategy with nuclear GPS2 levels being regulated by a fine balance between protein degradation and stabilization. GPS2 in the nucleus is readily degraded by E3 ubiquitin ligase, Siah2. TBL1 stabilizes ubiquitinated GPS2 from proteasomal degradation by interaction between both proteins' N terminal domains. In addition, we found that protein arginine methyltransferase 6 (PRMT6) methylates GPS2, which

contributes to the stability of the interaction between TBL1 and GPS2, preventing GPS2 from degradation.

Once it is stabilized in the nucleus, GPS2 regulates gene expression both as a repressor as part of the NCoR/SMRT complex and a coactivator as in the cases of LXR/PPAR and other transcription factor. Initial analysis of GPS2 genome wide localization in 3T3-L1 identified a subset of genes that are important for regulating lipid mobilization (i.e. ATGL/HSL), where GPS2 acts as a pioneer factor for PPAR γ recruitment (Cardamone, 2014). Here, further analysis of the same data set indicates that GPS2 binds to the proximal promoters of a large majority of mitochondrial genes, raising the possibility that GPS2 and associated cofactors modulate mitochondrial functions via regulation of the expression of the nuclear-encoded subunits of the electron transport chains.

MATERIALS AND METHODS

Cell Culture

3T3-L1 fibroblasts are maintained in DMEM (Corning, 10-016-CV) with 10% BCS (Thermo Scientific) at 37°C and 5% CO₂. Hela and HEK293T cell lines are maintained in DMEM with 10% FBS (Thermo Scientific) at 37°C and 5% CO₂.

Antibodies, siRNA and Reagents

In this work, commercial antibodies used in the experiments were: Anti-GPS2 that our lab generated using a peptide representing amino acid 1 to 22, Anti- β tubulin (TUB 2.1 clone, Sigma), Anti-HDAC2 (Santa Cruz Biotech sc-9959), Anti-PRMT6 (A300-929A, Bethyl Labs), Anti-HA-HRP (Roche), Anti-FLAG-HRP (Sigma), Anti-Siah2 (Santa Cruz Biotech sc-5507). SiRNAs against human TBL1 and PRMT6 and siRNA against human and mouse GPS2 were purchased from Ambion. MISSION[®] SiRNA Universal Negative Control (Sigma) is included in each experiment. Cells were treated with 10nM MG-132 (InSolution 474791, Calbiochem-EMD) for 4 hour to inhibit proteasomal degradation in cells. Cells were treated with 25nM FCCP (Sigma) to uncouple respiratory chain. Cells were treated with 10ng/ml TNF- α (Calbiochem) to induce inflammatory response.

Primers Design

Search for the DNA sequence of gene of interest using Ensembl database. Select two exons specific to the gene of interest and choose some regions at the end of the first exon and the beginning of the second exon. Paste the selected sequences into the primer selection tool, Primer3, and adjust the primer picking conditions as follows:

for primer oligo size in bases, minimum: 50, optimum: 150, max: 180; for melting temperature in Celsius, minimum: 62, optimum: 65, maximum: 68. After picking the forward and reverse primers, the gene sequence is confirmed using the UCSC Genome Bioinformatics' In-Silico PCR in which it uses the paired PCR primers to search for the gene against a sequence database.

RNA Isolation and qPCR Analysis

Total RNA was isolated by directly lysing cells on the plate adding a highly denaturing buffer and the lysate was homogenized through QIAshredder[®] (RNeasy Plus Mini Kit, QIAGEN). The homogenized lysate was centrifuged through gDNA Eliminator spin column (QIAGEN). Ethanol was added to provide proper binding condition and the sample was transferred to an RNeasy[®] spin column for binding of total RNA per manufacturer's protocol (QIAGEN). RNA concentration is determined using NanoDrop[™] 2000 (Thermo Scientific). cDNA was generated from the isolated total RNA using iScript[™] Reverse Transcriptase Supermix (Bio-Rad) per manufacturer's protocol using a Veriti[®] Thermal Cycler (Life Technologies), followed by SYBR[®] Green qPCR amplification on a Viia7[™] Real-Time PCR System (Life Technologies). The qPCRs were performed on 384-well PCR plates (GeneMate) or 96-well PCR plates (Thermo Scientific), with RealTime PCR adhesive films (Danville Scientific). qPCRs were performed in triplicate for each sample. All samples were normalized to Cyclophilin A. Analysis on the qPCR results was performed in Microsoft Excel.

Site Directed Mutagenesis and Bacterial Transformation

GPS2 mutation of arginine 323 (GPS2-R323A) was performed using QuikChange II XL Site-Directed Mutagenesis Kit per manufacturer's protocol (Agilent Technologies). To 50ul–100ul competent bacteria, 10–100pg of mutant DNA was added and mixture was kept on ice for 20 minutes. Heat shocked the bacteria at 42°C for 40 seconds, and then placed on ice for 2 minutes. Added bacteria to 1ml LB and incubate for 45 mins at 37°C at 200 rpm. Centrifuged the bacteria at 6000 rpm for 5 minutes, discarded supernatant, and plated on LB plates with ampicillin using glass beads. Incubated at 37°C at 200 rpm overnight. Next day, picked colonies and grew in LB broth at 37°C at 200 rpm overnight.

Mini Prep and Midi Prep

2mls of bacteria were pelleted for 30 seconds at max speed. Pellet was resuspended in 300ul of buffer P1 (Qiagen) and incubated for 5 minutes. Then, it was lysed with 300ul of buffer P2 (Qiagen) and inverted 5 times, incubated for 5 minutes. The lysate was neutralized with 300ul of buffer P3 (Qiagen), incubated for 5 mins on ice. Spun down lysate at full speed for 10 minutes. 800ul supernatant was transferred in to a new microcentrifuge tube, added 560ul of isopropanol, and mixed. Spun down the mixture for 15 minutes at full speed. Washed the pellet with 300–500ul 70% ethanol and spun down at max speed for 5 minutes. Air-dried DNA was resuspended in 40ul of water. After confirming the mutation of GPS2 arginine 323 in by sequencing, the plasmid DNA is purified by HiSpeed Plasmid Midi Kit (Qiagen).

Transient Transfection

Mammalian cells were grown to about 70% confluence. Transient transfection in cells was performed using the Lipofectamine[®] 2000 per manufacturer's protocol (Life Technologies) with 2µg plasmid DNA and/or with 10nM siRNA. Cells were incubated at for 24 hours at 37°C/5% CO₂ for transfection of plasmid DNA and for 48 hours for transfection of siRNA.

Protein Extraction and Western Blotting

To extract fractionated cytosolic and nuclear proteins, cells were washed with cold PBS, harvested and spun down for 5 minutes at 1000 rpm (4°C). Cell pellet was resuspended in cold hypotonic buffer (10mM Hepes pH 7.9, 1mM EDTA, 210mM Mannitol, 70mM sucrose, 0.1mM PMSF, and protease inhibitor mix (Roche), and lysed by 10 passages of syringe (26Gx3/8") homogenization. After precipitating the nuclei by 2000xg centrifugation for 10 minutes at 4°C, the supernatant containing the cytosolic proteins was recovered and the nuclear pellet was lysed for 20 minutes on ice with high salt buffer (20mM Tris-HCl pH 8.0, 25% glycerol, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 0.1mM PMSF, 10mM NEM, and protease inhibitor mix). Centrifuged at 20,000xg to remove membrane debris, the supernatant containing the nuclear proteins was recovered. Concentration of the fractionated protein extracts were measured using the Quick Start[™] Bradford 1X Dye Reagent per manufacturer's protocol (Bio-Rad) and the NanoDrop[™] 2000. Fractionated extracts were boiled with NuPAGE LDS Sample Buffer-4X (Life Technologies) and DTT at 100°C for 10 minutes. Extracts then were loaded onto 10% Mini-PROTEAN[®] TGX[™] Gel and separated by

electrophoresis with 1x Tris-Glycine-SDS Running Buffer (Boston BioProducts).

Precision Plus Protein™ Dual Color Standards was used as the ladder (Bio-Rad).

Proteins were then transferred onto PVDF membranes (EMD Millipore), followed by standardized western blotting protocol. Chemiluminescent western blot detection was performed using Clarity™ Western ECL Substrate and ChemiDoc™ XRS+ with Image Lab software per manufacturer's protocol (Bio-Rad).

