

L-Ornithine orchestrates transcription via alternative splicing regulation

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L-Ornithine orchestrates transcription via alternative splicing regulation

Running title: Novel ornithine signals regulate alternative splicing

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Abstract

Ornithine is a non-proteinogenic amino acid involved in the urea cycle and amino acid metabolism. We previously demonstrated that ornithine enhances the transcription in hepatocytes through a noncanonical pathway^{1,2}, although the underlying mechanism remains unclear. Little is known about how amino acids directly regulate transcriptional activity independently of canonical nutrient-sensing pathways.

Here, we identified heterogeneous nuclear ribonucleoproteins (hnRNPs), including hnRNP M and PTBP1, as L-ornithine-binding proteins that mediate transcriptional and alternative splicing responses.

Bead-based protein purification technology identified several hnRNP family proteins that selectively interacted with L-ornithine, whereas citrulline did not. RNA sequencing and RT-PCR analyses showed that L-ornithine altered alternative splicing patterns in multiple genes. Binding analyses revealed that hnRNP M and PTBP1 exhibited a higher affinity for L-ornithine than for D-ornithine, and that hnRNP M directly interacted with L-ornithine independently of RNA. Furthermore, PTBP1 knockdown abolished L-ornithine-dependent transcriptional activation and splicing changes.

These findings demonstrate that L-ornithine regulates gene expression through direct

interaction with splicing factors, revealing a previously unrecognized mechanism linking amino acids to RNA-processing-mediated transcriptional control.

This mechanism may represent a new layer of nutrient-dependent cellular regulation and provide insight into how amino acid availability shapes cellular function and adaptation.

Keywords: Ornithine signal, alternative splicing, heterogeneous nuclear ribonucleoproteins

Introduction

Protein nutrition has a considerable impact on a wide range of physiological processes in animals, and feeding them a low-protein diet induces various phenotypic changes³⁻⁹.

In particular, we and our colleagues have demonstrated that feeding rats a low-protein diet reduces serum insulin levels, increases systemic insulin sensitivity¹⁰, promotes ectopic fat accumulation in the liver and skeletal muscles, and decreases gluconeogenesis in the liver¹⁰⁻¹². Furthermore, some phenotypes induced by low-protein diets can be elicited by restricting specific amino acids. For instance,

methionine restriction extends the lifespan of various organisms, including rodents and fruit flies¹³⁻¹⁶. Moreover, arginine restriction induces hepatic steatosis¹², whereas lysine restriction leads to fat accumulation in skeletal muscles¹⁷. These findings suggest that different amino acids contribute to different phenotypic outcomes under low-protein conditions.

Additionally, we showed that culturing liver and muscle cell lines in media mimicking the serum amino-acid concentrations of rats with dietary intervention induced fat accumulation¹⁸ and uptake¹⁷, respectively, suggesting that amino acids themselves can directly act on cells as signaling molecules to regulate cellular metabolism. Consistently, a protein-deficient diet led to reductions in the hepatic mRNA expression of gluconeogenic enzymes, such as glucose phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*), and a subsequent decrease in gluconeogenic activity in rats¹⁰; culturing liver-derived H4IIE cells in an amino acid-free medium suppressed *G6pc* transcription. Interestingly, this suppression was relieved by ornithine treatment¹, and *G6pc* induction by ornithine was independent of mechanistic target of rapamycin complex 1 activity, which is a key nutrient-sensing molecule responsive to amino-acid stimulation^{19,20}. These results indicate that ornithine

may activate a noncanonical signaling pathway(s) to regulate cellular metabolism,
which warrants investigation of the unique molecular mechanism of ornithine signaling.

In this study, we focused on the transcriptional regulation of key gluconeogenic
enzymes that had their expression markedly enhanced by L-ornithine. To uncover the
relevant mediator(s) involved, we developed a bead-based protein purification method
and successfully identified several proteins that (semi-)specifically bind to ornithine.
Here we show, for the first time, that ornithine regulates gluconeogenic gene expression
by interacting with various splicing factors and modulating the alternative splicing of
multiple target genes.

Results

D-Ornithine does not upregulate gluconeogenic gene expression levels

H4IIE rat hepatoma-derived cells were cultured in Full (amino acid-sufficient medium),
Zero (amino acid-depleted medium), or Zero+Orn (Zero medium supplemented with 4
mM L-ornithine) medium for the indicated durations to analyze gluconeogenic gene
expression levels. Consistent with our previous results¹, *G6pc*, *Fbp1*, and *Pck1* mRNA
levels significantly increased when cultured in Full or Zero+Orn medium for 24 h
compared with those when cultured in Zero medium (Fig. 1a). When cells were cultured

95 in Zero medium supplemented with D-ornithine (enantiomer of L-ornithine), the mRNA
96 levels also increased, but to a lesser extent compared with those when cultured in
97 Zero+L-Orn medium (Fig. 1b), indicating that D-ornithine is the less-active enantiomer.

98

99 **Isolation of ornithine-binding proteins**

100 To identify the signal-mediating molecules that allow ornithine to upregulate
101 gluconeogenic gene expression levels, we developed a methodology to isolate proteins
102 that bind to ornithine. In this method, ornithine (or other target molecules) was
103 covalently conjugated to beads carrying amino moieties using a linker in the initial step.
104 We previously reported that ornithine has the ability to enhance gluconeogenic gene
105 expression levels, whereas citrulline (5-*N*-carbamoylated ornithine) does not¹; therefore,
106 L-citrulline-conjugated beads were also used as a control counterpart (Fig. 2a). H4IIE
107 cell lysates were incubated with these beads, and the bead-associated proteins then
108 eluted with L-ornithine or L-citrulline. Subsequent silver staining revealed many protein
109 bands in the eluate with L-ornithine, whereas only a few slight protein bands were
110 detected in the eluate with L-citrulline (Fig. 2b). By performing LC–MS/MS analysis
111 of the eluate with L-ornithine, various proteins were identified as candidate ornithine-

binding proteins. Gene Ontology analyses indicated that proteins involved in RNA splicing, processing, and metabolic processes were enriched among the ornithine-binding proteins identified (Fig. 2c). Remarkably, many RNA splicing factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs) were isolated (Fig. 2d). The interactions of hnRNP A2/B1, L, C, and I (PTBP1) with L-ornithine were confirmed via immunoblotting, but those with L-citrulline could not be detected (Fig. 2e).

