Riboregulation of Enolase 1 activity controls glycolysis and embryonic stem cell differentiation

Highlights

- Human ENO1 specifically binds cellular mRNAs
- RNA inhibits ENO1 activity and alters glycolytic metabolite concentrations
- Differentiation-regulated acetylation augments ENO1’s RNA binding
- ENO1’s riboregulation affects mESC differentiation

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In brief

Huppertz et al. report that ENO1, a glycolytic enzyme, is regulated by RNA. This form of biological control, called riboregulation, is shown to alter cell metabolism and stem cell differentiation. The authors discuss the implications of their findings for the control of other enzymes and cancer metabolism and treatment.

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Riboregulation of Enolase 1 activity controls glycolysis and embryonic stem cell differentiation

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SUMMARY

Differentiating stem cells must coordinate their metabolism and fate trajectories. Here, we report that the catalytic activity of the glycolytic enzyme Enolase 1 (ENO1) is directly regulated by RNAs leading to metabolic rewiring in mouse embryonic stem cells (mESCs). We identify RNA ligands that specifically inhibit ENO1’s enzymatic activity in vitro and diminish glycolysis in cultured human cells and mESCs. Pharmacological inhibition or RNAi-mediated depletion of the protein deacetylase SIRT2 increases ENO1’s acetylation and enhances its RNA binding. Similarly, induction of mESC differentiation leads to increased ENO1 acetylation, enhanced RNA binding, and inhibition of glycolysis. Stem cells expressing mutant forms of ENO1 that escape or hyper-activate this regulation display impaired germ layer differentiation. Our findings uncover acetylation-driven riboregulation of ENO1 as a physiological mechanism of glycolytic control and of the regulation of stem cell differentiation. Riboregulation may represent a more widespread principle of biological control.

INTRODUCTION

Glycolysis represents a central metabolic pathway in all living organisms. Regulation of glycolysis is critical for stem cell differentiation and cancer biology (Gu et al., 2016; Kondoh et al., 2007). Pluripotent stem cells must meet their requirements for continuous self-renewal utilizing glycolysis to provide building blocks for the biosynthesis of nucleotides, peptides, and lipids. While glycolysis is required for stemness, a shift toward oxidative phosphorylation (OXPHOS) accompanies lineage differentiation (Folmes et al., 2011). However, the mechanisms underlying this metabolic rewiring remain to be better understood.

RNA-binding proteins (RBPs) have been recognized for decades as regulators of metabolism (reviewed by Muckenthaler et al., 2017) but have only recently received broader attention (Adeli, 2011). RBPs facilitate short- and long-term metabolic adjustments of cells undergoing cell division and differentiation (reviewed by Esparza-Moltó and Cuevza, 2020) and can themselves integrate metabolic stimuli, for example, through post-translational modifications (Choudhary et al., 2014), changes in localization (Tischbein et al., 2019) or metabolite availability (Kim and Myong, 2016). Recently, many central metabolic enzymes, including glycolytic enzymes, have been identified to bind RNA in different cell types and organisms (reviewed by Hentze et al., 2018). One of these RNA-binding metabolic enzymes is Enolase 1 (ENO1, Castello et al., 2012; Beckmann et al., 2015; Matía-González et al., 2015), which catalyzes the reversible interconversion between 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP). ENO1 is commonly overexpressed in malignant cells (reviewed by Hentze et al., 2018). One of these RNA-binding metabolic enzymes is Enolase 1 (ENO1, Castello et al., 2012; Beckmann et al., 2015; Matía-González et al., 2015), which catalyzes the reversible interconversion between 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP). ENO1 is commonly overexpressed in malignant cells (reviewed by Hentze et al., 2018). One of these RNA-binding metabolic enzymes is Enolase 1 (ENO1, Castello et al., 2012; Beckmann et al., 2015; Matía-González et al., 2015), which catalyzes the reversible interconversion between 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP). ENO1 is commonly overexpressed in malignant cells (reviewed by Hentze et al., 2018).
RNA degradation (Kühnel and Luisi, 2001; Morita et al., 2004) and its role in tRNA targeting to mitochondria in yeast (Entelis et al., 2006; Baleva et al., 2015, 2017), relatively limited functional insights have been gained, especially in mammalian systems.

Here, we find that human ENO1 binds hundreds of mRNAs of the cellular transcriptome via specific binding regions. Synthetic RNA ligands corresponding to these regions inhibit ENO1’s enzymatic activity in vitro, diminish glycolysis in HeLa cells, and specifically alter glycolytic metabolite levels and serine synthesis in pluripotent mouse embryonic stem cells (mESCs). Under physiological conditions, the metabolic shift from glycolysis to OXPHOS during stem cell differentiation occurs concomitantly with an increase in ENO1’s RNA binding. However, when mutating ENO1 to be hyper-inhibited by RNA, stem cells are dramatically compromised in their differentiation toward the definitive endoderm, whereas RNA-binding-deficient ENO1 promotes endodermal differentiation. ENO1’s RNA binding is activated by acetylation, as shown by pharmacological, RNAi and cell differentiation experiments. RNA-mediated inhibition of enzymatic activity—riboregulation—constitutes a physiologically relevant form of metabolic control, which plays a relevant role during stem cell differentiation.

RESULTS

Human Enolase 1 is a bona fide RNA-binding protein

We first confirmed that human ENO1 binds RNA in HeLa cells (Figure 1A). To this end, we used the T4 polynucleotide kinase (PNK) assay (Richardson, 1965), which relies on UV crosslinking to establish a covalent bond between protein and RNA, combined with cell lysis and limited RNase treatment. RNA molecules crosslinked to ENO1 are immunoprecipitated and combined with cell lysis and limited RNase treatment. RNA molecules crosslinked to ENO1 are immunoprecipitated and used as the input for PNK-mediated 5’t RNA labeling with radioactive ATP. We observed that with an increasing RNase I concentration, the radioactive signal collapses to a sharp band just slightly exceeding the molecular mass of ENO1 (signal above 48 kDa in 32P-RNA autoradiograph; Figure 1A), indicating that ENO1 associates with RNA in HeLa cells.

ENO1 is among the top 100 most abundant proteins in the cell (Nagaraj et al., 2011). For the enzyme–RNA interaction to play a role, a considerable fraction of ENO1 needs to be associated with RNA. We addressed this question by exposing RNase-treated or untreated lysates to sucrose density gradient centrifugation and found around 10% of HeLa cell ENO1 to be sensitive to RNase treatment (Figure 1B), indicating that in highly glycolytic cells only a small fraction of ENO1 is bound by RNA. This raises the question of whether the fraction of RNA-bound ENO1 changes in response to physiological stimuli. We investigate this question further in Figure 4.

We next determined ENO1’s RNA ligands by applying the enhanced crosslinking and immunoprecipitation (eCLIP) protocol (Figures S1A–S1C, Van Nostrand et al., 2016). We ascertained that ENO1 interacts with a wide range of RNAs in HeLa cells with a preference toward the 5’ untranslated region (5’ UTR), 3’ UTR and coding region of spliced mRNAs (Figure S1D). Based on the DEWseq analysis (Sahadevan et al., 2022) of the RNA crosslink sites, we identified approximately two thousand direct ENO1-binding regions across the transcriptome (Figure 1C). Interestingly, the two top-scoring linear sequence motifs (Figure S1E) jointly account for only ~22% of all ENO1-binding sites. When assessing the identity of the proteins encoded by the ENO1 ligand mRNAs, we found that many encoded proteins are involved in biological processes and have molecular functions relevant for the RNA lifecycle such as splicing and translational initiation (Figure S1F and S1G). Principally, ENO1 interacts with a large pool of mRNAs without a prevalent linear RNA sequence motif and with a tendency for mRNAs that encode proteins involved in RNA-related processes. This led us to investigate whether the interactions between ENO1 and RNA are specific and subject to regulation.

Enolase 1 binds specific RNA ligands in vitro and in vivo

First, we set out to assess the specificity of ENO1’s RNA binding. To this end, we validated six ENO1 ligand RNAs, identified by eCLIP, in RNA immunoprecipitation (RIP) coupled with quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments, confirming their enrichment over a control IP with IgG antibody of the same isotype (Figures 1D and S1H). By contrast, RNAs of similar or higher abundance that were not previously identified by eCLIP are not significantly enriched, suggesting that ENO1 recognizes specific cellular RNAs.