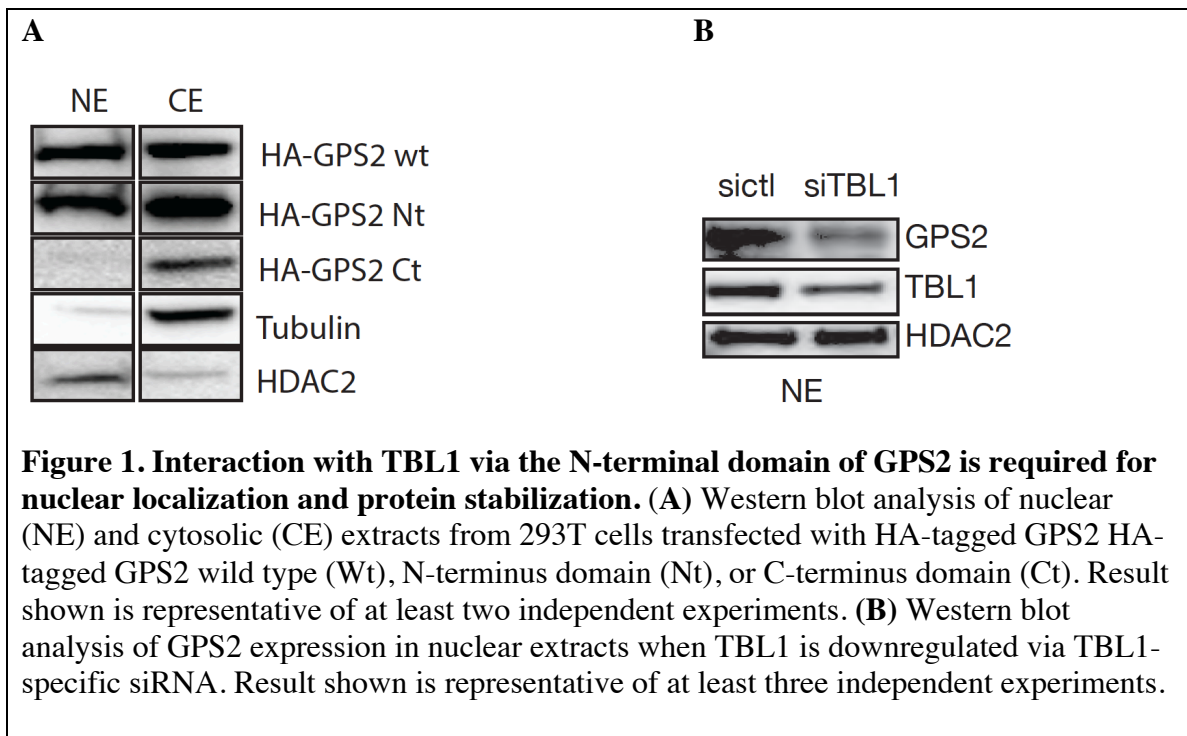
RESULTS

Interaction with TBL1 via the N-terminal domain of GPS2 is required for its nuclear localization and protein stabilization.

Preliminary experiments in the lab suggested that nuclear localization of GPS2 was determined by a sequence within the N-terminus. To investigate the molecular determinants of GPS2 nuclear localization, HA-tagged GPS2 full length (Wt), its N-terminus domain (Nt) or the C-terminus domain, were overexpressed in 293T cells. Western blot analysis shows that the full length GPS2 protein and the deletant containing only the N-terminus domain are both expressed in the nuclear (NE) and cytosolic fractioned (CE) extracts, whereas the C-terminus domain is found only in the cytosolic fraction (Fig. 1A). This indicates that the N-terminus domain is required for GPS2 nuclear localization. Unfortunately, further mapping of the N-terminus region could not be performed due to protein instability.

It is known that the N-terminal domain of GPS2 interacts with TBL1 (aa 1–53) and promotes the correct assembly of the NCoR/SMRT corepressor complex (aa 53–90) (Oberoi, 2011). Therefore, we hypothesize that binding to TBL1 is required for the stabilization of GPS2. Indeed, when I downregulate TBL1 via small interfering RNA GPS2 expression is decreased (Fig. 1B). My result confirms previous findings that GPS2 protein expression is decreased when TBL1 is genetically ablated in mouse embryonic stem cells (Perissi, 2004). In addition, deletion of amino acids 1-40 from the N-terminus of GPS2 leads to decreased expression compared to the wild type and deletion of amino acids 1-60 as well as 1-80, which showed no detectable protein expression (unpublished

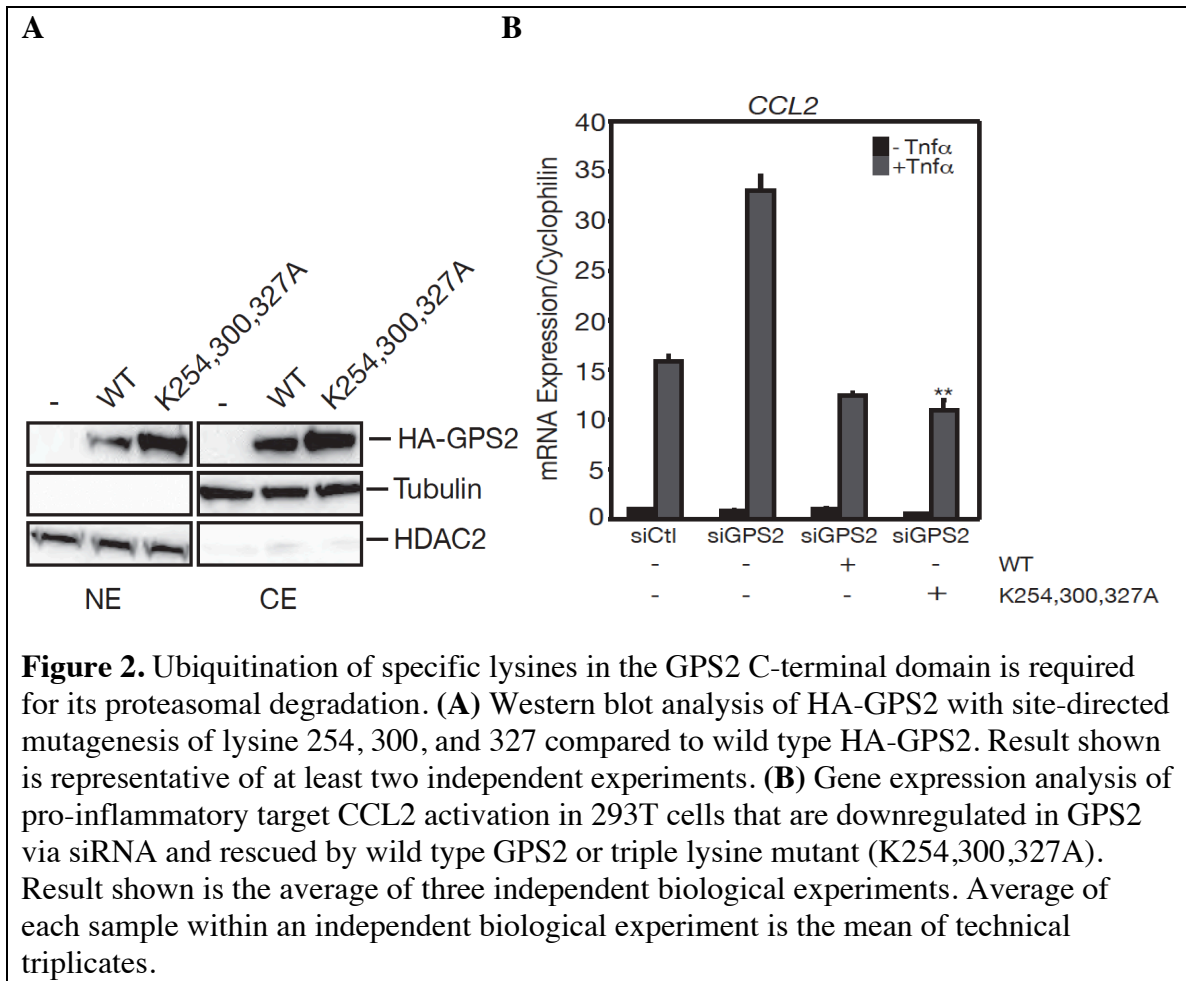
data). These results confirm that TBL1, which interacts with GPS2 1-52, is required for GPS2 protein stabilization, and further mapping revealed amino acid 40-60 in the N-terminus of GPS2 is the minimal nuclear localization domain required for its nuclear targeting. Interestingly, prediction analysis indicates the presence of a classic nuclear localization sequence (NLS) within this region. However, specific mutation of the NLS does not affect GPS2 localization to the nucleus, suggesting that GPS2 is targeted to the nucleus via interaction with other nuclear cofactors.



Ubiquitination of specific lysines in the GPS2 C-terminal domain is required for its proteasomal degradation.

It has been reported that GPS2 instability is due to ubiquitin-dependent degradation process (Peng et al., 2001; Cardamone et al., 2012; Bi et al., 2014; Jin et al.,