Next, we conducted these same experiments using D-ornithine because it had failed to increase the mRNA levels of gluconeogenic enzymes. The results showed that band patterns of silver staining were not noticeably different between lanes when using L-ornithine- or D-ornithine-conjugated beads (Fig. 2f). However, when eluates from these beads were subjected to immunoblotting, one of the hnRNP proteins (PTBP1) specifically bound to L-ornithine but not to D-ornithine or L-citrulline (Fig. 2g). We then attempted to isolate L-ornithine-specific binding proteins using the strategy shown in Fig. 2h. Briefly, L-ornithine-conjugated beads were prepared and incubated with H4IIE cell extracts, after which the beads were washed with excess D-ornithine to remove proteins that could bind to it. The remaining proteins were then eluted with L-ornithine and analyzed via LC-MS/MS (Fig. 2h). Consequently, the proteins shown in Fig. 2i

were detected. Among these, the interactions of L-ornithine with hnRNP M, hnRNP C, SRSF2, and hnRNP A1 were confirmed through immunoblotting (Fig. 2j). As these proteins contain RNA-binding domains and function by binding to pre-mRNA and small nuclear RNA²¹⁻²³, we examined whether their interaction with ornithine was dependent on RNAs by treating H4IIE cell extracts with RNase. As shown in Fig 2k, RNase treatment abolished the binding of most proteins; however, binding to hnRNP M was maintained even after RNase treatment, strongly suggesting that ornithine directly interacts with hnRNP M. Next, to examine the binding affinity for D-ornithine, beads covalently conjugated with L-ornithine were prepared and incubated with H4IIE cell extracts. The beads were then eluted with L-ornithine or D-ornithine, and the eluates analyzed for the presence of hnRNP M or PTBP1 (Fig. 2l). The results showed that hnRNP M and PTBP1 were eluted with ornithine, and the amount eluted with D-ornithine was lower than that eluted with L-ornithine, indicating that D-ornithine has the lower binding affinity.

L-Ornithine modulates alternative splicing of various genes

As ornithine interacted with various splicing factors, we tested whether alternative

146 splicing was regulated in response to ornithine treatment in H4IIE cells. Cells were
147 cultured in Full, Zero, and Zero+Orn media for 15 h, and RNA sequence analyses
148 subsequently performed. We found that the alternative splicing of various genes was
149 altered in Zero medium compared with that in Full medium. Importantly, many of these
150 alternative splicing changes were recovered following ornithine treatment. We selected
151 six of these alternatively spliced genes (*Insr*, *Dmtf1*, *Hibch*, *Tcerg1*, *Zmynd8*, and
152 *Tmed4*) and further examined alternative splicing using reverse transcription PCR (RT-
153 PCR). Primers were designed within exons flanking the alternatively spliced exon, and
154 RT-PCR performed using the RNAs extracted from H4IIE cells subjected to various
155 culture conditions as templates (Fig. 3a,b). The upper and lower bands represent exon
156 inclusion and skipping, respectively. For all genes, except *Tmed4*, exon exclusion was
157 enhanced in Zero medium compared with that in Full or 10% fetal bovine serum
158 medium. In contrast, exon inclusion was promoted by L-ornithine treatment compared
159 with that in the untreated Zero medium. Interestingly, supplementation with L-citrulline
160 (Fig. 3a) or D-ornithine (Fig. 3b) failed to restore the changes in alternative splicing
161 patterns induced by the Zero medium. In the case of *Tmed4*, exon inclusion and
162 exclusion showed opposite patterns. The splicing patterns of all tested genes gradually

changed over time after switching the medium, becoming particularly pronounced at 15 h, and the magnitude of their splicing changed in a chemical dose-dependent manner (Fig. 3c,d).

PTBP1 is required for ornithine-induced gluconeogenic gene expression

To examine the role that L-ornithine-specific binding proteins play in alternative splicing regulation and gluconeogenic gene expression, PTBP1 was knocked down using small interfering RNA (siRNA) in H4IIE cells (Fig. 4a). Notably, the L-ornithine-induced enhancement of exon inclusion in *Insr* was suppressed by PTBP1 knockdown in H4IIE cells (Fig. 4b). We then performed real-time quantitative PCR (qPCR) to quantify the relative abundance of the splicing variants of *Insr*, referred to as *Ir-a* and *Ir-b* (Fig. 4c). Results showed that the L-ornithine-induced decrease in the proportion of *Ir-a* was attenuated upon PTBP1 knockdown (Fig. 4c). Finally, we measured the expression levels of gluconeogenic genes in these cells and found that PTBP1 knockdown suppressed the L-ornithine-induced upregulation of *G6pc*, *Fbp1*, and *Pck* mRNA levels. These data suggest that PTBP1, as an L-ornithine-specific binding protein, is required for the transcriptional activation of gluconeogenic genes in response

to ornithine.

Discussion

We previously demonstrated that culturing H4IIE hepatoma cells in amino acid-free medium suppresses the expression of rate-limiting gluconeogenic enzymes, and that this effect is fully reversed by ornithine supplementation. Here, we uncovered the molecular basis of this phenomenon. By isolating ornithine-binding proteins, we identified a previously unrecognized amino-acid signaling pathway that directly links ornithine sensing to the control of alternative splicing. In line with this model, ornithine broadly regulated alternative splicing across multiple genes in H4IIE cells. Strikingly, both the activation of gluconeogenic gene expression and modulation of alternative splicing were strictly specific to L-ornithine and not recapitulated by the structurally related L-citrulline or D-ornithine, highlighting a stereospecific signaling mechanism. Although the mechanism by which amino acids regulate downstream signaling by binding to proteins has been elucidated in recent years^{24,25}, the present study is the first to reveal differences in protein binding and signal regulation between enantiomers. Mechanistically, we identified hnRNP M and PTBP1 as ornithine-

binding proteins with selective affinity for L-ornithine. Functional perturbation revealed that PTBP1 deletion not only abrogated ornithine-dependent splicing regulation but also disrupted the transcriptional activation of gluconeogenic genes. Together, our findings establish ornithine as a signaling metabolite that coordinates alternative splicing and transcription through specific RNA-binding proteins, revealing a previously unappreciated layer of amino acid-dependent gene regulation.

However, siRNAs targeting different regions of PTBP1 (Extended Data Fig. 1a), as well as hnRNP M knockdown, had no detectable effects on either the splicing patterns or transcriptional activity (Extended Data Fig. 1b–e). This discrepancy highlights an unexpected layer of complexity in the underlying regulatory mechanism. One possible explanation is that distinct PTBP1-targeting siRNAs differentially affect specific splicing isoforms, thereby masking their overall impact. Alternatively, other metabolites, as discussed below, may contribute to the regulation of transcription and splicing independent of PTBP1.