Next, we synthesized RNAs of 35 nucleotides in length that either correspond to ENO1-binding regions or GC content-matched controls, derived from the same mRNAs (schematic in Figure 1E; sequences are given in Table S1). We used these in an orthogonal assay to validate the eCLIP results and further assess the specificity of binding. Representative ligand and control RNAs, derived from the PABPC1 5’ UTR, were analyzed in a competition electromobility shift assay (EMSA) using recombinant human ENO1 (Figures 1F and 1G; Kd(target)=27±19 nM; Kd(control)=2,587±9 nM, Figure S1I). Highly consistent results were obtained for two additional ligand and control pairs derived from the FTH1 and PTP4A1 mRNAs, respectively (Figures 1G, S1J, and S1K). Using NMR, we observed RNA-induced chemical shift perturbations and line broadening of ENO1 resonances in 1H, 15N-HSQC spectra, confirming direct RNA binding in vitro (shortened FTH1 ligand RNA—18-mer, Figures 1H and S1L). Taken together, ENO1 binds RNA at numerous transcriptomic sites in human cells with two orders of magnitude difference between specific and non-specific interactions.

To explore the importance of ENO1’s RNA binding, we knocked down endogenous ENO1 by RNAi, and collected comparative RNA sequencing data to identify possible effects of ENO1 on its RNA targets (Figure S2A). Despite an efficient knockdown of ENO1 (Figures S2B and S2C), we could not obtain convincing evidence for a regulatory effect of ENO1 on the transcriptome in general or the ENO1 targets determined by eCLIP in particular (Figures S2A, S2D, and S2E). We cannot presently exclude that such effects may have been missed due to the limited engagement of ENO1 with cellular RNAs (Figure 1B) or ENO1 primarily affecting mRNA function without changes in abundance. Nonetheless, recent findings highlighted the regulatory potential of RNA on protein function, including vault RNA-mediated regulation of p62’s role in autophagy (Horos et al., 2019), the inhibition of SHMT1’s activity by SHMT2’s 5’ UTR (Guiducci et al., 2019), and classic work on the control of E. coli RNA polymerase by 6S RNA (Wassarman et al., 1992).
We therefore tested whether RNA binding might affect ENO1’s enzymatic activity.

RNA ligands inhibit ENO1’s activity in vitro
To test the impact of RNA on ENO1’s activity, we purified recombinant human ENO1 and assayed its enzymatic activity in vitro (Figures 2A, 2B, S2F, and S2G). While the three RNA ligands independently inhibit ENO1’s activity reproducibly and to a similar extent, the corresponding control RNAs do not elicit any significant changes in ENO1’s activity (Figures 2A and 2B, $K_i$ of PABPC1: 99.1 nM, 95% CI: 49.4–223.2 nM; Figures S2F, $K_i$ of FTH1: 150 nM, 95% CI: 86.4–310.9 nM; Figure S2G, $K_i$ of PTP4A1: 103 nM, 95% CI: 56.0–190.3 nM).

Instructed by RBDmap data (Castello et al., 2016; Horos et al., 2019), we generated an ENO1 mutant (K343A; ENO1down) with a 5- to 10-fold decreased RNA binding (Figure 2C; $K_i$ of target: 224 ± 12 nM; $K_i$ of control: 3,261 ± 12 nM; Figures S2H–S2J) compared to 300%–500% increase in the RNA–RBP complexes in HeLa cells with PC3 nuclei.
with ENO1wt as measured by competition EMSA (Figures 1F, 1G, and S1I–S1K).
Importantly, in contrast to its wild-type counterpart ENO1down displays no discernible alteration in its enzymatic activity with any of the RNAs tested (Figures 2D, S2K, and S2L). Thus, both the use of control RNAs and evaluation of the ENO1down mutant corroborate that RNA specifically riboregulates ENO1’s activity in vitro.

ENO1’s glycolytic substrates compete with RNA in vitro
Next, we tested whether ENO1’s enzymatic substrates and RNA compete for ENO binding by performing EMSA experiments using the two ENO1 substrates of the forward and backward reaction, respectively, 2-PG or PEP (Lebioda et al., 1993) as competitors. For these experiments, we used metabolite concentrations that are close to the expected concentrations in cells (Park et al., 2016). While both ENO1 substrates compete with RNA for ENO1 binding, 3-phosphoglycerate (3-PG), the immediate precursor of 2-PG with an identical molecular mass used as a specificity control, fails to compete (Figures 2E and 2F). Thus, we observe specific competition between substrates and an RNA ligand for binding to ENO1.

ENO1’s RNA binding affects glycolysis in HeLa cells
In addition to the ENO1down mutant, we next generated a version of ENO1 with increased RNA-binding capacity in cellulo, referred to here as ENO1up. The design of ENO1up was also guided by RBDmap data (Castello et al., 2016) and entails the change of three lysine residues (K89A/K92A/K105A).
Figure 3. Riboregulation of ENO1 in HeLa cells
(A) Anti-FLAG IP and PNK of transiently expressed ENO1-FLAG-HA proteins. RNA binding of tagged wild-type ENO1 (ENO1wt), a mutant with increased RNA binding (ENO1up) and reduced RNA binding (ENO1down) are being compared.
(B) Representative image of a proximity ligation assay (RNA-PLA) for ENO1wt with its mRNA target FTH1 (red dots). ENO1 is being detected using an anti-FLAG antibody and the endogenous ENO1 is knocked down using siRNAs. The red PLA signal is presented as a maximal projection of a z-stack (10 pictures for 10-μm stack). Pictures for the DAPI (nuclear staining) and cellular outline (gray) are taken in one plane. The scale bars represent 20 μm.
(C) Representative image of an RNA-PLA for ENO1down with its mRNA target FTH1.
(D) Representative image of an RNA-PLA for ENO1up with its mRNA target FTH1.
(E) Quantification of RNA-PLA signals as dots per cell from three biological replicates with at least 30 cells in total. Statistically significant differences were detected using one-way ANOVA and Tukey-correction for multiple comparison testing. Statistical significance is represented as follows: p value of <0.0001, **** and p value of 0.01 to 0.05, *. See also Figures S3 A–S3C.
(F) Comparison of ENO1’s RNA binding (PNK), and ENO1’s enzymatic activity in HeLa cells, indirectly measured by the accumulation of lactate in the medium (SD, n = 3). Endogenous ENO1 was knocked down using siRNAs to assess the effects of transfected ENO1 variants.
(G) Michaelis-Menten saturation curve of the basal enzymatic activity of recombinant ENO1wt, ENO1down and ENO1up in vitro in the absence of RNA using a non-linear curve fitting with least squares regression (SD, n = 3). V max and K m measurements for ENO1wt, ENO1down, and ENO1up as determined from the Michaelis-Menten saturation curve. The asymmetrical confidence interval (CI) is given (n = 3). See also Figures S3D–S3L.
(H) Immunoprecipitates of transiently expressed ENO1wt in siENO1-treated HeLa cells were exposed to RNase A or left untreated. The FLAG-HA tagged ENO1wt was eluted with FLAG peptide and used as input for ENO1 activity assays (SD, n = 3). The two-tailed Student’s t test is used to detect statistically significant differences. Statistical significance is represented as follows: p value of 0.01 to 0.05, * and p value of ≥0.05, not indicated. See also Figure S3J.
(I) Experimental setup as in H for FLAG-HA-tagged ENO1down.
(J) Experimental setup as in H for FLAG-HA-tagged ENO1up.
Figure 4. Acetylation activates ENO1’s RNA binding

A) PNK of ENO1 in HeLa cells after a 16-h treatment with the histone deacetylation (HDAC) inhibitor, sodium butyrate, with concentrations ranging from 2.5 to 10 mM. Western blotting was performed for ENO1 and acetylated lysine (acetyl(K)).

B) Mass spectrometry analysis of ENO1 IPs from HeLa cells treated with no or 10 mM sodium butyrate. Identification of the ENO1 peptide of acetylated lysine 5 normalized to the total IP efficiency (SD, n = 3). Statistical significance is represented as follows: p value of 0.001 to 0.01, ** and p value of ≥ 0.05, not significant.

C) Experimental setup as in (B) for the peptide of acetylated lysine 71.

D) Experimental setup as in (B) for the peptide of acetylated lysine 81/89.