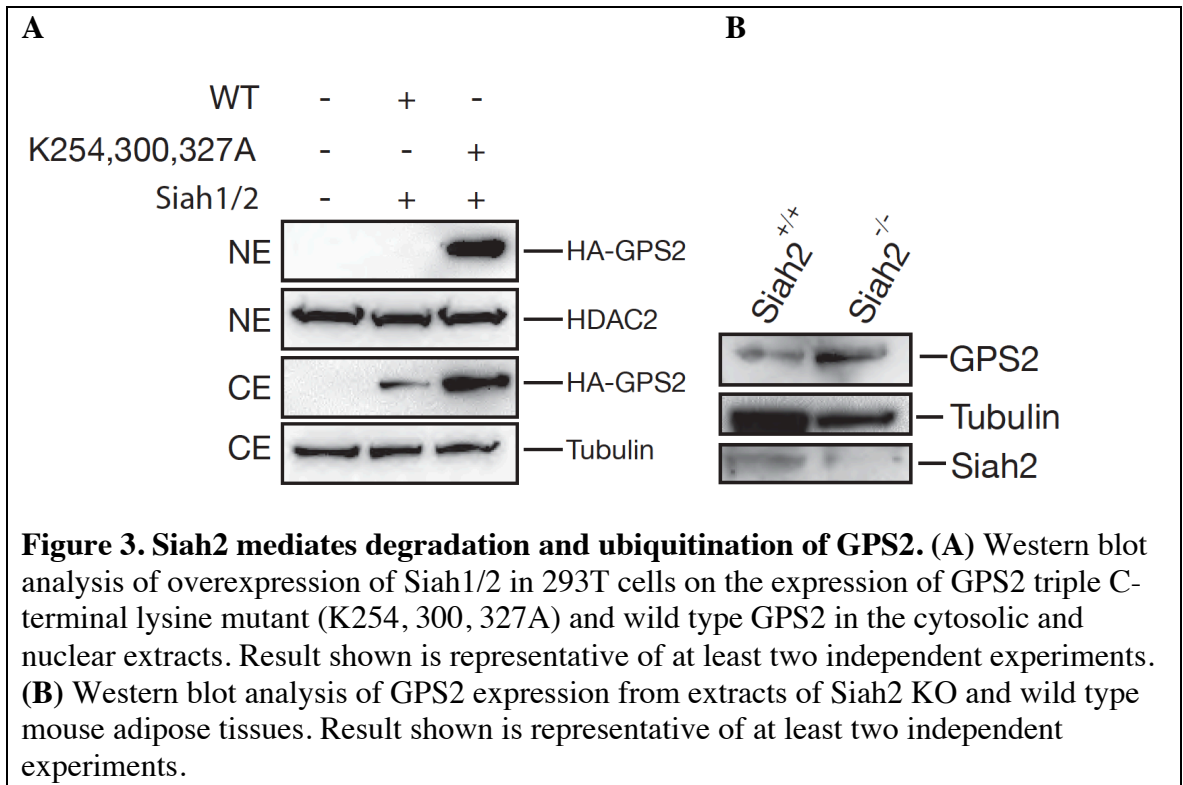
1997). While investigating whether GPS2 is destabilized and degraded via proteasomal degradation in the absence of TBL1, our unpublished data confirmed that destabilization of GPS2 is mediated through poly-ubiquitination-dependent degradation, as shown with immunoprecipitation of GPS2 from cells overexpressing HA-tagged ubiquitin or untransfected cells. Our preliminary data also suggested that the sites of poly-ubiquitination are located in the C-terminal domain. To confirm the role of C-terminus in regulating GPS2 stability, I performed western blot analysis of HA-GPS2 with overexpression of GPS2 with specific mutation of three lysines (K254, 300, 327A) in the C-terminal domain compared to wild-type GPS2 (Wt). The result indicates the triple mutant shows increased GPS2 stabilization when compared to wild type (Fig. 2A). This suggests that these lysines play a degradative function for GPS2. As expected, considering the increased expression, the triple lysine mutant (K234, 300, 327A) is also slightly more efficient than the wild type in rescuing hyperinflammatory activation by specific downregulation of GPS2 (Cardamone, 2012) (Fig. 2B). The results together reveal that GPS2 instability is promoted by poly-ubiquitination dependent degradation, within the C-terminal domain.



GPS2 ubiquitination and degradation by Siah2.

The next question we wanted to address was which is the ubiquitin ligase responsible for GPS2 ubiquitination-dependent degradation. Previous reports by Zhang et al. (1998) and our lab have shown that *Drosophila* seven-in-absentia homolog 2 (Siah2)-mediated ubiquitination promotes the dismissal of the NCoR/SMRT corepressor complex from targeted genes and is necessary for the degradation of NCoR. Since GPS2 is a core component of the NCoR/SMRT corepressor complex, we reasoned that Siah2, or the highly conserved homolog Siah1, could potentially be responsible for catalyzing the

poly-ubiquitination-dependent degradation of GPS2. This hypothesis was validated by previous work in the lab, confirming that in 293T cells, endogenous and overexpressed GPS2 are significantly downregulated with the overexpression of HA-Siah1 and HA-Siah2. To further confirm that the effect of the Siah proteins is mediated by the ubiquitination of the lysines (K254, 300, 327A) in the C-terminal domain of GPS2, I overexpressed Siah1/2 in 293T cell and showed that Siah1/2 prevents the expression of the wild type GPS2 construct but it does not induce degradation of GPS2 triple mutant in nuclear protein extracts (Fig. 3A). In addition, we were able to obtain adipose tissue samples of Siah2 knock out mice (Frew et al., 2003), and I found that that GPS2 protein level is significantly higher in the fat of the KO mice compared to that of the wild type littermates (Fig. 3B). In conclusion, these data indicate that GPS2 proteasomal degradation is mediated by Siah2 dependent poly-ubiquitination of three lysines located within GPS2 C-terminus.



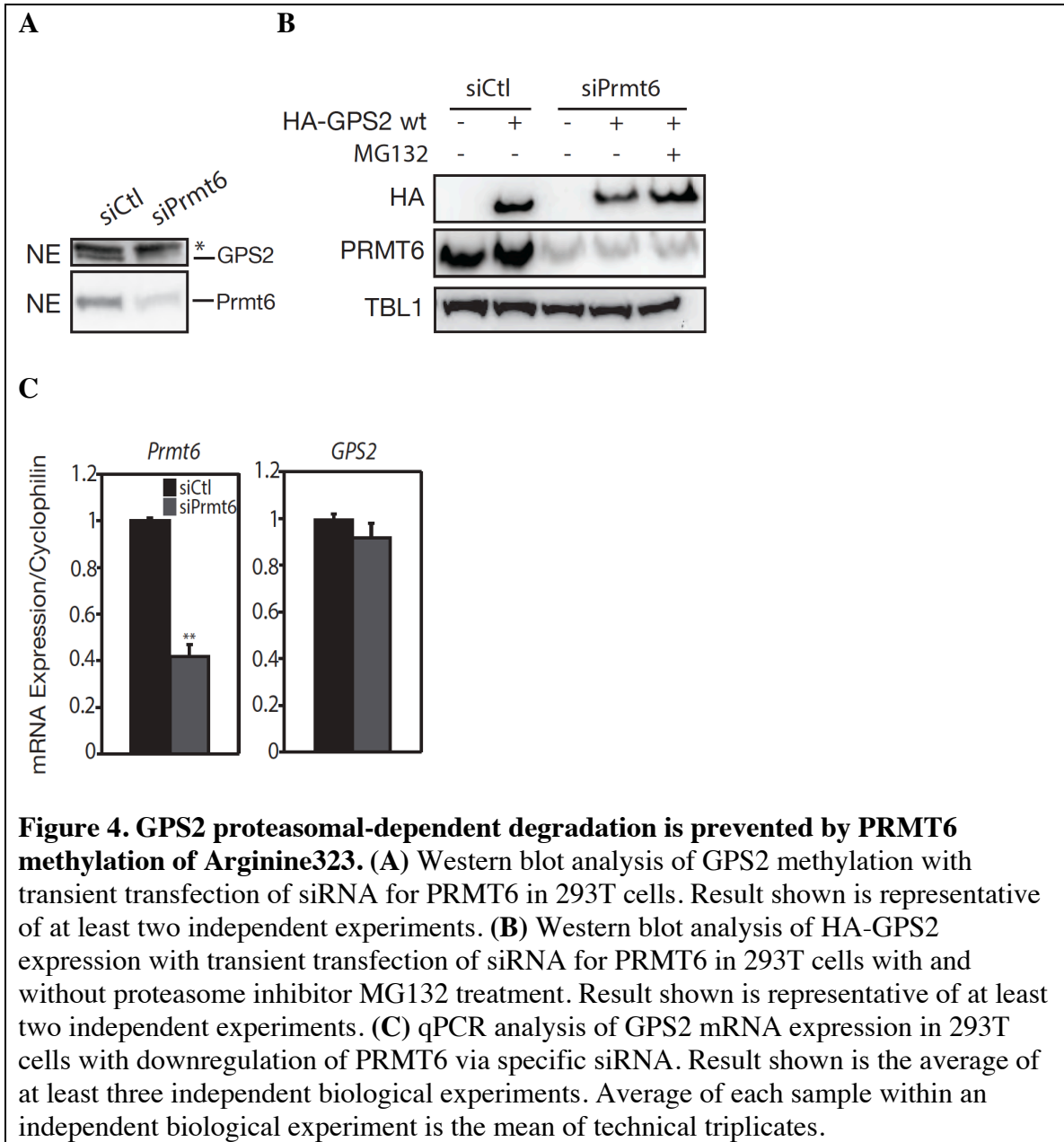
PRMT6-mediated methylation of arginine323 prevents proteasomal-dependent degradation of GPS2.

Interestingly, the three lysines required for GPS2-dependent poly-ubiquitination are flanking arginine 323 (R323), which has been recently reported as a site of differential demethylation in melanoma cells (Jarmalavicius, 2010). Thus, we hypothesized that methylation of R323 could affect GPS2 protein stability and degradation. Previous work in our lab has shown that the R323A mutant construct of GPS2 that cannot be methylated is highly unstable, unless the expression is rescued by inhibiting its degradation via the specific proteasome inhibitor MG132 (unpublished data). In addition, the ablation of the methylation site impaired GPS2 binding to TBL1 in

immunoprecipitation (unpublished data). Specific motifs, such as WD40 domains like the one present in the C-terminus of TBL1, have been reported to possess selective affinity for methylated residues (Migliori, 2012). This suggests that TBL1-mediated protective effect against GPS2 degradation might be modulated by post-transcriptional modifications.