D-Ornithine failed to induce either gluconeogenic gene expression or alternative splicing changes, suggesting that metabolites specifically generated from L-ornithine may mediate these effects. Although polyamines are well-known ornithine-derived

metabolites (Extended Data Fig. 2a) that have been implicated in splicing regulation²⁶, neither intracellular polyamine levels nor pharmacological inhibition of their synthesis affected splicing or gluconeogenic gene expression (Extended Data Fig. 2b–e), arguing against their involvement in this context. Metabolomic analyses instead identified several amino acids, including proline, alanine, aspartate, and glutamate, whose intracellular levels were selectively increased by L-ornithine but not by D-ornithine (Extended Data Fig. 3a–f). Functional analyses revealed that alanine and aspartate modulate alternative splicing (Extended Data Fig. 3g,h), and that aspartate particularly enhances gluconeogenic gene expression (Extended Data Fig. 3i). Notably, these effects occurred independently of the changes in intracellular ornithine levels, suggesting that aspartate acts as a downstream effector of ornithine signaling.

These findings may account for the lack of a pronounced phenotype observed upon hnRNP M or PTBP1 knockdown. Even if ornithine-mediated signaling is attenuated, compensatory signaling by aspartate may function redundantly, thereby masking the effects of gene suppression. These observations suggest that multiple

230 amino acid-derived signals operate in parallel to coordinately regulate alternative
231 splicing and transcription.

232 Our results demonstrate that ornithine regulates both alternative splicing and
233 transcription, and that these activities are closely correlated. This raises the possibility
234 that splicing regulation acts upstream to drive the observed transcriptional changes.
235 Therefore, we examined the transcription factors whose expression is known to be
236 regulated by alternative splicing and identified several candidates. However, we have
237 yet to identify or validate the transcription factors responsible for regulating the
238 expression of rate-limiting gluconeogenic enzymes. These findings highlight an
239 important area for future investigation and suggest that further studies are required to
240 elucidate the mechanistic link between splicing regulation and transcriptional control.

241 Recently, we demonstrated that ornithine exerts physiological effects, as dietary
242 supplementation with ornithine suppresses hepatic steatosis induced by an arginine-
243 restricted diet in which only arginine is limited²⁷. In contrast, this protective effect
244 was not observed in fatty liver induced by a low-protein diet. Arginine restriction also
245 regulates very-low-density lipoprotein secretion²⁸, and ornithine has been implicated
246 as a molecular regulator of this process. Collectively, these findings suggest that the

modulation of ornithine signaling may influence not only hepatic steatosis but also circulating cholesterol and triglyceride levels. Notably, this effect was not observed following intravenous administration²⁷, suggesting that ornithine does not act solely within the liver but may instead exert its effects through coordinated signaling across multiple cell types and tissues. In line with this notion, the present study demonstrated that ornithine interacts with a variety of proteins to mediate intracellular signaling. These observations suggest that ornithine functions as a multifunctional nutrient capable of inducing diverse phenotypic outcomes both in vivo and in vitro, warranting further investigation into its underlying mechanisms and broader physiological roles.

Tables

Table 1. Experimental media compositions (mg/L)

	Full	Zero
Glycine	30.0	0
L-Alanine	35.6	0
L-Serine	42.0	0
L-Threonine	95.0	0
L-Cystine	48.0	0

L-Methionine	30.0	0
L-Glutamine	584.0	0
L-Asparagine•H ₂ O	60.0	0
L-Glutamic acid	58.8	0
L-Aspartic acid	53.2	0
L-Valine	94.0	0
L-Leucine	105.0	0
L-Isoleucine	105.0	0
L-Phenylalanine	66.0	0
L-Tyrosine	72.4	0
L-Tryptophan	16.0	0
L-Lysine•HCl	146.0	0
L-Arginine•HCl	84.0	0
L-Histidine	31.0	0
L-Proline	46.0	0
EBSS	10%	10%
Vitamin solution	1%	1%
NaHCO ₃	2200	2200
D-Glucose	4500	4500

259

260

261 **Table 2. Primer list**

Gene	Primer sequence (5'–3')
rat <i>Insr</i>	F: GTCCCCACCTTTTGAGTCTG
	R: GTCACATTGCCCACCTCTTC
rat <i>Gapdh</i>	F: TCTCTGCTCCTCCCTGTTCTA
	R: GGTAACCAGGCGTCCGATAC
rat <i>Dmtf1</i>	F: TGGGATCTTCTAGCTGAGGG
	R: ATGTTTGCAGTGTTCCACTGTT
rat <i>Fip1l1</i>	F: AGGATCCGAATGGGACTGGA
	R: TGCTGTTCTCATTTCACGC
rat <i>Hibch</i>	F: TCAGCTGAGGATGTTGCTGG
	R: CAGCTGGTTTCCATTTTGGAGT
rat <i>Tcerg1</i>	F: GGCATGCCAATTCCACTTCC
	R: TCCACTCAGAAACTGCGGTG
rat <i>Tmed4</i>	F: CACATCGGCGAGACTGAGAA
	R: TCTGTTCCACCTGGTCAAGC
rat <i>Zmynd8</i>	F: ACTTGGGCTTGGATTCGGAC
	R: GTGATGGTGGATGTGGACGG

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Extended data figure legends

Fig 1. L-ornithine upregulates expression levels of gluconeogenic genes, but D-ornithine did not.

(a): H4IIE rat hepatoma cells were cultured in a medium containing 20 amino acids (Full), in a medium with no amino acids (Zero), or in a medium Zero supplemented with 4 mM L-ornithine for indicated hours. *G6pc* mRNA levels were quantified using real-time qPCR and normalized against 18S rRNA levels. *Fbp1* and *Pck* were analyzed using the $\Delta\Delta C_t$ method due to the lack of reliable standard curves. **(b):** H4IIE cells were cultured in Zero, Zero supplemented with 4 mM L-ornithine, or Zero supplemented with 4 mM D-ornithine for 15 hours. *G6pc*, *Fbp1*, or *Pck* mRNA levels were quantified using real-time qPCR and normalized against 18S rRNA levels. (a, b): Bar: mean $\pm$ S.E.M., a false discovery rate- (FDR-) adjusted *p*-value, (a): **p* < 0.05 (between Full and Zero), #*p* < 0.05 (between Full and Zero+Orn), \$*p* < 0.05 (between Zero and Zero+Orn), (b): **p* < 0.05, ***p* < 0.01, ****p* < 0.001. *n* = 3, Welch's *t* test.