(legend continued on next page)
After reducing endogenous ENO1 levels in HeLa cells by RNAi and exogenously expressing the respective FLAG-tagged ENO1 variants, ENO1up displays increased RNA binding compared with ENO1wt (PNK assay; Figures 3A and 3F), while the recombinant ENO1up has a comparable Kᵢ value for target and control PABPC1 to ENO1wt (EMSA; Figures 1G and S3D). We further investigate this difference between the in cellulo and in vitro RNA-binding of ENO1up in Figure 4.

In line with the in vitro data (Figures 2C and S2H–S2J), ENO1down displays substantially decreased RNA binding in HeLa cells relative to ENO1wt (Figures 3A and 3F). We independently confirmed the differential RNA binding of the ENO1up and ENO1down mutants using an immunofluorescence-based, UV crosslinking-independent RNA proximity ligation assay (RNA-PLA; Zhang et al., 2016) that enables the in situ detection of endogenous or tagged proteins with their RNA targets (see STAR Methods). ENO1’s association with the FTH1 mRNA ligand was validated by the combination of an antisense probe hybridizing close to ENO1’s FTH1 mRNA-interaction region and an antibody specifically recognizing the FLAG-tagged ENO1 variants. Using this orthogonal assay, we validated the differential RNA binding of ENO1wt (Figures 3B and 3E), ENO1down (Figures 3C and 3E), and ENO1up (Figures 3D and 3E) in HeLa cells, ensuring that the expression levels and localization of the ENO1 variants were comparable (Figure S3A) and that the PLA signal is specific (Figures S3B and S3C).

When we tested these mutants for their ability to rescue glycolysis (lactate accumulation in the medium) in HeLa cells after knockdown of the endogenous ENO1, ENO1wt unexpectedly rescued lactate production (Figure 3F). ENO1down, the RNA-binding-deficient mutant, has a comparable activity to the wild-type protein in HeLa cells as expected when only 10% of endogenous ENO1 is RNA bound (Figure 1B), showing that the K343A mutation does not incapacitate the enzyme. In contrast, ENO1up fails to rescue the knockdown-induced inhibition of lactate accumulation (Figure 3F), although it is fully active when tested in the absence of RNA in vitro (Figures 3G, S3H, and S3I). The activity measurements in vitro were controlled with a mutant lacking enzymatic activity (ENO1as, E295A/D320A/K394A, Figures S3H and S3I, Kang et al., 2008).

We next probed whether RNA directly interferes with ENO1’s activity in HeLa cells and whether differential RNA binding of ENO1 is the cause of the differential capacity of the ENO1 variants to rescue lactate production. We immunoprecipitated the three ENO1 variants from lysates of cells lacking the endogenous protein. We then treated these immunoprecipitates with RNase or left them untreated, eluted the ENO1 variants with FLAG peptide, and assayed their enzymatic activity (Figures 3H–3J and S3J). While the activity of ENO1down is irreversible to the RNase treatment, ENO1wt is more active after RNase treatment. Furthermore, ENO1up also responds to RNase treatment and displays a tendency to be affected by the RNase treatment even more strongly. These experiments strongly support the concept that endogenous cellular RNAs interfere with ENO1’s activity and that the ENO1 variants represent a powerful tool to assess the importance of these regulatory interactions.

We complemented these findings by nucleofection experiments with synthetic 35-mer ligand and control RNAs into HeLa cells (Figures S3K and S3L). The results unambiguously confirm specific riboregulation of lactate production by ENO1’s RNA ligands and fully support the notion that RNA binding interferes with ENO1’s enzymatic activity in cells.

**Acetylation augments ENO1’s RNA binding**

What may explain the difference between the enhanced RNA binding of the ENO1up mutant in cellulo (Figures 3E and 3F) and the normal RNA binding of the recombinant protein in vitro (Figure S3D)? Considering that the ENO1up mutant represents a change of three lysine residues to alanine, we hypothesized that a post-translational lysine modification such as ubiquitination or acetylation in cellulo could activate ENO1’s RNA binding. Multiple experimental interrogations yielded no evidence for relevant ubiquitination (data not shown). By contrast, treatment of HeLa cells with sodium butyrate, an inhibitor of protein deacetylases, profoundly induced ENO1’s acetylation and RNA binding (Figure 4A). When we subjected ENO1’s IPs from HeLa cells treated with sodium butyrate to mass spectrometry and compared them to untreated samples, we identified several acetylated lysine residues. While the expected general trend of increased lysine acetylation of ENO1 following sodium butyrate treatment is apparent (Figures 4B–4D), only the peptide encompassing K81 and K89 was found to be significantly more acetylated (Figure 4D). Interestingly, K89 is one of the amino acids mutated in ENO1up (K89A/K92A/K105A).

SIRT2 had previously been implicated in ENO1’s deacetylation (Cha et al., 2017; Hamaidi et al., 2020). Thus, we knocked down SIRT2’s expression with siRNAs (Figures S4A and S4B) and assessed the consequences on the RNA binding of...
wild-type ENO1 and our ENO1 mutants. RNA binding of ENO1wt and ENO1down increased when knocking down ENO1’s putative deacetylase, while ENO1up remained unaffected (Figure 4F). This result implicates SIRT2 as the relevant deacetylase involved in the regulation of ENO1’s RNA binding and suggests that the ENO1up mutation mimics acetylated ENO1.

Sucrose density gradient centrifugation analysis (experimental setup as in Figure 1B) was then used to further quantify the impact of the ENO1 mutations on RNA binding and to assess the role of acetylation for ENO1’s RNA binding in cells (Figure 4G). To this end, we digested lysates from HeLa cells treated with control siRNAs or siRNAs targeting SIRT2 mrNA and expressing ENO1wt, ENO1up, or ENO1down with RNase or left them undigested. We then assessed the differences by western blotting and detected that ENO1wt is more sensitive to RNase treatment than ENO1down, as expected (Figure 4G, top and middle panel). By contrast, ENO1up is nearly 4 times more sensitive to RNase treatment, confirming that these mutations enhance RNA binding (Figure 4G, bottom panel).

Modulating the acetylation status of these ENO1 mutants by knocking down SIRT2 confirms the PNK results (Figure 4F) and shows that increased acetylation profoundly augments ENO1wt’s RNA binding (from 11% ± 6% to 34% ± 4%; Figure 4G, top panel), and ENO1down’s by ~3.5-fold (from 0% ± 7% to 26% ± 3%; Figure 4G, middle panel). As predicted, ENO1up fails to increase its RNA binding upon SIRT2 KD (Figure 4G, bottom panel), in agreement with our PNK results (Figure 4F). These data support the hypothesis that one (or more) of the amino acids K89, K92, and K105 are controlled by SIRT2 activity.

To exclude the possibility that the increase in RNA binding upon SIRT2 KD is caused by off-target effects, we validated our findings using two different pharmacological inhibitors of SIRT2, SirRea1 (Rumpf et al., 2015), and Thiomyristoyl (Jing et al., 2016; Figures S4C and S4D). Treatment of HeLa cells with either of these two inhibitors consistently increases ENO1’s RNA binding. Thus, three different modes of SIRT2 modulation confirm SIRT2 as an important modulator of ENO1’s RNA binding.

To further test the concept that ENO1up mimics the acetylated state of ENO1, we mutated the lysines that are changed to alanine in ENO1up (K89, K92, and K105) to the more conventionally used acetylation-mimic, glutamine (ENO1KtoQ). In support of the above-mentioned results and interpretation, ENO1KtoQ shows the same enhancement of RNA binding as ENO1up compared to ENO1wt (Figure 4H). To examine the effect of a K89, K92, and K105 mutation that maintains the positive charge but alters the identity of the amino acids, we replaced the three lysines with arginines, generating ENO1KtoR. We tested its RNA binding in comparison with the other ENO1 variants (Figure 4I). ENO1KtoR phenocopies ENO1wt, indicating that ENO1up mimics the acetylated form of the protein by loss of the positive charge.