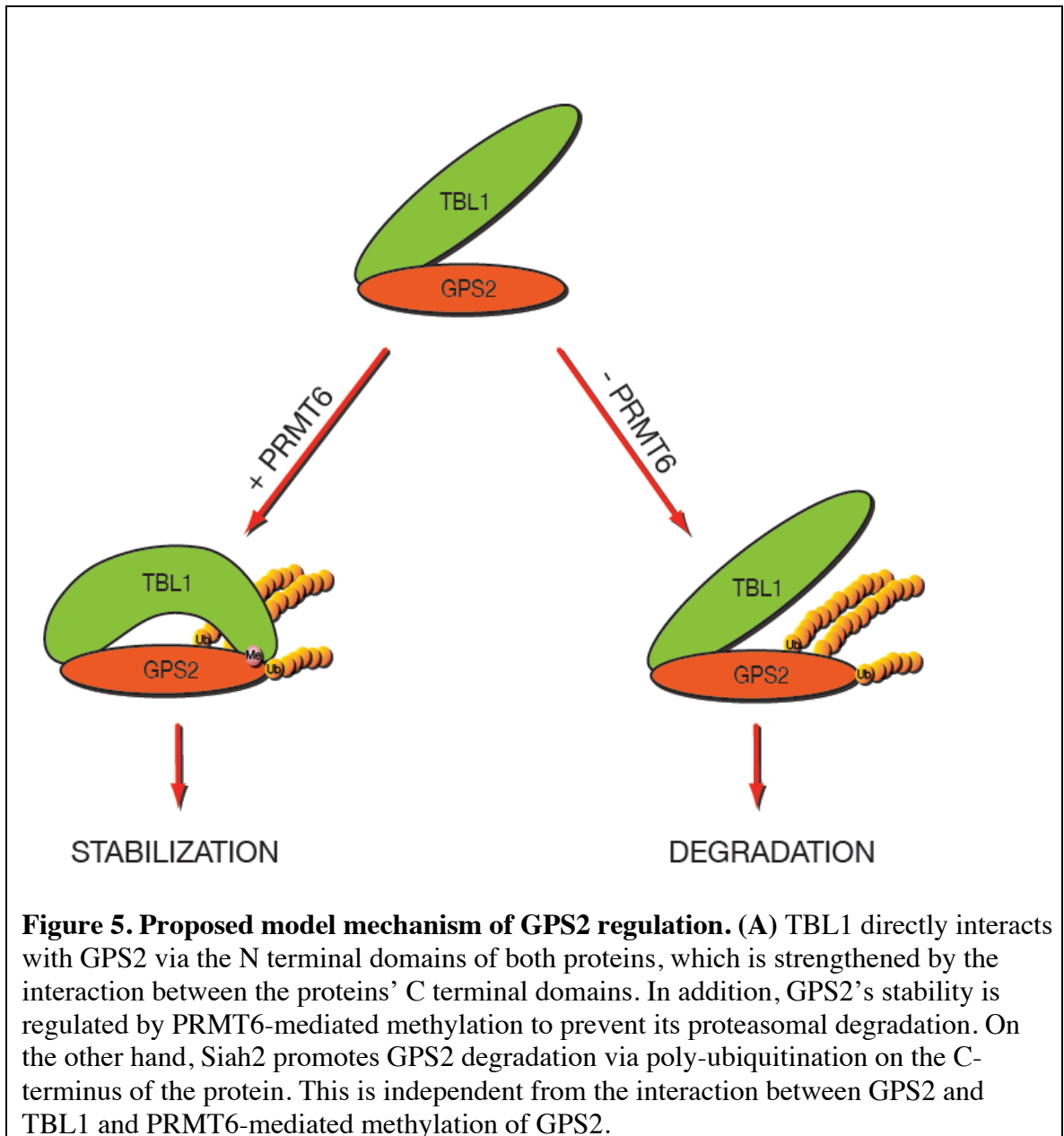
In order to further investigate this potential regulatory mechanism, we looked for the specific enzymes responsible for R323 methylation of GPS2. Protein arginine N-methyltransferases (PRMTs) that can catalyze mono-methylation and asymmetric demethylation belong to the Type I PRMT family (Zhang and Reinberg, 2006). Although PRMT1 is the major source of Type I methyltransferase activity in mammalian cells, previous work had eliminated PRMT1 as a candidate enzyme for GPS2 (Jarmalavicius, 2010). Among the rest of the Type I PRMTs, only PRMT6 is exclusively a nuclear enzyme, whereas the others are mainly cytosolic or membrane associated (Bedford, 2009; Frankel, 2002; Harrison, 2010; Lee, 2009). Therefore, I downregulated PRMT6 in HeLa cells to test whether GPS2 methylation would be impaired. As shown in Figure 4A, siPRMT6 transfection leads to significant reduction of GPS2 methylation in the nucleus since decrease in methylation subsequently leads to decreased protein level. In addition, specific downregulation of PRMT6 in 293T cells results in reduced expression of HA-tagged GPS2 (Fig. 4B). Importantly, the reduction in protein expression is due to proteasomal degradation and not to changes in gene expression as shown in the following findings: i) protein expression of HA-tagged GPS2 is rescued with proteasome inhibition

by MG132 (Fig. 4B); ii) mRNA expression of endogenous GPS2 is not affected by specific downregulation of PRMT6 as measured by RT-qPCR (Fig. 4C).



Proposed model mechanism of GPS2 regulation.

Next, our lab set out to investigate whether TBL1 protects GPS2 from degradation by preventing its poly-ubiquitination mediated by Siah2. Without TBL1, GPS2 is rapidly degraded. This suggests that TBL1 plays a protective role towards GPS2 in preventing it from degradation. However, using MG132 to inhibit proteasomal degradation revealed that GPS2 ubiquitination is still present with downregulation of TBL1 (unpublished data). This suggests that Siah2-mediated poly-ubiquitination and degradation of GPS2 is independent of the presence of TBL1. Altogether, our results indicate that interaction with TBL1 stabilizes GPS2 between the N terminal domains of both proteins, and secondary interaction between the C terminal domains via PRMT6-mediated methylation further promotes GPS2 stability. This interaction prevents GPS2 proteasomal degradation, but is independent of GPS2 poly-ubiquitination on the C terminal domain by Siah2.



GPS2 is required for transcriptional regulation of nuclear encoded mitochondrial gene in response to cellular perturbation.

Our lab has previously performed GPS2 chromatin immunoprecipitation sequencing (ChIP-seq) in 3T3-L1 cells (Cardamone, 2014). Bioinformatic analysis of this

data indicates that GPS2 peaks are localized within the vicinity of a number of nuclear-encoded mitochondrial genes. Since a majority of mitochondrial genes are nuclear-encoded (Garesse and Vallejo, 2001), this suggests the possibility that GPS2, along with associated cofactors, can potentially modulate mitochondrial functions through regulating the expression of nuclear-encoded mitochondrial genes. Because we expect that altering the stability and/or the nuclear localization of GPS2 would affect the expression of its target genes, including mitochondrial genes, I set out to optimize the conditions to assess changes in gene expression in response to FCCP, which is a potent proton ionophore that can uncouple ATP synthesis and oxidative phosphorylation of the electron transport chain in the mitochondria (MeSH, 2007). First, I performed time course experiments to establish the optimal time point that FCCP induces changes in mitochondrial gene expression in 3T3-L1 using qPCR. The nuclear-encoded mitochondrial genes of interest are presented in Table 1. These genes are targets of GPS2 binding in ChIPseq that were selected to represent important mitochondrial function.

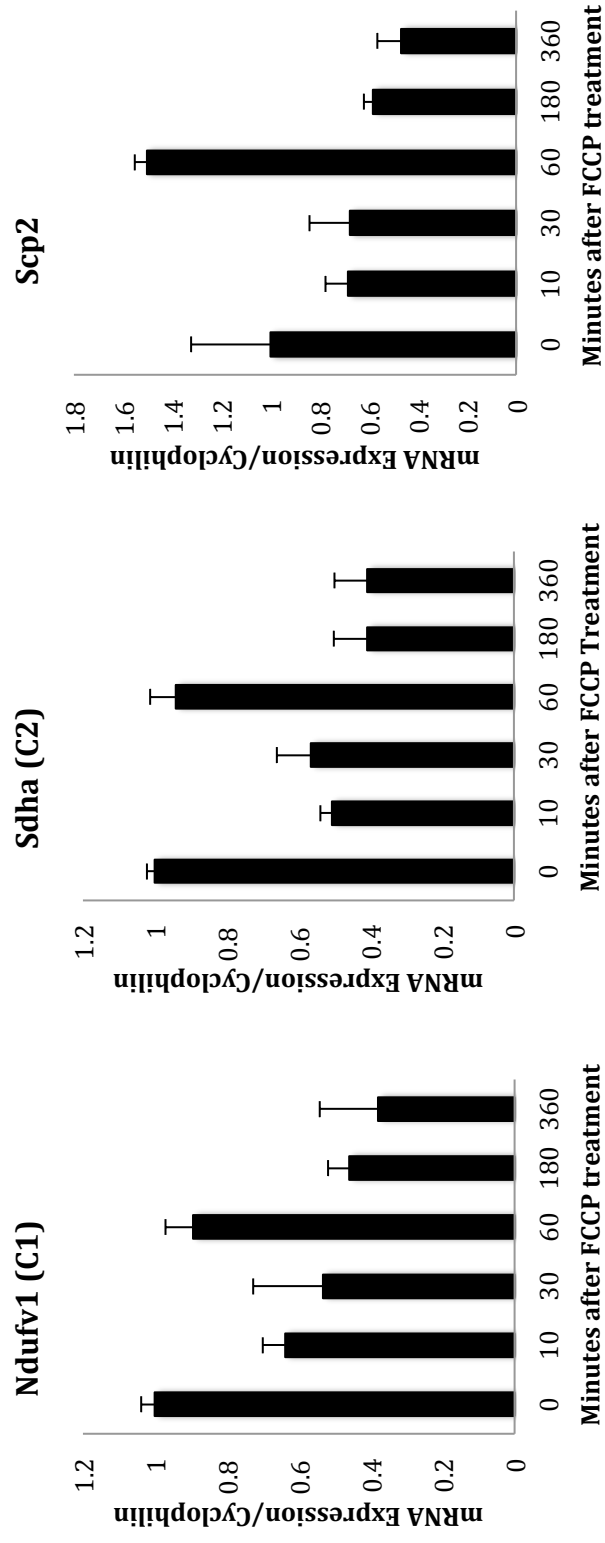
My results indicate that treating 3T3-L1 with FCCP leads to depressed expression of these genes after 10 minutes and 30 minutes, which then return to the level comparable to the untreated cells after 60 minutes. Subsequently, the gene expressions are reduced after 3 hours and 6 hours of FCCP treatment (Fig. 6A). According to these results, we chose 30-minute and 3-hour time points for subsequent experiments to test GPS2 regulation of nuclear-encoded mitochondrial gene transcription in presence of FCCP. As shown in Figure 6B, gene expression levels for GPS2 and subunits of complexes I, II, III, and V of the electron transport chain are reduced upon FCCP treatment in 293T cells