Fig 2. Isolation of L-ornithine binding proteins

(a): Schematic representation of the chemical structure of beads covalently conjugated

366 with L-ornithine, D-ornithine, or L-citrulline. **(b)**: Dialyzed H4IIE cell extracts, from
367 which ornithine or citrulline had been removed by overnight dialysis, were incubated
368 with L-ornithine-conjugated or L-citrulline-conjugated beads. The bound proteins were
369 then eluted with L-ornithine or L-citrulline, separated by SDS-PAGE, and visualized
370 by silver staining. **(c)**: Proteins contained in the eluates from Fig. 2B were identified by
371 LC-MS/MS analysis, and Gene Ontology (GO) analysis was performed on the
372 identified proteins. **(d)**: Lists of proteins identified as L-ornithine-binding proteins with
373 high Mascot scores. **(e)**: Immunoblotting analysis was performed on the eluates from
374 Fig. 2b using the indicated antibodies. **(f)**: H4IIE cell extracts were incubated with free-,
375 L-ornithine-, D-ornithine-, and L-citrulline-conjugated beads at 4C, and bound proteins
376 were eluted with excess L-ornithine, D-ornithine, or L-citrulline. Eluates were carried
377 out for the SDS-PAGE and bound proteins were visualized by silver staining. **(g)**:
378 Immunoblotting analysis was performed on the eluates from Fig.2f using the indicated
379 antibodies. **(h)**: Isolation of L-ornithine-specific binding proteins. H4IIE cell extracts
380 were incubated with beads covalently conjugated with L-ornithine to allow binding of
381 L-ornithine interacting proteins. After extensive washout with excess D-ornithine to
382 remove proteins that also bind D-ornithine, L-ornithine specific proteins were eluted

with L-ornithine, and the eluates were subjected to LC-MS/MS analysis. **(i)**: Lists of the L-ornithine-specific binding proteins. **(j)**: Immunoblotting analysis was performed on the eluates from Fig. 2F using the indicated antibodies. **(k)**: To examine whether each protein binds via RNA, H4IIE cell extracts were treated with RNase prior to the binding experiments. **(l)**: To compare the binding affinities of binding proteins for L-ornithine and D-ornithine, H4IIE cell extracts were incubated with L-ornithine-conjugated beads, and the bound proteins were eluted with either L-ornithine or D-ornithine to assess their relative binding affinities.

Fig 3. Ornithine regulates alternative splicing of various genes.

(a): H4IIE cells were cultured in Full, Zero, Zero+L-Orn, Zero+Cit for 15 h. RT-PCR analyses were carried out to detect the change in alternative splicing. Primers were designed within the exons flanking the exon whose inclusion or exclusion was expected to be regulated. **(b)**: H4IIE cells were cultured in Full, Zero, Zero+L-ornithine, or Zero+D-ornithine. RT-PCR analyses were carried out to detect the change in alternative splicing. **(c)**: H4IIE cells were cultured in Zero or Zero supplemented with indicated concentration of L-ornithine for 15 hours. RT-PCR analyses were carried out to detect

the change in alternative splicing. **(d)**: H4IIE cells were cultured in Zero or Zero supplemented with L-ornithine for indicated hours. RT-PCR analyses were carried out to detect the change in alternative splicing.

Fig 4. Transcriptional and alternative splicing regulation by ornithine binding proteins.

(a): Schematic overview of the experimental design. **(b)**: H4IIE cells were transfected with siRNA against with NC or siPTBP1 #1. RT-PCR analyses were carried out to detect the change in alternative splicing. For PTBP1 and β -actin, protein levels were analyzed by immunoblotting. **(c)**: *Ira*- and *Irb*-specific primers were designed and used for qPCR analysis. Based on these results, the mRNA ratio of *Ira*/(*Ira*+*Irb*) was calculated. **(d)**: H4IIE cells were transfected with siRNA against with NC or PTBP1. *G6pc*, *Fbpl*, or *Pck* mRNA levels were quantified using real-time qPCR and normalized against 18S rRNA levels. **(c)**, **(d)**: Bar: mean $\pm$ S.E.M., a false discovery rate- (FDR-) adjusted *p*-value, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. *n* = 3, Welch's *t* test.

Extended Data Fig. 1.

(a): Schematic diagram of the target sites of siRNA against PTBP1 are shown. **(b):**

H4IIE cells were transfected with siRNA against with NC or siPTBP1 #2. PTBP1 and

β -actin, protein levels were analyzed by immunoblotting. *Ira*- and *Irb*-specific primers

were designed and used for qPCR analysis. Based on these results, the mRNA ratio of

Ira/(Ira+Irb) was calculated. **(c):** *G6pc* mRNA levels were quantified using real-time

qPCR and normalized against 18S rRNA levels. **(d):** H4IIE cells were transfected with

siRNA against with NC or sihnRNP M. hnRNP M and β -actin protein levels were

analyzed by immunoblotting. *Ira*- and *Irb*-specific primers were designed and used for

qPCR analysis. Based on these results, the mRNA ratio of *Ira/(Ira+Irb)* was calculated.

(e): *G6pc*, *Fbp1*, or *Pck* mRNA levels were quantified using real-time qPCR and

normalized against 18S rRNA levels. (b) – (e): Bar: mean $\pm$ S.E.M., a false discovery

rate- (FDR-) adjusted *p*-value, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. *n* = 3, Welch's *t*

test.

Extended Data Fig. 2. Transcriptional and splicing regulation by L-ornithine

metabolites.

(a): A schematic diagram illustrating ornithine metabolites. (b): The intracellular polyamine levels were measured in H4IIE cells cultured for 15 h in 10%FBS medium, Full, Zero, Zero supplemented with L-ornithine, or Zero supplemented with L-citrulline. (c): The intracellular polyamine levels were measured in H4IIE cells cultured for 48 h in 10%FBS medium containing 0, 0.1, 1.0 mM DFMO. (d): H4IIE cells were cultured for 48 hours in 10% FBS medium containing 0, 0.1, 1.0 mM DFMO. RT-PCR analyses were carried out to detect the change in alternative splicing of various genes. (e): *G6pc*, *Fbp1*, or *Pck* mRNA levels were quantified using real-time qPCR and normalized against 18S rRNA levels. Bar: mean $\pm$ S.E.M., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$, Welch's t test.

Extended Data Fig. 3. Transcriptional and splicing regulation by L-ornithine metabolites.

(a): H4IIE cells were cultured in Zero, Zero+L-ornithine, Zero+D-ornithine for 1, 3, 15 hours. Intracellular metabolites were quantified by LC-MS/MS analysis. (b-f): Intracellular levels of ornithine (b), proline (c), alanine (d), aspartic acid (e), glutamic acid (f) were shown. Y-axis indicates the fold change of intracellular levels of those at

451 time point = 0 (hr). A false discovery rate- (FDR-) adjusted p -value, $*p < 0.2$ compared
452 to Zero, $n = 3$, Welch's t test. **(g)**: H4IIE cells were cultured in Full, Zero, Zero+L-
453 ornithine, or Zero+L-proline. RT-PCR analyses were carried out to detect the change in
454 alternative splicing. **(h)**: H4IIE cells were cultured in Full, Zero, Zero+L-ornithine, or
455 Zero+L-alanine, Zero+L-aspartate, Zero+L-glutamate. RT-PCR analyses were carried
456 out to detect the change in alternative splicing. **(i)**: H4IIE cells were cultured in Full,
457 Zero, Zero+L-ornithine, or Zero+L-alanine, Zero+L-aspartate, Zero+L-glutamate.
458 *G6pc*, *Fbp1*, or *Pck* mRNA levels were quantified using real-time qPCR and
459 normalized against 18S rRNA levels. Bar: mean $\pm$ S.E.M., a false discovery rate-
460 (FDR-) adjusted p -value, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. $n = 3$, Welch's t test.
461

462 **Methods**

463 **Materials**

464 Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui
465 Pharmaceutical Co. (Tokyo, Japan). Earle's buffered salt solution (10×), MEM vitamin
466 solution (100×), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St.
467 Louis, MO, USA). Difluoromethylornithine (DFMO) was purchased from TCI (Tokyo,
468 Japan). All other reagent-grade chemicals used in this study are commercially available.