**ENO1’s RNA association increases during differentiation**

To explore physiological functions of the ENO1-RNA interaction, we chose mESCs. Similar to cancer cells, mESCs utilize glucose as a major energy source in the undifferentiated state (Gu et al., 2016; Kondoh et al., 2007). To directly test the inhibitory effect of RNA on ENO1’s enzymatic activity in mESCs and to explore how ENO1’s riboregulation alters cellular metabolism and metabolites, we nucleofected control or ligand PABPC1 RNA. As previously seen in HeLa cells (Figures S3K and S3L), nucleofected ligand RNAs specifically inhibit lactate accumulation in the medium (Figure 5D). 13C glucose tracing experiments show that glucose progresses through the first steps of glycolysis and its branching pathways without significant differences imposed by ENO1’s ligand RNA (see levels of fructose-1,6-bisphosphate, ribose-5-phosphate, and glycerol-3-phosphate, Figure 5A). However, metabolites downstream of ENO1 are significantly diminished when the specific ENO1 RNA ligand was used (see levels of PEP, pyruvate, and lactate, Figure 5A), demonstrating...
a clear, direct, and specific effect of riboregulation on the metabolome of mESCs. Interestingly, we also detected that 3-PG is reduced upstream of the ENO1 “block,” suggesting that ENO1 inhibition may serve to supply serine biosynthesis with 3-PG, which branches off from glycolysis just upstream of ENO1. To investigate this further, we measured serine levels after nucleofection with either of two ENO1 ligands (PABPC1, Figure 5B; FTH1, Figure 5C) and found that in both instances serine levels are increased following ENO1’s riboregulation while other, non-essential amino acids are not impacted. These data represent direct evidence for ENO1’s ligand RNAs rewiring mESCs’ carbon metabolism.

Removal of the leukemia inhibitory factor (LIF) from the culture medium of mESCs induces differentiation, accompanied by a decrease in glycolysis and increased respiration (Figures 5E and 5F, Williams et al., 1988; Gu et al., 2016). Of note, the decrease in glycolysis is accompanied by increased RNA binding after LIF withdrawal for 7 days (PNK; Figure S5A). To temporally resolve the increase in RNA binding over the course of mESC differentiation, we performed RIP-qRT-PCR experiments for the ligand and control mRNAs previously validated in HeLa cells (Figure 1D).

Unfortunately, the RNA nucleofection protocol is incompatible with meaningful mESC differentiation analyses. To test the importance of ENO1’s riboregulation in mESC differentiation, we therefore first used unperturbed mESCs (Sladitschek and Neveu, 2019), withdrew LIF for a period of 7 days, and sorted cells that were positive for the expression of Brachyury (blue fluorescent protein [BFP]-positive), which is primarily found in the mesoderm (Arnold and Magnuson, 2005), or Eomes (mCherry-positive), which is predominantly expressed in the definitive endoderm (Arnold et al., 2008). We detected that lactate accumulation in the medium of Eomes+ cells significantly exceeds that of Brachyury+ cells (Figure S5C), suggesting that the differentiation to the definitive endoderm may require sustained glycolysis in comparison to the primitive streak. Of note, ENO1 binding to RNA correlates inversely (compare Figure S5D). Sorted Eomes-expressing cells show decreased RNA binding and trend toward decreased ENO1 acetylation in comparison to the Brachyury-expressing cells (Figures S5E and S5F).

Diffrential RNA association of ENO1 alters stem cell differentiation

To examine whether the correlation between ENO1’s RNA binding, its acetylation state and lactate accumulation in cells reflects a causal requirement for riboregulation of ENO1 during ESC differentiation, we introduced murine versions of the ENO1 variants characterized before (Figure 3) into both alleles of the Rosa26 locus by CRISPR-Cas9 genome editing and subsequently knocked out endogenous ENO1. The different heterologous forms of ENO1 are expressed at similar levels to each other (Figure S6A). The lactate accumulation in the medium of ENO1wt cells is somewhat less than seen in control cells, indicating potential differences in expression levels between the endogenous ENO1 protein and the protein expressed from a different locus and promoter (Figure 6B). As previously observed in HeLa cells, ENO1up displays increased RNA binding leading to a decreased enzymatic activity as reflected by a reduction in lactate production; likewise, ENO1down shows a decrease in RNA binding compared with ENO1wt (Figure 6A, compare Figure 3F).

Independent clones of these cell lines were subjected to LIF withdrawal and analyzed for differentiation into the different germ layers. Engineered mESCs expressing ENO1wt differentiated normally into the distinct germ layers, as assessed by qRT-PCR analysis of the expression of respective marker genes (Figures 5C and S6B). By contrast, ENO1up-expressing cells fail profoundly in their differentiation to definitive endoderm and neuroectoderm (Figure 6E), while the expression of primitive streak and mesodermal markers was quite variable and statistically not significantly affected. We also noticed that ENO1 down cells, where ENO1’s activity escapes riboregulation, conversely show increased differentiation toward the definitive endoderm (Figure 6D).

To corroborate that the phenotypic changes of ENO1up-expressing cells is a consequence of diminished ENO1 activity, we fused an auxin-inducible degron tag to the C terminus of endogenous ENO1 of both alleles in mESCs carrying the OsTir1 receptor in the TIGRE locus. We then triggered ENO1 degradation by the addition of auxin for 48 h at the previously determined critical point of differentiation of 4 days, where we detected an increase in ENO1’s RNA association (Figure 5G). When depleting ENO1 from differentiating mESCs at this point, we observe specific, defective differentiation toward neuroectoderm and definitive endoderm, phenocopying cells expressing ENO1up (Figures 6D and 6E). This experiment shows that the expression of ENO1, which is strongly inhibited by RNA (Figure 3J), phenocopies a loss of ENO1 at a critical time point during differentiation.

Taken together, our experiments demonstrate the physiological importance of ENO1’s riboregulation for mESC differentiation, and especially for the formation of the endodermal germ layer. As such, they uncover a central form of regulated stem cell differentiation.

DISCUSSION

Here, we elucidate a physiological role of the RNA-binding activity of mammalian ENO1. Through multiple lines of evidence, both in vitro and in cell systems, we show that RNA specifically controls ENO1’s activity. We also provide direct experimental evidence for a physiological role of ENO1’s riboregulation during mESC differentiation, especially for endoderm formation. Future experiments will directly examine how the resulting alterations in energy metabolism and metabolites affect cell differentiation.

Regulation of glycolysis by the collective mRNA transcriptome (“crowd control”)

Our biochemical studies and the data using the ENO1 mutants show that RNA directly interacts with ENO1 (Figure 1) in a way that is mutually exclusive with substrate binding (Figure 2) and...
inhibits ENO1’s activity in vitro (Figure 2) and in cells (Figures 3 and 5). The inhibitory constant (K_i) in the mid to low nanomolar range makes ENO1’s RNA ligands plausible regulators of glycolysis. Acetylation-mediated changes in RNA binding (Figure 4) serve to modulate ENO1’s enzymatic activity during cell differentiation and riboregulation directly controls the metabolite profile of mESCs (Figure 5).

The conventional function of the mRNA transcriptome is based on its protein-encoding potential. Our data show that many, possibly thousands of sites within mammalian cells’ transcriptomes display specific ENO1 binding. The PABPC1, FTH1, and PTP4A1 ligands—derived from eCLIP targets selected from a cloud of hundreds of similar ones (Figure 1C), largely mRNAs (Figure S1D)—riboregulate ENO1 activity
These data suggest an additional function that is shared between these ENO1-binding mRNAs, and hence a collective function of the mRNA transcriptome, namely "crowd-controlled" riboregulation. We do not know formally whether all ENO1 eCLIP targets function in the same way, but the data presented here would not favor an opposing view. With numerous other glycolytic enzymes exhibiting RNA-binding activity (Castello et al., 2012; Beckmann et al., 2015; Matia-González et al., 2015), the example of ENO1’s riboregulation calls for detailed exploration of the other enzymes.

**A mechanistic model for ENO1’s riboregulation**

Based on RBDmap data (Castello et al., 2016; Horos et al., 2019), the ENO1down mutant is RNA-binding-deficient likely because K343 directly interacts with RNA; this hypothesis awaits structural elucidation. ENO1up has led us on the path of identification of the role of acetylation as a regulatory modification for ENO1’s RNA binding and function. The lysines changed in ENO1up may not be in direct contact with RNA, but their acetylation neutralizes the positive charge and in turn may induce a conformational change that facilitates RNA binding. Our experiments targeting SIRT2 (Figures 4F and 4G) also identify a deacetylase able to regulate ENO1’s RNA association. Future experiments will identify the ENO1 acetylases and deacetylases involved in the regulation of mESC differentiation. Interestingly, recent findings show that the glycolytic flux is rerouted in T cells depleted for SIRT2 (Hamaidi et al., 2020), which in turn promotes serine production and nucleotide synthesis. We find that riboregulation of ENO1, which is supported by increased ENO1 acetylation, also alters glycolytic metabolites and increases cellular serine levels (Figure 5).