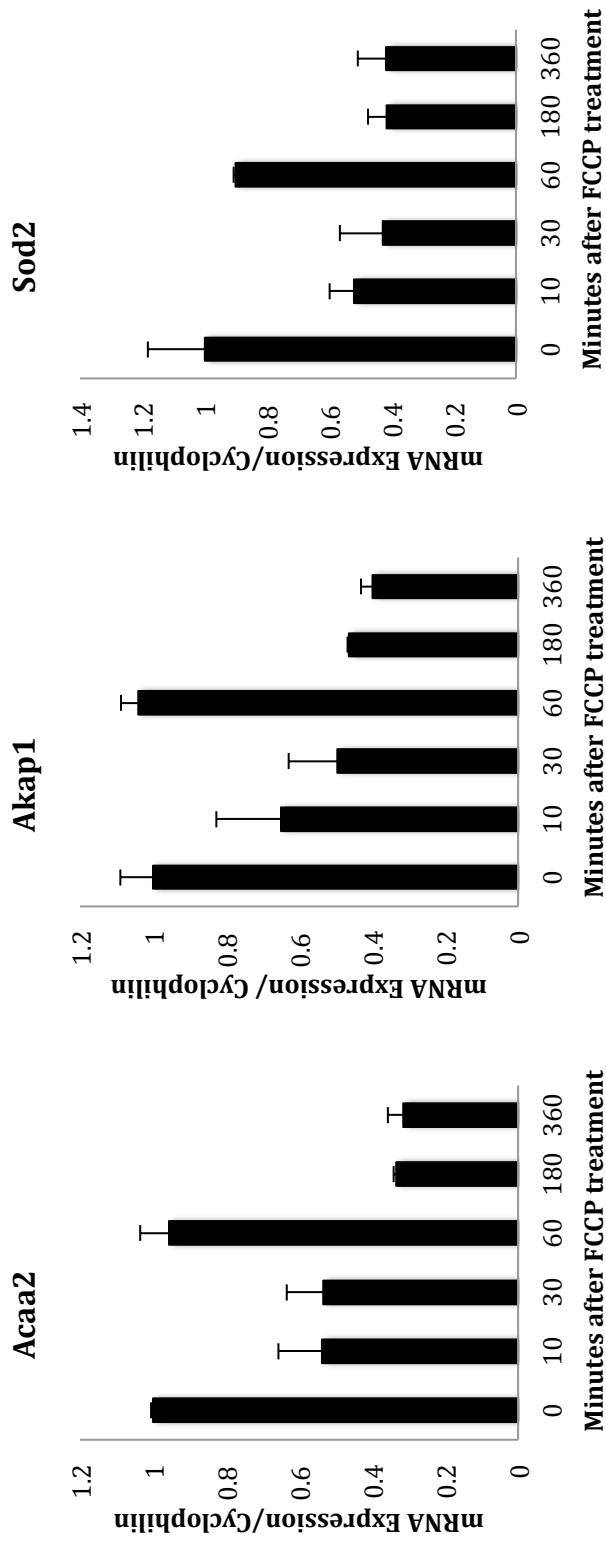
after 30 minutes, then return to initial levels after 3 hours. Downregulation of GPS2 via specific siRNA transient transfection leads to significant reduction in gene expression levels of complex I and III subunits of electron transport chain. Similar patterns of reduction in expression with downregulation of GPS2 are observed in selected genes of fatty acid oxidation/synthesis, mitochondrial fusion/fission, and mitochondrial anchor/translocase, with the exception of cholesterol synthesis (*idi1*) and superoxide dismutase (*sod2*) (Fig. 6C, 6D, 6E).

Gene	Protein Function
akap1	protein kinase A anchor protein
tomm20	translocase of outer mitochondrial membrane
sod2	superoxide dismutase
ndufv1	complex I of electron transport chain
sdha	complex II of electron transport chain
uqcrc1	complex III of electron transport chain
cox7a	complex IV of electron transport chain
atp5a1	complex V of electron transport chain
scp2	intracellular lipid transfer protein
cpt1	mitochondrial oxidation of long chain fatty acid
acaa2	fatty acid beta-oxidation
idi1	involved in synthesis of cholesterol
dnm1	mitochondrial fusion protein
opa1	mitochondrial fission protein

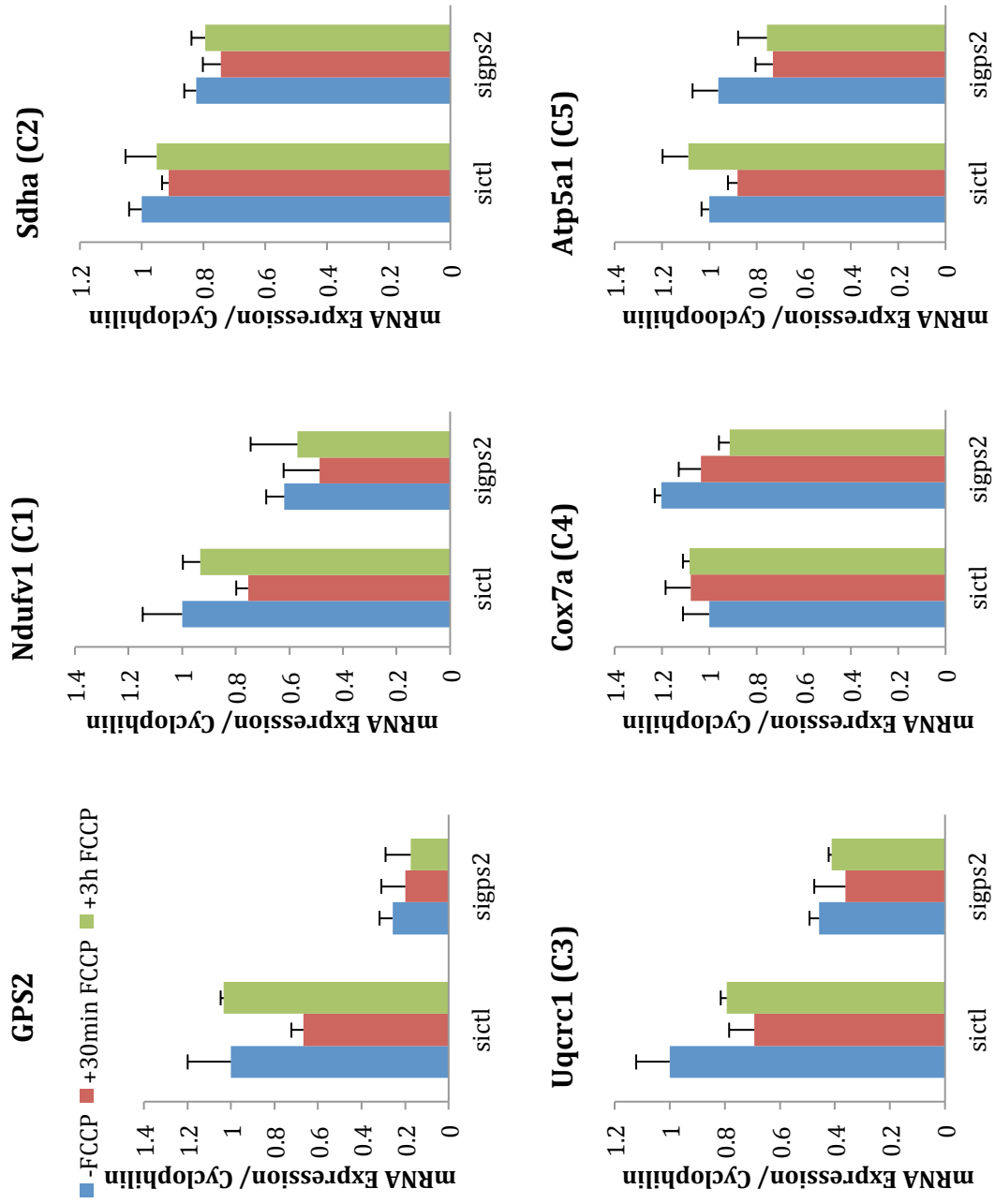
Table 1. Nuclear-encoded mitochondrial genes of interest.