469

470 **Cell culture**

471 H4IIE-C3 cells (rat hepatoma cell line; ATCC CRL-1600) were grown in DMEM
472 supplemented with 10% FBS and antibiotics under 5% CO₂ at 37°C. When the cells
473 reached sub-confluency, the media were changed to the experimental media (Table 1),
474 and the cells then cultured for the indicated durations.

475

476 **RNA extraction and real-time quantitative PCR**

477 Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA,
478 USA), and cDNA synthesis performed using the ReverTra Ace qPCR RT Master Mix

(Toyobo, Osaka, Japan). The cDNAs were subjected to qPCR using the THUNDERBIRD SYBR qPCR Mix (Toyobo) and a 7500TH Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). For all samples, 18S ribosomal RNA was measured as an internal control and used for data normalization. Relative expression levels were primarily quantified using instrument-derived quantity values based on standard curve calibration. However, for *Fbp1* and *Pck* shown in Fig. 1a, reliable standard curves could not be obtained; therefore, relative expression levels were calculated using the $\Delta\Delta C_t$ method²⁹, assuming comparable amplification efficiencies between the target and reference genes. The primer sets used are listed in Table 2.

To detect changes in alternative splicing patterns, primers were designed to flank the exon of interest, and PCR then performed using cDNA as a template. The PCR products were separated via polyacrylamide gel electrophoresis (PAGE), and expression levels of the two splicing isoforms generated by alternative splicing subsequently compared.

Preparation of ornithine-conjugated, citrulline-conjugated, and free beads

N- α ,*N*- δ -Di(9-fluorenylmethoxycarbonyl)-L-ornithine (Fmoc-Orn(Fmoc)-L-OH;

496 Watanabe Chemical Industries, Hiroshima, Japan), *N*- α ,*N*- δ -di(9-
497 fluorenylmethoxycarbonyl)-D-ornithine (Fmoc-Orn(Fmoc)-D-OH; Watanabe
498 Chemical Industries), and *N*- α -(9-fluorenylmethoxycarbonyl)-L-citrulline (Fmoc-Cit-
499 OH; Watanabe Chemical Industries) were dissolved in dehydrated *N,N*-
500 dimethylformamide (DMF) to prepare 40 mM ligand solutions. *N*-Hydroxysuccinimide
501 (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were separately
502 dissolved in DMF to prepare 400 mM solutions. To 500 μ L of 40 mM ligand solution,
503 400 μ L DMF, 50 μ L of 400 mM NHS solution, and 50 μ L of 400 mM EDC solution
504 were added, and the mixture incubated at room temperature for 2 h using a microtube
505 mixer to obtain activated ligand solutions. NH₂ beads (2.5 mg; Tamagawa Seiki,
506 Nagano, Japan) were washed three times with DMF and mixed with 500 μ L of the
507 activated ligand solution to enable the coupling reaction, which was allowed to proceed
508 overnight at room temperature. The resulting beads were washed three times with DMF,
509 mixed with 20% acetic anhydride, and reacted for 2 h at room temperature to mask the
510 unreacted residual amino moieties. After washing thrice with DMF, the beads were
511 incubated with 50% piperidine for 30 min at room temperature to deprotect the Fmoc
512 group. The supernatant was collected, and its absorbance measured at 301 nm to assess

513 the free Fmoc concentration and calculate the reaction efficacy. The average efficacy
514 of all beads used in this study was approximately 30%. The final beads were washed
515 three times with DMF and again three times with 50% methanol diluted in distilled
516 water. Specimens were made shortly before and stored at 4°C until use. Free beads were
517 prepared by following the same procedure above, except without including ligands.

518

519 **Detection of ornithine- and citrulline-binding proteins**

520 Free, ornithine-conjugated, or citrulline-conjugated beads were washed three times
521 with 500 µL dialysis buffer (10 mM HEPES-KOH, 150 mM KCl, 1 mM EDTA, 10%
522 glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). H4IIE cells
523 were lysed by adding lysis buffer. The lysate was collected in a 1.5 mL tube and
524 sonicated until no visible cell clumps remained. The samples were then centrifuged at
525 $15,000 \times g$ for 20 min at 4°C, and the supernatant transferred into a Cellu-Sep T1
526 Tubular Membrane (nominal MWCO: 3,500; Membrane Filtration Products, Seguin,
527 TX, USA) and dialyzed overnight at 4°C against 250 mL dialysis buffer. Each bead
528 preparation (2.5 mg) was added to 500 µL dialyzed cell extract and incubated
529 overnight at 4°C. The beads were then washed three times with dialysis buffer and

530 three more times with 500 μ L elution buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM
531 NaF, 1 mM EGTA, 1 mM EDTA, pH 6.8). Finally, 20 μ L of the elution buffer
532 containing 250 mM ornithine or citrulline was added and incubated for 30 min at 4°C
533 using a microtube mixer. After centrifugation at 15,000 $\times$ g for 10 min at 4°C, the
534 supernatant was collected and subjected to silver staining or LC-MS/MS analysis.
535 The samples were mixed with half the volume of 3 $\times$ Laemmli sample buffer and
536 boiled for 5 min. Sodium dodecyl sulfate (SDS)-PAGE was performed using Extra
537 PAGE One Precast Gels (Nacalai Tesque, Kyoto, Japan). Silver staining was
538 performed using a SilverQuest Staining Kit (Invitrogen), according to the
539 manufacturer's protocol.

540

541 **LC-MS/MS analysis of amino acid-binding proteins**

542 LC-MS/MS analysis of amino acid-binding proteins was outsourced to Prof. Akihiro
543 Ito at the Tokyo University of Pharmacy and Life Sciences (Tokyo, Japan). MS and
544 MS/MS data were analyzed against the Swiss-Prot database using Proteome Discoverer
545 (Thermo Fisher Scientific, Waltham, MA, USA) and the MASCOT search engine
546 (Matrix Science, London, UK). Gene Ontology enrichment analysis of the identified

547 proteins was performed using Enrichr (<https://maayanlab.cloud/Enrichr/>).