How does acetylation activate ENO1’s RNA binding, especially since the loss of a positive charge more often diminishes protein interactions with negatively charged nucleic acids (reviewed by Blee et al., 2015)? We envisage that acetylation of the lysines altered in ENO1up induces a conformational change to a more "open state" that facilitates RNA binding and thus promotes inhibition of enzyme function. While such a model is plausible, alternative scenarios cannot be excluded at this stage, and structural investigations should help to resolve this question.

**Riboregulation as a path to different forms of regulatory drugs for glycolysis?**

For the past decade, research groups have set out to identify ENO1 inhibitors for their use in the treatment of cancer, type 2 diabetes, and infectious diseases (Cho et al., 2017; Jung et al., 2013; Leonard et al., 2016; Pietkiewicz et al., 2009; Satani et al., 2016). Many of the inhibitors interact with the magnesium ion in the active site of ENO1 or chelate the metal ion rendering the enzyme inactive (Jung et al., 2013; Lebioda et al., 1993; Poyner and Reed, 1992). Our findings could pave the way toward a different class of compounds that exploit the cells’ endogenous riboregulatory mechanisms for therapeutic intervention (Yu et al., 2018).

**Limitations of the study**

Although many metabolic enzymes and nearly all glycolytic enzymes have recently been found to bind RNA, this study is focused on ENO1. Future work will have to examine other enzyme RBPs to identify common features and distinctions. We describe a specific function of ENO1’s RNA-binding activity, riboregulation, and we do not exclude alternative or additional functions of ENO1’s RNA-binding activity. Since metabolic remodeling and regulation of glycolysis represents a common feature of cell differentiation and in cancer biology, it will be important to explore the riboregulation of ENO1, described here, in these other biological and pathophysiological contexts.

The structural basis of the specific interaction between ENO1’s ligand RNAs and the enzyme also remains to be defined to better understand how RNA binding inhibits the catalytic function of the enzyme and to determine how acetylation controls ENO1’s RNA binding. In addition to K89, it remains to be determined whether acetylation of other amino acids or other post-translational modifications of ENO1 contribute to the regulation of ENO1’s RNA binding.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2022.05.019.

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AUTHOR CONTRIBUTIONS

I.H. contributed to conceptualization, formal analysis, investigation, methodology, project administration, visualization, and writing (original draft and editing). J.P.-P. contributed to investigation and methodology (mESC). P.M., T. Sekaran, and T. Schwarz contributed to formal analysis and software (eCLIP, RNA-seq). F.R. contributed to investigation (AID-targeted ENO1 mESC lines). D.F.-A. contributed to investigation (PLA). Z.K. contributed to investigation (SIRT2 inhibitor experiments). L.D.-P. and J.H. contributed to conceptualization, formal analysis, and visualization (NMR). E.K. and K.P. contributed to conceptualization and formal analysis (13C-glucose tracing experiment and SIRT2 inhibitor experiments). L.D.-P. and J.H. contributed to conceptualization, project administration, visualization, and writing (original draft and editing). M.W.H. contributed to conceptualization, formal analysis, investigation, methodology (mESC), and writing (original draft and editing). I.H. contributed to conceptualization, formal analysis, investigation, methodology, project administration, visualization, and writing (original draft and editing).

DECLARATION OF INTERESTS

The authors declare the following competing interests: the European Molecular Biology Laboratory with the inventors M.W. Hentze and I. Huppertz applied for a patent (WO2022074066) on the use the ENO1-RNA interaction as a novel therapeutic compounds. The authors declare the following competing interests: the European Molecular Biology Laboratory with the inventors M.W. Hentze and I. Huppertz applied for a patent (WO2022074066) on the use the ENO1-RNA interaction as a novel therapeutic compounds.