A

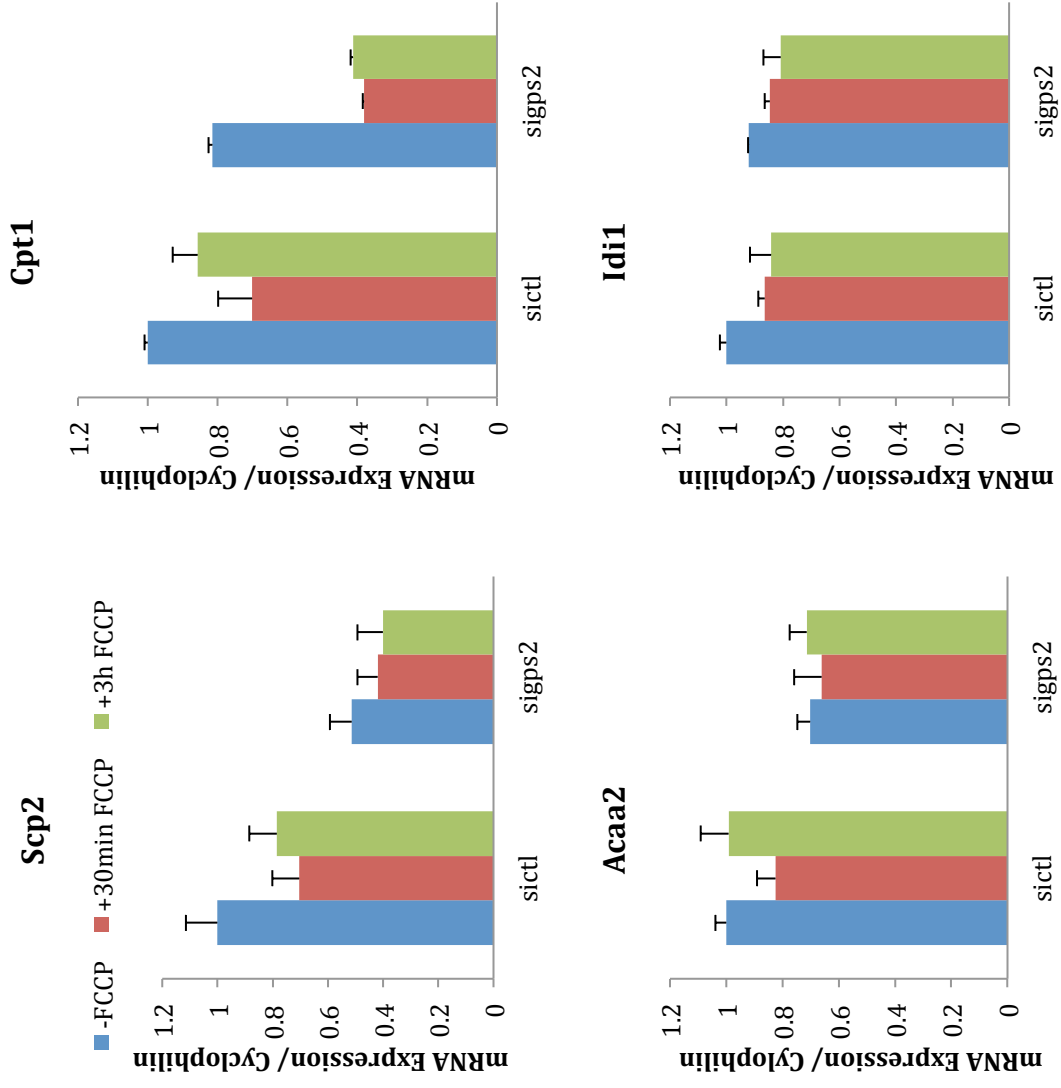




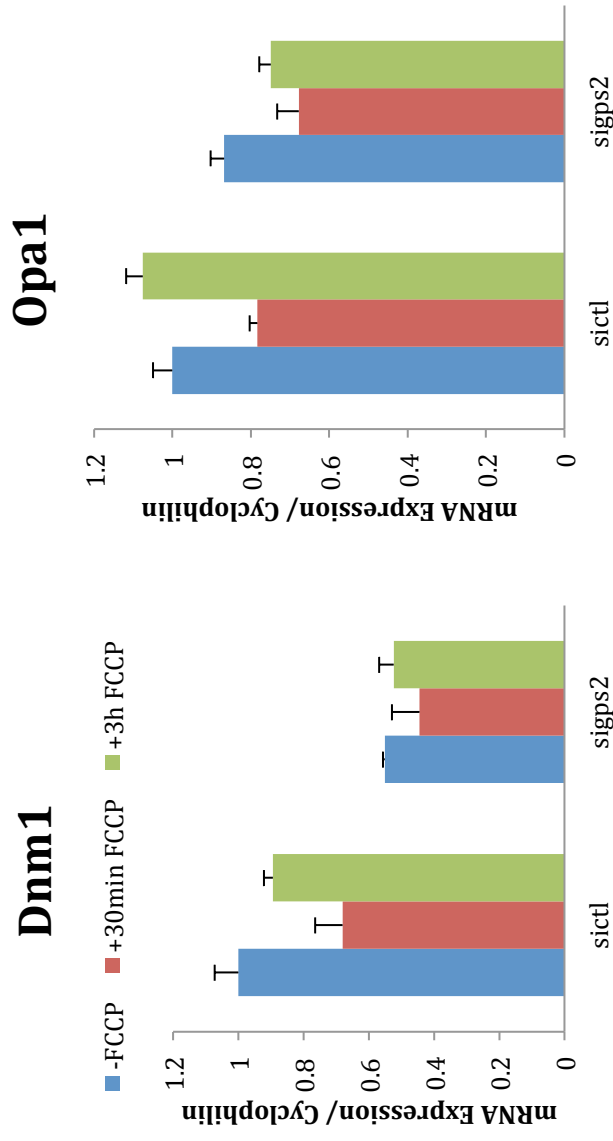
B



C



D



E

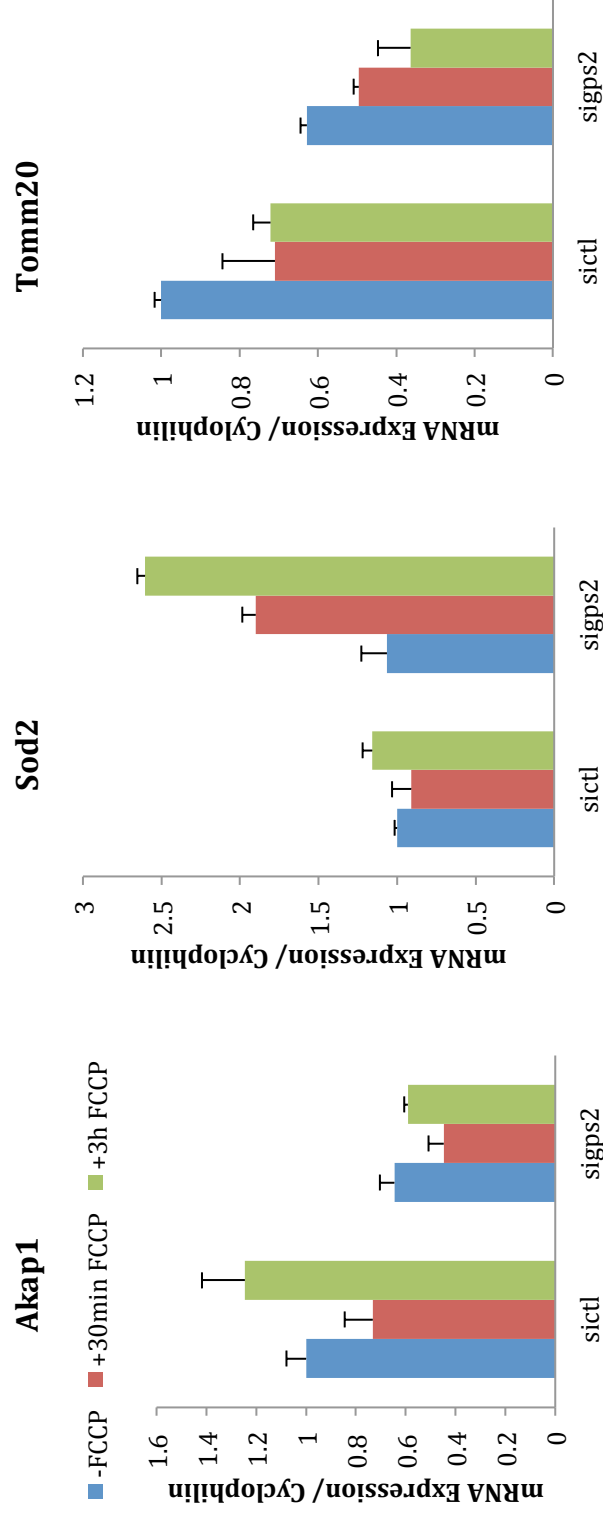


Figure 6. GPS2 is required for transcriptional regulation of nuclear-encoded mitochondrial gene in response to cellular stress. (A) qPCR analysis of gene expression in response to FCCP at various time points. (B) qPCR analysis of ETC subunit gene expression upon FCCP treatment and downregulation of GPS2 via specific siRNA. (C) qPCR analysis of fatty acid oxidation and cholesterol synthesis gene expression of upon FCCP treatment and downregulation of GPS2 via specific siRNA. (D) qPCR analysis of mitochondrial fusion and fission gene expression of upon FCCP treatment and downregulation of GPS2 via specific siRNA. (E) qPCR analysis of other mitochondrial related gene expression of upon FCCP treatment and downregulation of GPS2 via specific siRNA.