548

549 **Transfection with siRNAs**

550 The siRNAs used in this study were obtained from Nippon Gene Materials Co. (Tokyo,
551 Japan). They comprised the following sequences: PTBP1 #1, 5'-
552 GUCUCAACGUCAAAUACAA-3'; PTBP1 #2, 5'-GUGGUACAAUUGUAUGUGU-
553 3'; and hnRNP M #1, 5'-AGACCUGGUUAAAGAAAAA-3'; The siRNAs were
554 transfected into H4IIE cells using Lipofectamine RNAiMAX Transfection Reagent
555 (Thermo Fisher Scientific) via reverse transfection, following the manufacturer's
556 protocol.

557

558 **Western blotting analysis**

559 Cell lysates were prepared using standard methods and subjected to SDS-PAGE. Gel-
560 separated proteins were transferred onto polyvinylidene fluoride membranes (Merck
561 Millipore, Billerica, MA, USA), and protein bands visualized using a Western
562 Lightning Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham,
563 MA, USA). The following antibodies were used: anti-hnRNP A1 (#06-248; Sigma-

564 Aldrich), anti-hnRNP L (4D11) (mouse, #sc-32317, 1:200; Santa Cruz Biotechnology,
565 Dallas, TX, USA), anti-hnRNP C1/C2 (4F4) antibody (SANTA CRUZ, mouse, #sc-
566 32308, 1:200), anti-hnRNP U (3G6) (mouse, #sc-32315, 1:200; Santa Cruz
567 Biotechnology), anti-PTBP1 (rabbit, #12582-1-AP, 1:3,000; Proteintech, Rosemont,
568 IL, USA), anti- β -actin (mouse, #A5441, 1:20,000; Sigma-Aldrich, HRP-conjugated
569 streptavidin (#N100; Thermo Fisher Scientific), HRP-conjugated anti-mouse IgG
570 (#NA931V; GE Healthcare, Chicago, IL, USA), and HRP-conjugated anti-rabbit IgG
571 (#NA934V; GE Healthcare, Chicago, IL, USA) antibodies (Table 2).

572

573 **Measurement of intracellular polyamine levels**

574 After aspirating the culture medium from cells grown in a 60-mm dish, 1 mL trypsin
575 solution was added, and the cells incubated at 37°C with 100% humidity and 5% CO₂
576 for 5–10 min. Once the cells detached, 3 mL of 10% FBS/DMEM was added and
577 pipetted to disperse the cells, whereafter the suspension was transferred to a 15-mL
578 centrifuge tube. The cells were centrifuged at 1,000 $\times$ g at room temperature for 5 min,
579 and the supernatant then aspirated. The pellet was gently resuspended by tapping the
580 tube, and cell counting performed after adding fresh 10% FBS/DMEM. The collected

cells were then processed using the Total Polyamine Assay Kit (ab239828; Abcam, Cambridge, UK), according to the manufacturer's protocol. Fluorescence was measured using an ARVO X3 plate reader (PerkinElmer) at an excitation wavelength of 535 nm and emission wavelength of 591 nm.

Metabolome analysis

Samples were prepared as previously described¹⁸ with minor modifications. Four dishes of cells per condition were prepared, three of which were subjected to metabolite extraction, and the remaining dish used to count cell numbers. After culturing in experimental media, the cells were washed with phosphate-buffered saline (PBS). Then, the cells were scraped with methanol containing 10 μ M 2-(*N*-morpholino)ethanesulfonic acid and methionine sulfone as an internal control. The lysates were collected in test tubes and centrifuged at $14,000 \times g$ for 10 min at 4°C. The supernatant was transferred to chloroform, ultrapure water added, and the mixture then centrifuged for 3 min. Thereafter, the upper aqueous layer was mixed with ultrapure water and centrifuged for 3 min. The supernatant was subjected to ultrafiltration using 3-kDa cutoff filters (Amicon Ultra 3 K device; Merck, Darmstadt, Germany), followed by 6 h

of lyophilization. The metabolites were reconstituted in ultrapure water and subjected to LC–MS/MS (LCMS-8030; Shimadzu, Kyoto, Japan) using the Method Package for Primary Metabolites ver. 2 (Shimadzu), according to the manufacturer’s protocol. The relative amount of each metabolite was evaluated by quantifying the corresponding peak area, and data normalization performed by dividing the relative amount of metabolites by the number of cells.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (S.E.M.), with the number of samples indicated in the figure. Depending on the experiment, a two-group comparison using Welch’s *t*-test was performed. The *p*-values were further adjusted using the Benjamini–Hochberg criterion, and a *p*-value < 0.05 considered statistically significant. All statistical analyses were conducted using Python software (v.3.12).

Data availability

615 All the original data for this manuscript are available upon reasonable request.

616

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625

626 **Author contributions** M.I., S.F., W.S., N.M., H.S., M.A., S.F., N.H., and F.H. designed
627 the experiments and critically discussed the findings. M.I., S.F., W.S., N.M., H.S., M.A.,
628 S.F., and N.H. performed the experiments. S. F., and D.Y. measured the amino acid
629 concentrations in H4IIE cells. Y.M., and A.I. identified the ornithine-binding proteins.
630 D.Y., A.I., S.F., H.N., and F.H. contributed materials and analytical tools. S.F., H.N.,
631 and F.H. wrote the manuscript.

632

633 **Competing interests** The authors declare no conflicts of interest.

634

635 **Additional information**

636 **Correspondence and requests for materials** should be addressed to Fumihiko

637 Hakuno.

638 **Reprints and permissions information** is available at

639 <http://www.nature.com/reprints>.