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## STAR+METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal ENO1 antibody | Abcam | Cat# ab112994; RRID: AB_10858766 |
| Rabbit monoclonal ENO1 antibody | Abcam | Cat#ab155102 |
| Rabbit polyclonal Nucleolin antibody | Abcam | Cat#ab50279; RRID: AB_881762 |
| Rabbit monoclonal Biotin antibody | Abcam | Cat#ab234284 |
| Mouse monoclonal Biotin antibody | Abcam | Cat#ab201341; RRID: AB_2861249 |
| Goat anti-mouse IgG H&L (HRP) | Abcam | Cat#ab6789; RRID: AB_955439 |
| Goat anti-rabbit IgG H&L (HRP) | Abcam | Cat#ab97051; RRID: AB_10679369 |
| Rabbit polyclonal LARP1 antibody | Bethyl Laboratories | Cat#A302-088A; RRID: AB_1604275 |
| Mouse monoclonal HA.11 epitope tag antibody | Biolegend | Cat#MMS-101P; RRID: AB_2314672 |
| Normal Rabbit IgG | Cell Signaling Technology | Cat#2729; RRID: AB_591709 |
| Rabbit monoclonal SIRT2 antibody | Cell Signaling Technology | Cat#12650S; RRID: AB_2716762 |
| Mouse monoclonal LC3 antibody | Cosmo Bio | Cat#cac-ctb-lc3-2-ic; RRID: AB_10707197 |
| Rabbit Acetyl Lysine antibody | Immunechem | Cat#C0P380; RRID: AB_2801477 |
| Rabbit IgG isotype control | Invitrogen | Cat#10500C; RRID: AB_2532981 |
| Mouse monoclonal anti-rabbit light chain antibody | Millipore | Cat#MAB201P; RRID: AB_827270 |
| Goat polyclonal anti-mouse light chain antibody | Millipore | Cat#M AP200P; RRID: AB_805324 |
| Mouse monoclonal ENO1 antibody | Novus | Cat# H00002023-M01; RRID: AB_463881 |
| Mouse monoclonal ENO3 antibody | Novus | Cat#H00002027-M01; RRID: AB_509354 |
| Rabbit polyclonal ENO1 antibody | Proteintech | Cat#11204-1-AP; RRID: AB_2099064 |
| Rabbit polyclonal SLC3A2 antibody | Santa Cruz | Cat#sc-9160; RRID: AB_638288 |
| Normal mouse IgG | Santa Cruz | Cat#sc-2025; RRID: AB_737182 |
| Mouse monoclonal β-Actin antibody | Sigma | Cat#A5441; RRID: AB_476744 |
| Mouse monoclonal FLAG antibody | Sigma | Cat#F3165; RRID: AB_259529 |
| Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488 | Termo Fisher Scientific | Cat#A-21206; RRID: AB_2535792 |
| Donkey anti-Mouse IgG (H+L) Alexa Fluor 488 | Termo Fisher Scientific | Cat#A-21202; RRID: AB_141607 |
| **Bacterial and virus strains** |        |            |
| E.coli BL21(DE3) CodonPlus-RIL | Agilent | Cat#: 230240 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| 1,4 Dithiothreitol (DTT) | Biomol | Cat#: 04010.10 |
| 40% Acrylamide/Bis Solution, 19:1 | BioRad | Cat#: 1610144 |
| Protein Assay Dye Reagent Concentrate | BioRad | Cat#: 5000006 |
| 4–15% TGX Precast Midi Protein Gel, 18 well | BioRad | Cat#: 5671084 |
| 4–15% TGX Precast Midi Protein Gel, 12+2 well | BioRad | Cat#: 5671083 |
| 4–15% TGX Precast Midi Protein Gel, 26 well | BioRad | Cat#: 5671085 |
| 4–12% XT Bis-Tris Precast Midi Protein Gel, 18 well | BioRad | Cat#: 3450123 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Trans-Blot Turbo Midi 0.2 μm Nitrocellulose Transfer Packs | BioRad | Cat#: 1704159 |
| SureBeads Protein G magnetic beads | BioRad | Cat#: 161-4023 |
| Ammonium chloride (15N, 99%) | Cambridge Isotope Laboratories | Cat#: NLM-467 |
| Deuterium oxide (D, 99.9%) | Cambridge Isotope Laboratories | Cat#: DLM-4 |
| D-Glucose (U-13C6, 99%) | Cambridge Isotope Laboratories | Cat#: CLM-1396-MPT-PK |
| [γ-32P] ATP, 6000 Ci/mmol, 10 mCi/ml | Hartmann | Cat#: SRP-501 |
| TruSeq Stranded mRNA Library Preparation kit | Illumina | Cat#: 20020594 |
| EmbryoMax ES Cell Qualified FBS, sterile-filtered, suitable for stem cell culture | Merck | Cat#: ES-009-B |
| ESGRO Leukemia Inhibitory Factor (LIF) supplement | Merck | Cat#: ESG1106 |
| N,N,N',N'-Tetramethylethylenediamine (TEMED) | Merck | Cat#: T9281 |
| Anti-FLAG M2 Magnetic Beads | Merck | Cat#: M8823 |
| Benzonase, 25 U/μL | Merck Millipore | Cat#: 71206 |
| Trichloroacetic acid | Merck Millipore | Cat#: 100807 |
| Electrochemiluminescence (ECL) solution | Merck Millipore | Cat#: WBKLS0500 |
| T4 Polynucleotide Kinase (PNK) | New England BioLabs | Cat#: M0201L |
| Proteinase K | New England BioLabs | Cat#: P8107S |
| HRV 3C protease | Protein Purification Core Facility (EMBL) | N/A |
| Ni-NTA Agarose | Qiagen | Cat#: 30210 |
| cOmplete Protease Inhibitor Cocktail | Roche | Cat#: 11697498001 |
| DAPI | Roche | Cat#: 10236276001 |
| Imidazole | Roth | Cat#: 3899.4 |
| Gelatine | Sigma | Cat#: G1890 |
| 2-Mercaptoethanol | Sigma | Cat#: M6250 |
| Isopropyl β-D-1-thiogalactopyranoside (IPTG) | Sigma | Cat#: I5502 |
| Adonitol, 99% | Thermo Fisher Scientific | Cat#: L03253 |
| Methoxylamine hydrochloride, 25-30% aq. soln. | Thermo Fisher Scientific | Cat#: L08415 |
| Pyridine, 99% | Thermo Fisher Scientific | Cat#: A12005 |
| N-Methyl-N-(trimethylsilyl) trifluoroacetamide, 97% | Thermo Fisher Scientific | Cat#: A13141 |
| DMEM media, high glucose | Thermo Fisher Scientific | Cat#: 11965092 |
| DMEM, low glucose, no glutamine, no phenol red | Thermo Fisher Scientific | Cat#: 11054001 |
| Fetal Bovine Serum, qualified | Thermo Fisher Scientific | Cat#: 10270106 |
| Trypsin | Thermo Fisher Scientific | Cat#: 25300054 |
| TrypLE Express | Thermo Fisher Scientific | Cat#: 12604021 |
| MEM Non-Essential Amino Acids Solution (100X) | Thermo Fisher Scientific | Cat#: 11140050 |
| Sodium Pyruvate (100 mM) | Thermo Fisher Scientific | Cat#: 11360070 |
| Penicillin-Streptomycin | Thermo Fisher Scientific | Cat#: 15140122 |
| L-Glutamine | Thermo Fisher Scientific | Cat#: 25030081 |
| Lipofectamine RNAiMAX Transfection Reagent | Thermo Fisher Scientific | Cat#: 13778030 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Lipofectamine 2000 Transfection Reagent | Thermo Fisher Scientific | Cat#: 11668030 |
| ProLong Diamond Antifade Mountant | Thermo Fisher Scientific | Cat#: P36961 |
| TURBO DNase, 2 U/μL | Thermo Fisher Scientific | Cat#: AM2239 |
| RNase I, cloned, 100 U/μL | Thermo Fisher Scientific | Cat#: AM2295 |
| RNase T1/A | Thermo Fisher Scientific | Cat#: EN0551 |
| SuperScript IV Reverse Transcriptase Thermo Fisher Scientific | Cat#: 18090010 |
| Maxima First Strand cDNA Synthesis Kit for RT-qPCR Thermo Fisher Scientific | Cat#: K1671 |
| Random Hexamers (50 μM) | Thermo Fisher Scientific | Cat#: N8080127 |
| dNTP Mix (10 mM ea) | Thermo Fisher Scientific | Cat#: 18427013 |
| 16% Formaldehyde (w/v), Methanol-free | Thermo Fisher Scientific | Cat#: 28906 |
| SYBR Green PCR Master Mix | Thermo Fisher Scientific | Cat#: 4309155 |
| Phire Tissue Direct PCR Master Mix | Thermo Fisher Scientific | Cat#: F170L |
| Spectrum Spectra/Por 2 RC Dialysis Membrane Tubing 12,000 to 14,000 Dalton MWCO | Thermo Fisher Scientific | Cat#: 11495849 |
| Oyntiva HisTrap FF Columns | Zeiss | Cat#: 10539438 |
| Immersol 518F | Zeiss | Cat#: 10539438 |
| Critical commercial assays | | |
| Enolase 1 activity assay | Abcam | Cat#: ab241024 |
| Lactate colorimetric/fluorometric assay kit | Biovision | Cat#: K607 |
| P3 Primary Cell 4D-Nucleofector | Lonza | Cat#: V4XP-3024 |
| Nucleofector 4D Kit (SE Cell Line) | Lonza | Cat#: VXO-1012 |
| Duolink in situ red starter kit rabbit | Merck | Cat#: DUO92101 |
| Direct-zol RNA miniprep | Zymogen | Cat#: R2051 |
| RNA Clean & Concentrator-5 kit | Zymogen | Cat#: R1013 |
| Deposited data | | |
| eCLIP of ENO1 in HeLa cells | This manuscript | Array Express: E-MTAB-9031 |
| RNAseq data | This manuscript | Array Express: E-MTAB-9032 |
| Imaging and Western blotting data | This manuscript | Mendeley Data: https://doi.org/10.17632/wc7f54nc5d.1 |
| Metabolomics data | This manuscript | Mendeley Data: https://doi.org/10.17632/wc7f54nc5d.1 |
| Experimental models: cell lines | | |
| HeLa (female origin) | (Castello et al., 2012) | N/A |
| ES-R1 from mouse (male origin) | Lab of Alexander Aulehla (EMBL) (Nagy et al., 1993) | N/A |
| Triple Knock-In ES-R1 from mouse (male origin) | (Sladitschek and Neveu, 2019) | N/A |
| ENO1wt in ROSA locus ES-R1 from mouse (male origin) | This manuscript | N/A |
| ENO1up in ROSA locus ES-R1 from mouse (male origin) | This manuscript | N/A |
| ENO1down in ROSA locus ES-R1 from mouse (male origin) | This manuscript | N/A |
| ENO1-AID, OsTir1 in TIGRE locus ES-R1 from mouse (male origin) | This manuscript | N/A |
| Oligonucleotides | | |
| ENO1 pool: SMARTpool ON-TARGETplus | Dharmaco | Cat#: L-004034-00-0005 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SIRT2 pool: SMARTpool ON-TARGETplus | Dharmacon | Cat#: L-004826-00-0005 |
| Control pool: SMARTpool ON-TARGETplus | Dharmacon | Cat#: D-001810-10-20 |
| sgRNAs, RNA oligonucleotides and qPCR | This manuscript | N/A |
| primers–please refer to Table S1 | | |

### Recombinant DNA

| pcDNA5/FRT/TO FLAG-HA c-term | This manuscript | N/A |
|---|---|---|
| pcDNA5/FRT/TO human ENO1wt FLAG-HA c-term | This manuscript | N/A |
| pcDNA5/FRT/TO human ENO1up FLAG-HA c-term | This manuscript | N/A |
| pcDNA5/FRT/TO human ENO1down FLAG-HA c-term | This manuscript | N/A |
| pcDNA5/FRT/TO murine ENO1wt FLAG-HA c-term | This manuscript | N/A |
| pcDNA5/FRT/TO murine ENO1up FLAG-HA c-term | This manuscript | N/A |
| pcDNA5/FRT/TO murine ENO1down FLAG-HA c-term | This manuscript | N/A |
| pETM-22-TRX-1-His6-tagged human ENO1wt | This manuscript | N/A |
| pETM-22-TRX-1-His6-tagged human ENO1up | This manuscript | N/A |
| pETM-22-TRX-1-His6-tagged human ENO1down | This manuscript | N/A |
| pSpCas9(BB)-2A-GFP sgRNA 1–3 for ENO1 knockout | This manuscript | N/A |
| pSpCas9(BB)-2A-RFP/Cer3 sgRNA 4&5 for ENO1 knockout | This manuscript | N/A |
| pSpCas9(BB)-2A-RFP/Cer3 sgRNA for ROSA26 locus | This manuscript | N/A |
| pDONOR Rosa 26 EF1_MCS_ires_Cer3_murine ENO1wt | This manuscript | N/A |
| pDONOR Rosa 26 EF1_MCS_ires_Cer3_murine ENO1up | This manuscript | N/A |
| pDONOR Rosa 26 EF1_MCS_ires_Cer3_murine ENO1down | This manuscript | N/A |
| TIGRE-targeting plasmids OsTir1-V5_PuroResistance | (Dossin et al., 2020) | N/A |

### Software and algorithms

| R | R project | https://www.r-project.org/ |
| ggplot2 | https://www.tidyverse.org/ | https://ggplot2.tidyverse.org |
| Prism GraphPad | GraphPad Software | Version 8 |
| Image Lab | BioRad | https://www.bio-rad.com/de-de/product/image-lab-software?ID=KRE6P5E8Z |
| ImageJ | NIH | https://imagej.net/Welcome |
| CellProfiler | (Carpenter et al., 2006) | https://cellprofiler.org/ |
| htseq-clip | (Sahadevan et al., 2022) | https://htseq-clip.readthedocs.io/en/latest/overview.html |

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**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Matthias W. Hentze (hentze@embl.org).