DISCUSSION

GPS2 is involved in a range of important cellular processes, including cell division, cell apoptosis, transcriptional regulation, and transduction signaling pathways (Spain et al., 1996; Peng et al., 2000, 2001; Lee et al., 2006; Cheng et al., 2009; Sanyal et al., 2007; Venteclef et al., 2010). It has been recently identified that GPS2 is linked to the regulation of metabolic and inflammatory pathways (Sanyal et al., 2007; Jakobsson et al., 2009; Cardamone et al., 2012; Toubal et al., 2013; Venteclef et al., 2010). Previous reports from our lab indicate that GPS2 participates in these cellular processes due to its ability to regulate ubiquitin signaling in different cellular compartments (Cardamone et al., 2012, 2014). This suggests that the regulation of GPS2 intracellular localization could have an important implication on cellular homeostasis. In this study, we set out to identify such mechanism and found that GPS2 nuclear localization depends on a minimal nuclear localization domain located at the N-terminus, which is mediated via interaction with TBL1 (Fig. 1).

While investigating the degradative mechanism for GPS2 instability in the absence of TBL1, we discovered that GPS2 is poly-ubiquitinated and is subjected to proteasomal-dependent degradation (Fig. 2). Interestingly, we identified *Drosophila* seven-in-absentia homolog 2 (Siah2) is responsible for the ubiquitination of GPS2 (Fig. 3). Siah2 is an E3 ubiquitin ligase that is involved in the ubiquitination of the nuclear receptor corepressor NCoR and HDAC3 (Zhang et al., 1998; Zhao et al., 2010), among other targets. Our previous work has shown TBL1 is necessary for the ubiquitination and the dismissal of the NCoR/SMRT corepressor complex upon gene activation. Based on

the above observations and literature, we deduce that TBL1's F box domain is required for the recruitment of Siah2-UbcH5 to the NCoR complex, in a similar fashion to Ebi's regulation of Tramtrack88 in *Drosophila* and β -catenin in mammalian cells (Perissi et al., 2004,2008; Dong et al., 1999; Matsuzawa et al., 2001). However, another study reports that TBL1 acts to protect β -catenin in Wnt signaling, suggesting that Siah1 can independently ubiquitinate β -catenin (Dimitrova et al., 2010). These opposing observations pose the question of whether TBL1 acts to promote or prevent poly-ubiquitination and degradation mediated by Siah2-UbcH5. Our results indicate that in the case of GPS2 TBL1 is not required for Siah-dependent ubiquitination, but rather plays a protective role against protein degradation. Further studies are required to address the mechanism as to how interaction with TBL1 prevents GPS2 from becoming degraded.

Lastly, our work identifies arginine methyltransferase PRMT6 as an important regulator of GPS2 protein stabilization and finds that the protective effect of TBL1 is modulated by post-translational modification of GPS2. The local nuclear environment stringently controls this regulatory mechanism. It has been reported that asymmetric demethylation of GPS2 occurs on R323 while identifying modifications that are specific to human leukocyte antigen peptidome of melanoma cells (Jarmalavicius, 2010), however the enzyme that is responsible for the modification of GPS2 is unknown. In our study, results indicate that PRMT6 methylation is required for GPS2 protein stabilization promoted by TBL1 (Fig. 4). Our data suggests that PRMT6 is the main enzyme that methylates and stabilizes GPS2 in the nuclear compartment. However, we cannot exclude the possibility that other Type I PRMTs are able to methylate GPS2 in other cellular

compartments or in different tissues (Bedford and Clark, 2009). Interestingly, PRMT6 has been found to be involved in processes that are related to GPS2 functions, such as chromatin remodeling, cell cycle regulation, p53 activity, transcriptional regulation by NFkB pathway (Di Lorenzo et al., 2014; Kleinschmidt et al., 2012; Stein et al., 2012; Neault et al., 2012). This suggests that some of the effects observed with modulation of PRMT6 may be due to changes in GPS2-mediated functions.

Altogether, while our results confirm that GPS2 directly interacts with TBL1 via the N terminal domain of GPS2 (Oberoi et al., 2011), the data also show that the methylation of arginine 323 on the C terminal domain may be necessary for promoting a stable interaction between GPS2 and TBL1. This may be due to the fact that the WD40 domain of TBL1 acts as the reader of the methylated arginine on GPS2 (Migliori, 2012). In addition, the results indicate that PRMT6 mediates the methylation of GPS2, possibly on arginine 323, which prevents its proteasomal-dependent degradation that is regulated by Siah2.

In conclusion, this study elucidates a tightly regulated mechanism that defines GPS2 protein level in the nucleus, in which the E3 ubiquitin ligase Siah2 promotes the degradation of GPS2 and TBL1 stabilizes the protein in a PRMT6-dependent fashion. Our results show the downregulation of GPS2 protein levels in the nucleus directly lead to reduce expression of nuclear-encoded mitochondrial genes. This suggests GPS2 might play an important role in regulating mitochondrial oxidative capacity, whose imbalance has been linked to chronic inflammation and insulin resistance seen in people with Type II Diabetes (Patti and Corvera, 2010).

REFERENCES

- Bedford, M. T., and Clarke, S. G. (2009) Protein arginine methylation in mammals: who, what, and why. *Molecular Cell* **33**, 1–13.
- Bi, H., Li, S., Wang, M., Jia, Z., Chang, A. K., Pang, P., and Wu, H. (2014) SUMOylation of GPS2 protein regulates its transcription-suppressing function. *Molecular Biology of the Cell* **25**, 2499–2508.
- Cardamone, M. D., Kronen, A., Tanasa, B., Taylor, H., Ricci, L., Ohgi, K. A., Glass, C. K., Rosenfeld, M. G., and Perissi, V. (2012) A protective strategy against hyperinflammatory responses requiring the nontranscriptional actions of GPS2. *Molecular Cell* **46**, 91–104.
- Cardamone, M. D., Tanasa, B., Chan, M., Cederquist, C. T., Andricovich, J., Rosenfeld, M. G., and Perissi, V. (2014) GPS2/KDM4A pioneering activity regulates promoter-specific recruitment of PPARgamma. *Cell Reports* **8**, 163–176.
- Cheng, X., and Kao, H. Y. (2009). G protein pathway suppressor 2 (GPS2) is a transcriptional corepressor important for estrogen receptor alpha-mediated transcriptional regulation. *Journal of Biological Chemistry* **284**, 36395–36404.
- Cheng, D., Vemulapalli, V., and Bedford, M. T. (2012) Methods applied to the study of protein arginine methylation. *Methods in Enzymology* **512**, 71–92.
- Choi, H. K., Yoo, J. Y., Jeong, M. H., Park, S. Y., Shin, D. M., Jang, S. W., Yoon, H. G., and Choi, K. C. (2013) Protein kinase A phosphorylates NCoR to enhance its nuclear translocation and repressive function in human prostate cancer cells. *Journal of Cellular Physiology* **228**, 1159–1165.
- Choi, H. K., Choi, K. C., Yoo, J. Y., Song, M., Ko, S. J., Kim, C. H., Ahn, J. H., Chun, K. H., Yook, J. I., and Yoon, H. G. (2011) Reversible SUMOylation of TBL1-TBLR1 regulates beta-catenin-mediated Wnt signaling. *Molecular Cell* **43**, 203–216.
- Di Lorenzo, A., Yang, Y., Macaluso, M., and Bedford, M. T. (2014) A gain-of-function mouse model identifies PRMT6 as a NF-kappaB coactivator. *Nucleic Acids Research* **42**, 8297–8309.
- Dimitrova, Y. N., Li, J., Lee, Y. T., Rios-Esteves, J., Friedman, D. B., Choi, H. J., Weis, W. I., Wang, C. Y., and Chazin, W. J. (2010) Direct ubiquitination of beta-catenin by Siah-1 and regulation by the exchange factor TBL1. *Journal of Biological Chemistry* **285**, 13507–13516.