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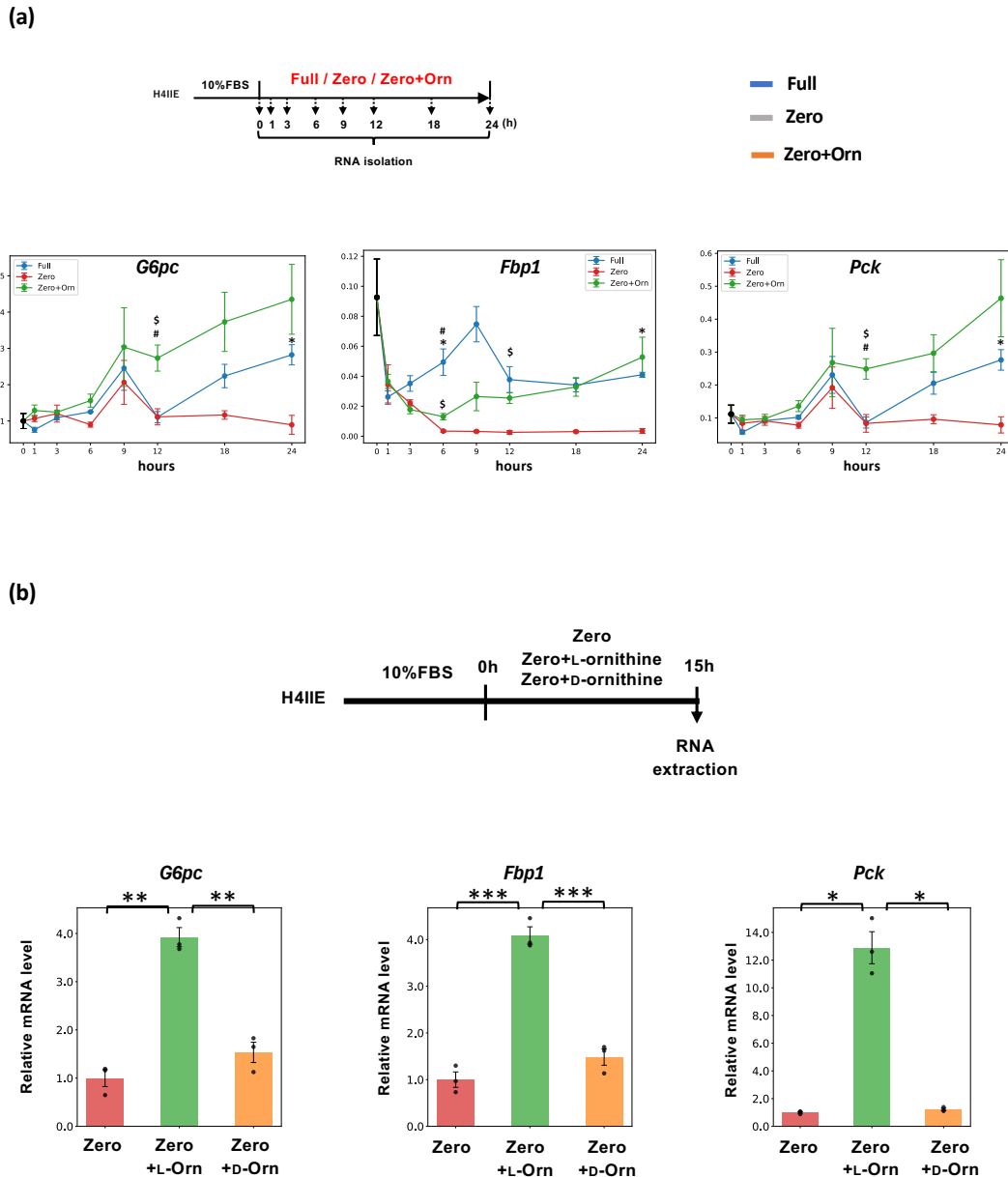


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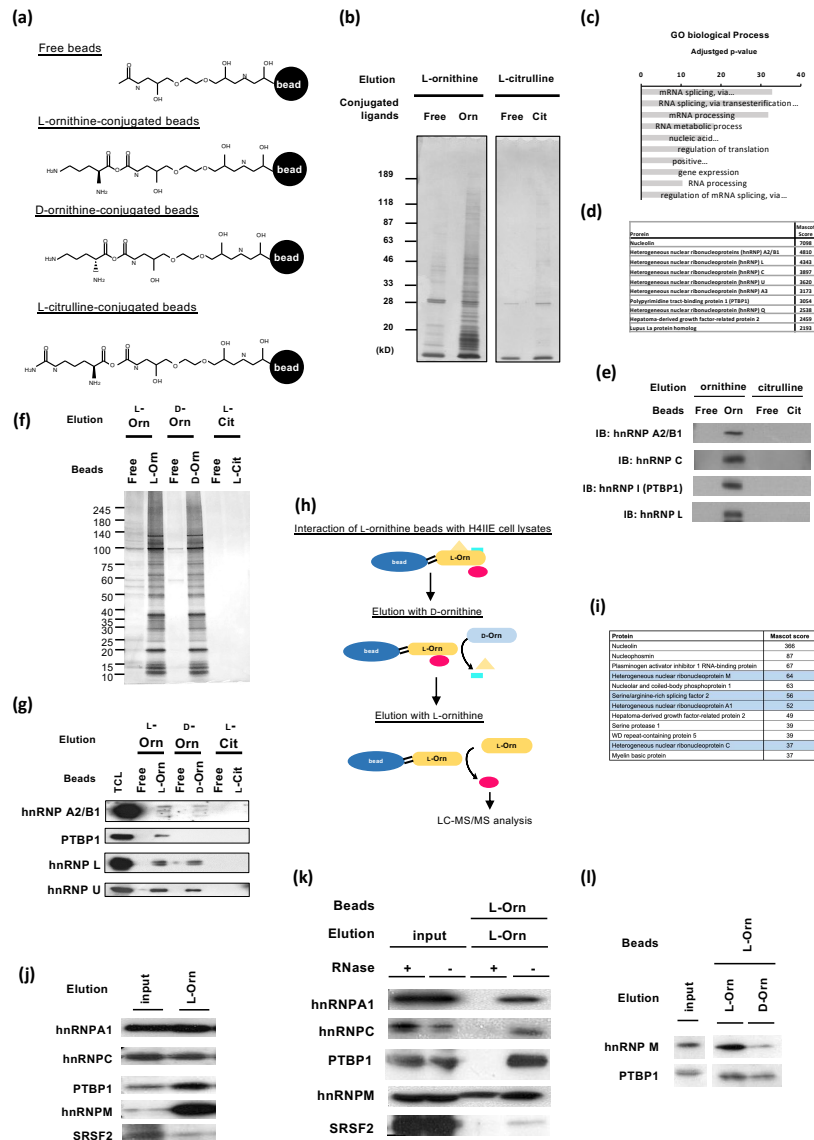