**Materials availability**
Plasmids and cell lines generated in this study are available upon request from the lead contact.

**Date and code availability**
- The RNA sequencing and eCLIP data have been deposited at EMBL-EBI’s ArrayExpress. The metabolomics data, raw imaging, and blotting data have been deposited at Mendeley Data. Accession numbers are listed in the key resources table and all data are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

HeLa cells (human female origin) were cultured in high-glucose (10 mM) Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml Pen/Strep. For the transfection of HeLa cells with siRNAs, cells were grown until 70%–80% confluent, trypsinized, counted and 200,000 cells were seeded per well in a 6-well dish.
for reverse transfection of siRNAs (ENO1 pool, SIRT2 pool or control pool, Dharmacon) following the manufacturer’s instructions (Lipofectamine RNAiMAX Transfection Reagent). For the transfection of plasmids, 5 μL of Lipofectamine 2000 reagent were used combined with 1 μg of plasmid DNA. In case of combined knock-down and rescue experiments, the cells were seeded, and siRNA transfection was performed. After 24 hours, the plasmid was transfected followed by an additional 24-hour incubation. The mouse embryonic stem cells (R1) were a donation from the group of Alexander Aulehia. Cell culture dishes were coated with 0.1% gelatin in PBS for 15 minutes prior to the addition of mESCs. The mESCs were cultured in high-glucose (10 mM) DMEM, supplemented with 15% FBS (EmbryoMax ES Cell Qualified), 2 mM L-glutamine, 100 U/ml Pen/Strep, 100 μM MEM non-essential amino acid solution, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol. If the mESCs were maintained to remain pluripotent, the leukaemia inhibitory factor (100 units/ml) was added to the medium. The triple knock-in cell line was a donation from the group of Pierre Neveu (Sladitschek and Neveu, 2019) and was cultured under the same conditions. The cell lines were not authenticated.

METHOD DETAILS

CRISPR/Cas9 gene insertion of ENO1 mutants

Single guide RNAs targeting the ROSA locus were predicted using the CRISPR online tool (Haeussler et al., 2016; Ran et al., 2013), ordered from Merck, annealed and cloned into the pSpCas9(BB)-2A-GFP (kindly provided by Kyung-Min Noh, EMBL) using the BbsI restriction sites (kindly cloned by David Kuster). The template plasmids for the knock-in of murine ENO1 mutants were prepared by introducing homology arms complementary to the ROSA locus upstream and downstream of the ENO1 wild-type or mutant DNA. While ENO1 is expressed under an EF1α promoter, the fluorescent protein cerulean is expressed under an additional, separate IRES promoter to facilitate an efficient cell sort. The generated plasmids were nucleofected into mESCs using the Nucleofector 4D system according to the manufacturer’s guidelines (Nucleofector Kit P3, program CG-104, one million cells, 16 μg template vector and 4 μg Cas9 vector per nucleofection). Single cell sorting of double positive cells (RFP and cerulean) was performed 48 hours after nucleofection (FACS Aria Fusion Sorter). Upon clonal expansion, successful insertion was tested by PCR to screen for homozygous insertions of the ENO1 wild-type or mutant DNA sequence in the ROSA locus (Phire Tissue Direct PCR Master Mix). The mutants of ENO1 were based on the findings of RBDmap data (Castello et al., 2016; Horos et al., 2019).

CRISPR/Cas9 gene deletion of ENO1

Using the ENO1 wildtype or mutant-expressing cell lines, we knocked out ENO1 in a subsequent step. Guide RNAs targeting the ENO1 locus were predicted using the CRISPR online tool (Haeussler et al., 2016; Ran et al., 2013), ordered from Merck, annealed and cloned into the pSpCas9(BB)-2A-GFP (PX458), or pSpCas9(BB)-2A-RFP using the BbsI restriction sites (kindly cloned by David Kuster). The template plasmids for the knock-in of murine ENO1 mutants were prepared by introducing homology arms complementary to the ROSA locus upstream and downstream of the ENO1 wild-type or mutant DNA. The generated plasmids were nucleofected into mESCs using the Nucleofector 4D system according to the manufacturer’s guidelines (Nucleofector Kit P3, program CG-104, one million cells, 16 μg template vector and 4 μg Cas9 vector per nucleofection). Single cell sorting of double positive cells (RFP and cerulean) was performed 48 hours after nucleofection (FACS Aria Fusion Sorter). Upon clonal expansion, successful insertion was tested by PCR to screen for homozygous deletion of ENO1 (Phire Tissue Direct PCR Master Mix). Furthermore, we tested the expression of ENO1 on a Western blot.

CRISPR/Cas9 gene insertion of the OsTIR1 receptor and the AID tag

Single guide RNAs targeting the TIGRE locus for insertion of the OsTIR1 gene were used as described in Dossin et al. (2020). Furthermore, constructions for insertion of the AID tag with either a blasticidin or neomycin resistance gene were provided by François Dossin (Group of Edith Heard). The ENO1 homology arms were introduced into both constructs using restriction-free cloning. The generated plasmids were nucleofected into mESCs using the Nucleofector 4D system according to the manufacturer’s guidelines (Nucleofector Kit P3, program CG-104, one million cells and 5 μg of each of the guide RNA-containing plasmids). Single cell sorting of double positive cells (RFP and GFP) was performed 48 hours after nucleofection. Upon clonal expansion, successful insertion was tested by PCR to screen for homozygous insertions of the TIGRE locus in the OsTIR1 gene (Phire Tissue Direct PCR Master Mix). The mutants of TIGRE were based on the findings of RBDmap data (Castello et al., 2016; Horos et al., 2019).

Differentiation and Fluorescence-activated cell sorting

To induce differentiation, LIF was removed from the medium for a period of seven days before harvesting (15 cm dishes). The cells were then trypsinized using 5 ml TrypLE Express at 37°C for five minutes and sorted using a FACS Aria Fusion Sorter. Cell populations of BFP-positive and mCherry-positive cells were respectively plated on gelatine-coated 10 cm dishes using LIF-free medium and cultured for five additional days before performing further experiments.