- Dong, X., Tsuda, L., Zavitz, K. H., Lin, M., Li, S., Carthew, R. W., and Zipursky, S. L. (1999) *ebi* regulates epidermal growth factor receptor signaling pathways in *Drosophila*. *Genes & Development* **13**, 954–965.
- Frankel, A., Yadav, N., Lee, J., Branscombe, T. L., Clarke, S., and Bedford, M. T. (2002) The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *Journal of Biological Chemistry* **277**, 3537–3543.
- Frew, I. J., Hammond, V. E., Dickins, R. A., Quinn, J. M., Walkley, C. R., Sims, N. A., Schnall, R., Della, N. G., Holloway, A. J., Digby, M. R., Janes, P. W., Tarlinton, D. M., Purton, L. E., Gillespie, M. T., and Bowtell, D. D. (2003) Generation and analysis of *Siah2* mutant mice. *Molecular and Cellular Biology* **23**, 9150–9161.
- Garesse, R. and Vallejo, C. (2001) Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. *Gene* **263**, 1–16.
- Guo, A., Gu, H., Zhou, J., Mulhern, D., Wang, Y., Lee, K. A., Yang, V., Aguiar, M., Kornhauser, J., Jia, X., Ren, J., Beausoleil, S. A., Silva, J. C., Vemulapalli, V., Bedford, M. T., and Comb, M. J. (2014) Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Molecular & Cellular Proteomics: MCP* **13**, 372–387.
- Guo, C., Li, Y., Gow, C. H., Wong, M., Zha, J., Yan, C., Liu, H., Wang, Y., Burris, T. P., and Zhang, J. (2014) The optimal corepressor function of nuclear receptor corepressor (NCoR) for peroxisome proliferator-activated receptor gamma requires G-protein pathway suppressor 2. *Journal of Biological Chemistry* **290**(6), 3666–3679. doi: 10.1074/jbc.M114.598797.
- Harrison, M. J., Tang, Y. H., and Dowhan, D. H. (2010) Protein arginine methyltransferase 6 regulates multiple aspects of gene expression. *Nucleic Acids Research* **38**, 2201–2216.
- Hotamisligil, G.S. (2006). Inflammation and metabolic disorders. *Nature* **444**, 860–867.
- Hotamisligil, GS, Shargill, NS, Spiegelman, BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*. 1993. **259**, 87–91.
- Huang, W., Ghisletti, S., Perissi, V., Rosenfeld, M. G., and Glass, C. K. (2009) Transcriptional integration of TLR2 and TLR4 signaling at the NCoR derepression checkpoint. *Molecular Cell* **35**, 48–57.

- Huang, W., Ghisletti, S., Saijo, K., Gandhi, M., Aouadi, M., Tesz, G. J., Zhang, D. X., Yao, J., Czech, M. P., Goode, B. L., Rosenfeld, M. G., and Glass, C. K. (2011) Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. *Nature* **470**, 414–418.
- Jakobsson, T., Venteclef, N., Toresson, G., Damdimopoulos, A. E., Ehrlund, A., Lou, X., Sanyal, S., Steffensen, K. R., Gustafsson, J. A., and Treuter, E. (2009) GPS2 is required for cholesterol efflux by triggering histone demethylation, LXR recruitment, and coregulator assembly at the ABCG1 locus. *Molecular Cell* **34**, 510–518.
- Jarmalavicius, S., Trefzer, U., and Walden, P. (2010) Differential arginine methylation of the G-protein pathway suppressor GPS-2 recognized by tumor-specific T cells in melanoma. *FASEB Journal* **24**, 937–946.
- Jin, D. Y., Teramoto, H., Giam, C. Z., Chun, R. F., Gutkind, J. S., and Jeang, K. T. (1997) A human suppressor of c-Jun N-terminal kinase 1 activation by tumor necrosis factor alpha. *Journal of Biological Chemistry* **272**, 25816–25823.
- Kleinschmidt, M. A., de Graaf, P., van Teeffelen, H. A., and Timmers, H. T. (2012) Cell cycle regulation by the PRMT6 arginine methyltransferase through repression of cyclin-dependent kinase inhibitors. *PloS One* **7**, e41446.
- Lee, Y. H., and Stallcup, M. R. (2009) Minireview: protein arginine methylation of nonhistone proteins in transcriptional regulation. *Molecular Endocrinology* **23**, 425–433.
- Lee, T. H., Yi, W., Griswold, M. D., Zhu, F., and Her, C. (2006) Formation of hMSH4-hMSH5 heterocomplex is a prerequisite for subsequent GPS2 recruitment. *DNA Repair* **5**, 32–42.
- Matsuzawa, S. I., and Reed, J. C. (2001) Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Molecular Cell* **7**, 915–926.
- MeSH Descriptor Data (2007) Carbonyl Cyanide P-Trifluoromethoxyphenylhydrazone. *U.S. National Library of Medicine*.
- Migliori, V., Mapelli, M., and Guccione, E. (2012) On WD40 proteins: propelling our knowledge of transcriptional control? *Epigenetics* **7**, 815–822.
- Neault, M., Mallette, F. A., Vogel, G., Michaud-Levesque, J., and Richard, S. (2012) Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor. *Nucleic Acids Research* **40**, 9513–9521.

- Oberoi, J., Fairall, L., Watson, P. J., Yang, J. C., Czimmerer, Z., Kampmann, T., Goult, B. T., Greenwood, J. A., Gooch, J. T., Kallenberger, B. C., Nagy, L., Neuhaus, D., and Schwabe, J. W. (2011) Structural basis for the assembly of the SMRT/NCoR core transcriptional repression machinery. *Nature Structural & Molecular Biology* **18**, 177–184.
- Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G., and Glass, C. K. (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759–763.
- Patti, ME, Corvera, S. (2010) The Role of Mitochondria in the Pathogenesis of Type 2 Diabetes. *Endocrine Reviews* **31(3)**, 364–395.
- Peng, Y. C., Breiding, D. E., Sverdrup, F., Richard, J., and Androphy, E. J. (2000) AMF1/Gps2 binds p300 and enhances its interaction with papillomavirus E2 proteins. *Journal of Virology* **74**, 5872–5879.
- Peng, Y. C., Kuo, F., Breiding, D. E., Wang, Y. F., Mansur, C. P., and Androphy, E. J. (2001) AMF1 (GPS2) modulates p53 transactivation. *Molecular and Cellular Biology* **21**, 5913–5924.
- Perissi, V., Aggarwal, A., Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (2004) A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* **116**, 511–526.
- Perissi, V., Scafoglio, C., Zhang, J., Ohgi, K. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (2008) TBL1 and TBLR1 phosphorylation on regulated gene promoters overcomes dual CtBP and NCoR/SMRT transcriptional repression checkpoints. *Molecular Cell* **29**, 755–766.
- Sanyal, S., Bavner, A., Haroniti, A., Nilsson, L. M., Lundasen, T., Rehnmark, S., Witt, M. R., Einarsson, C., Talianidis, I., Gustafsson, J. A., and Treuter, E. (2007) Involvement of corepressor complex subunit GPS2 in transcriptional pathways governing human bile acid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15665–15670.
- Spain, B. H., Bowdish, K. S., Pacal, A. R., Staub, S. F., Koo, D., Chang, C. Y., Xie, W., and Colicelli, J. (1996) Two human cDNAs, including a homolog of Arabidopsis FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. *Molecular and Cellular Biology* **16**, 6698–6706.

- Stein, C., Riedl, S., Ruthnick, D., Notzold, R. R., and Bauer, U. M. (2012) The arginine methyltransferase PRMT6 regulates cell proliferation and senescence through transcriptional repression of tumor suppressor genes. *Nucleic Acids Research* **40**, 9522–9533.
- Toubal, A., Clement, K., Fan, R., Ancel, P., Pelloux, V., Rouault, C., Veyrie, N., Hartemann, A., Treuter, E., and Venteclef, N. (2013) SMRT-GPS2 corepressor pathway dysregulation coincides with obesity-linked adipocyte inflammation. *Journal of Clinical Investigation* **123**, 362–379.
- Uysal, K.T., et al. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature*. 1997. **389**, 610–614.
- Venteclef, N., Jakobsson, T., Ehrlund, A., Damdimopoulos, A., Mikkonen, L., Ellis, E., Nilsson, L. M., Parini, P., Janne, O. A., Gustafsson, J. A., Steffensen, K. R., and Treuter, E. (2010) GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. *Genes & Development* **24**, 381–395.
- Wellen, K. E. & Hotamisligil, G. S. Inflammation, stress, and diabetes. *Journal of Clinical Investigation* **115**, 1111–1119 (2005).
- Zhang, J., Guenther, M. G., Carthew, R. W., and Lazar, M. A. (1998) Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes & Development* **12**, 1775–1780.
- Zhang, D., Harry, G. J., Blackshear, P. J., and Zeldin, D. C. (2008) G-protein pathway suppressor 2 (GPS2) interacts with the regulatory factor X4 variant 3 (RFX4_v3) and functions as a transcriptional co-activator. *Journal of Biological Chemistry* **283**, 8580–8590.
- Zhang, J., Kalkum, M., Chait, B. T., and Roeder, R. G. (2002) The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. *Molecular Cell* **9**, 611–623.
- Zhang, X., Ozawa, Y., Lee, H., Wen, Y. D., Tan, T. H., Wadzinski, B. E., and Seto, E. (2005) Histone deacetylase 3 (HDAC3) activity is regulated by interaction with protein serine/threonine phosphatase 4. *Genes & Development* **19**, 827–839.
- Zhang, Y. and Reinberg, D. (2006) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes & Development* **15**, 2343–2360.

- Zhang, D., Yang, Q., Ding, Y., Cao, X., Xue, Y., and Cheng, Z. (2008) Cytological characterization of the tandem repetitive sequences and their methylation status in the *Antirrhinum majus* genome. *Genomics* **92**, 107–114.
- Zhao, H. L., Ueki, N., and Hayman, M. J. (2010) The Ski protein negatively regulates Siah2-mediated HDAC3 degradation. *Biochemical and Biophysical Research Communications* **399**, 623–628.

VITA

Jiawen Huang was born in the People's Republic of China in 1989. She moved to San Diego, California at the age of twelve. After she graduated from Scripps Ranch High School in 2008, Jiawen attended the University of California–Berkeley, where she earned her B.S. degree in Nutritional Sciences in 2012. Jiawen worked in an ecology lab at the Lawrence Berkeley National Laboratory from 2011 to 2013, where she learned about environmental microbiology and discovered her interest in research, leading her to attend Boston University School of Medicine's Master's program in nutrition and metabolism.