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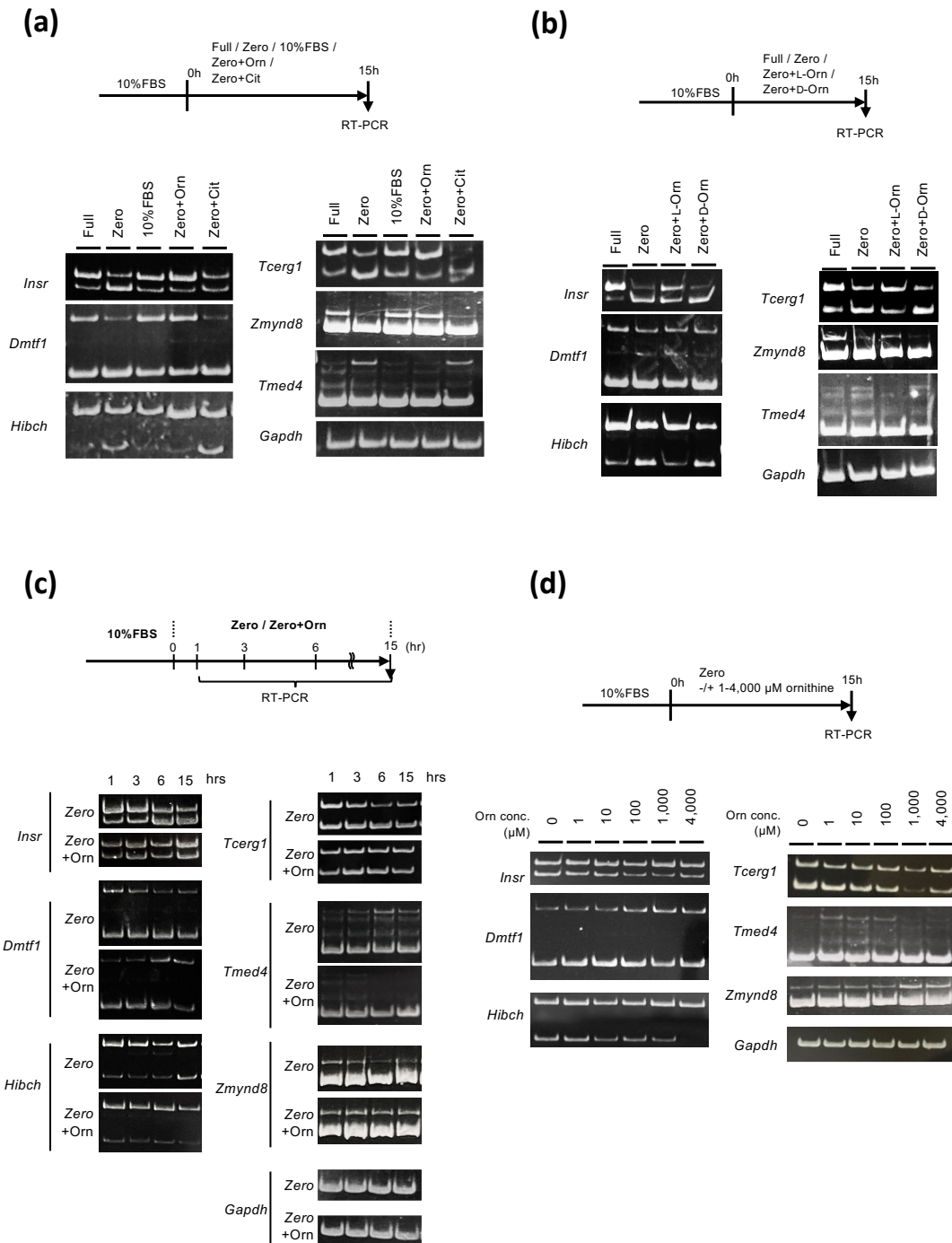


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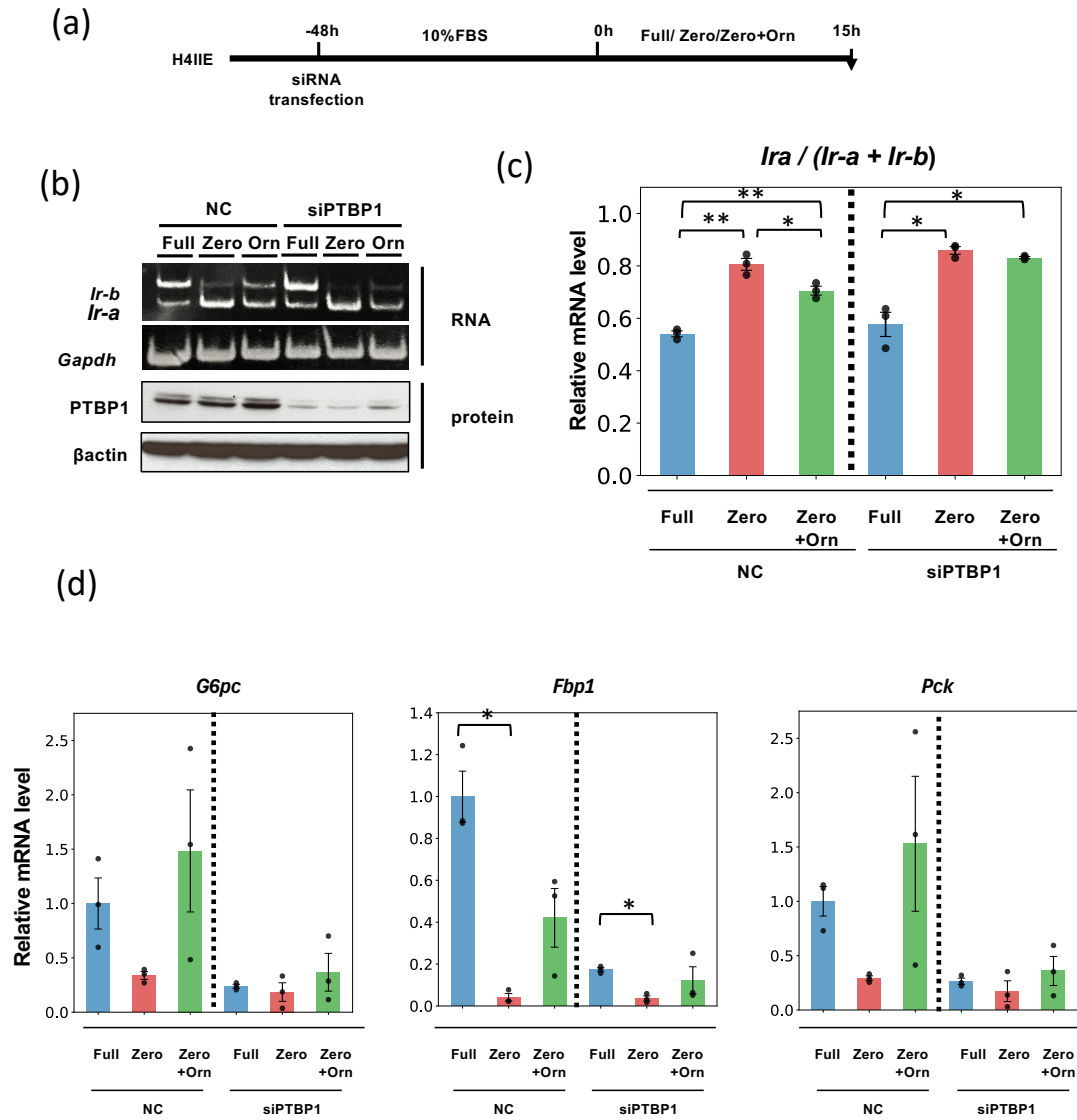


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Supplementary Files

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- [OrnithineG6pcExtendedData.pdf](#)