Protein extracts, SDS-PAGE and Western blotting

For Western blotting, cells were washed twice with ice-cold PBS and lysed on the plate using RIPA lysis buffer, supplemented with protease inhibitor. Lysates were treated with benzamidine for 15 minutes on ice and the protein concentrations were measured using the Protein Assay Dye Reagent Concentrate. 4× loading buffer was added to the lysates, boiled for five minutes and typically 15 μg of lysate was used for SDS-PAGE. Samples were run on 4–15% TGX Precast Midi Protein Gels (12+2, 18 or 26 wells). Proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Trans-Blot Turbo Midi 0.2 μm Nitrocellulose Transfer Packs) and blocked for 1 hour at room temperature with 5% milk in TBST. Primary antibodies were incubated in 5% milk TBST either overnight at 4°C or one hour at room temperature (RT), followed by 3×TBST washes, secondary antibody incubation...
in 5% milk in TBST for one hour at RT, 3xTBST washes and developed using the ECL solution. The antibodies used for Western blotting are listed here: Rabbit polyclonal ENO1 antibody (1:10,000; ab112994, Abcam), mouse monoclonal ENO1 antibody (1:5,000; ab155102, mouse embryonic stem cells, Abcam) was coupled with SureBeads Protein G magnetic beads for one hour at room temperature. As a control, rabbit IgG isotype control (Invitrogen, 10500C), normal rabbit IgG (Santa Cruz, sc-5205) was used with the beads at the same concentration. Typically, 1 µg was coupled with 30 µl of bead slurry. After coupling, the magnetic beads were washed twice with lysis buffer (100 mM NaCl; 50 mM Tris-HCl pH 7.5; 0.1% SDS; 1 mM MgCl₂; 0.1 mM CaCl₂; 1% NP-40; 0.5% sodium deoxycholate; protease inhibitors). Alternatively, pre-coupled FLAG M2 magnetic beads were washed twice with lysis buffer (50 µl per IP). HeLa cells or mESCs were washed twice with ice-cold PBS. Subsequently, the cells were UV-crosslinked at 150 mJ/cm² on ice and lysed in lysis buffer (Stratalinker 1800 UV Crosslinker). The lysates were centrifuged full speed at 4°C for 15 minutes and 15 µl were saved as inputs for a Western blot. The remainder of the lysates were then utilized for IPs at 4°C for two hours. After the IP and three times three-minute washes with lysis buffer at room temperature, beads were washed additionally twice with PNK buffer (50 mM NaCl; 50 mM Tris-HCl pH 7.5; 10 mM MgCl₂; 0.5% NP-40; protease inhibitors). The magnetic beads were resuspended in PNK buffer containing 0.1 mCi/mL [³²P] ATP, 1 U/ml T4 PNK, 1mM DTT and labelled for 15 minutes at 37°C. After four washes with PNK buffer, proteins were eluted at a low pH (0.1 M glycine, pH 2.0), neutralized with 1.5 M Tris-HCl, pH 8.5, and mixed with 4x sample loading buffer. The samples were heated to 95°C for five minutes and quickly spun down. The samples were resolved by SDS-PAGE and blotted on a nitrocellulose membrane using the Trans-Blot Turbo Transfer System. The membrane was exposed overnight to a phosphorimaging screen and scanned with a Typhoon FLA 9500, followed by immunoblotting. The files were processed with the Image Lab or ImageJ software.

**Polynucleotide kinase assay**

Prior to harvesting cells, ENO1 antibody (ab112994 for HeLa cells or ab155102 for mouse embryonic stem cells, Abcam) was coupled with SureBeads Protein G magnetic beads for one hour at room temperature. As a control, rabbit IgG isotype control (Invitrogen, 10500C), normal rabbit IgG (Cell Signaling, 2729) or normal mouse IgG (Santa Cruz, sc-2052) was coupled to the beads at the same concentration. Typically, 1 µg was coupled with 30 µl of bead slurry. After coupling, the magnetic beads were washed twice with lysis buffer (100 mM NaCl; 50 mM Tris-HCl pH 7.5; 0.1% SDS; 1 mM MgCl₂; 0.1 mM CaCl₂; 1% NP-40; 0.5% sodium deoxycholate; protease inhibitors). Alternatively, pre-coupled FLAG M2 magnetic beads were washed twice with lysis buffer (50 µl per IP). HeLa cells or mESCs were washed twice with ice-cold PBS. Subsequently, the cells were UV-crosslinked at 150 mJ/cm² on ice and lysed in lysis buffer (Stratalinker 1800 UV Crosslinker). The lysates were sonicated full speed at 4°C for 15 minutes and 15 µl were saved as inputs for a Western blot. The remainder of the lysates were then utilized for IPs at 4°C for two hours. After the IP and three times three-minute washes with lysis buffer at room temperature, beads were washed additionally twice with PNK buffer (50 mM NaCl; 50 mM Tris-HCl pH 7.5; 10 mM MgCl₂; 0.5% NP-40; protease inhibitors). The magnetic beads were resuspended in PNK buffer containing 0.1 mCi/mL [³²P] ATP, 1 U/ml T4 PNK, 1mM DTT and labelled for 15 minutes at 37°C. After four washes with PNK buffer, proteins were eluted at a low pH (0.1 M glycine, pH 2.0), neutralized with 1.5 M Tris-HCl, pH 8.5, and mixed with 4x sample loading buffer. The samples were heated to 95°C for five minutes and quickly spun down. The samples were resolved by SDS-PAGE and blotted on a nitrocellulose membrane using the Trans-Blot Turbo Transfer System. The membrane was exposed overnight to a phosphorimaging screen and scanned with a Typhoon FLA 9500, followed by immunoblotting. The files were processed with the Image Lab or ImageJ software.

**eCLIP**

eCLIP was performed as previously published (Van Nostrand et al., 2016), with minor adaptations. 1-3 µg of ENO1 antibody (ab112994, Abcam) or appropriate control IgG (rabbit) was coupled for 1 hour at RT to 30 µl of Protein G coupled magnetic beads. The cell lysates were treated with 0.1 U/ml RNase I for five minutes. One ml of lysate was used for each IP (concentration of 2 mg/ml) for two hours at 4°C. Complexes were eluted at low pH (0.1 M glycine, pH 2.0) and neutralized with 1.5 M Tris-HCl pH 8.5, and mixed with 4x sample loading buffer. The samples were heated to 95°C for five minutes and quickly spun down. The samples were resolved by SDS-PAGE and blotted on a nitrocellulose membrane using the Trans-Blot Turbo Transfer System. The membrane was exposed overnight to a phosphorimaging screen and scanned with a Typhoon FLA 9500, followed by immunoblotting. The files were processed with the Image Lab or ImageJ software.

**Data processing for eCLIP and statistical analysis**

The quality check of the eCLIP data was performed using fastqc (Andrews, 2010). The Unique Molecular Identifier (UMI) barcodes that are attached during library preparation were extracted and appended to the read name using umi-tools extract (Smith et al., 2017). The ligated adapters were trimmed and the reads shorter than 18 nucleotides were discarded using the cutadapt tool (Martin, 2011). The adapter-trimmed reads were aligned to the human genome (GRCh38.v23 from GENCODE) with STAR (Dobin et al., 2013), and the resulting annotation was pre-processed with the htseq-clip suite (available at https://htseq-clip.readthedocs.io/en/latest/overview.html; accessed 21 May 2020) into overlapping windows of 50 nucleotides in size with a step size of 20 nucleotides. The truncation site (position -1 relative to the start site of a read, also called crosslink site) was extracted and quantified usinghtseq-clip. We used the R/Bioconductor DESeq2 package (Sahadevan et al., 2022) to detect significantly enriched windows in IP samples over the corresponding size-matched input control samples (log₂ fold change >0.5, p-adjusted <0.1). IHW (Ignatiadis et al., 2016) was used for multiple hypothesis correction. Overlapping significant windows were merged to binding regions.
ENO1 Motif analysis
The previously obtained significant binding regions of ENO1 were filtered to retain regions with a minimum average of 30 normalized crosslink counts in the IP samples of protein-coding genes. We retained 472 protein coding exon regions, which were used for motif analysis. DREME(v4.11.3) (Bailey, 2011) from the MEME suite package (Machanick and Bailey, 2011) was used to identify the de novo motifs with parameters maxk set to 20 and norc.

Proximity Ligation Assay
A probe was designed based on ENO1’s identified and validated eCLIP binding site in the FTH1 mRNA. The probe is composed of a 20mer of DNA complementary to the RNA region proximal to ENO1’s binding site. Furthermore, the probe has a 3’biotinylation tag [BtN/Tg] to be recognized with an anti-biotin antibody (rabbit: ab234284 and mouse: ab201341).

Cells were grown on cover slips and fixed with 100% ice-cold acetone at room temperature for 20 minutes. The fixed cells were washed with PBS and permeabilized with PBS containing 1% BSA and 0.1% Triton X-100. To prepare the cells for the hybridization with the probe, the cells were washed with 0.1M Triethanolamine containing acetic anhydride. After washing twice with PBS-T (0.02% Tween20), the cells were washed twice with hybridization buffer (1x Denhardt’s solution, 0.1% (v/v) Tween20, 0.1% (w/v) CHAPS, 5 mM EDTA, 1 mg/ml RNase-free tRNA, 100 µg/ml heparin) and then incubated in hybridization buffer containing 100 nM of the relevant probe in a wet chamber at 37°C overnight. The probe was boiled for five minutes at 95°C prior to addition. Subsequently, Duolink in situ red starter kit rabbit (Sigma) was followed using the anti-ENO1 antibody (rabbit: 1:400; 11204-1-AP, Proteintech) or anti-FLAG antibody (mouse: 1:400; F3165, Sigma) for detection of the protein–RNA signal. To measure ENO1 expression by fluorescence imaging, the same primary antibody concentrations were used. For detection, the Alexa 488-conjugated secondary antibodies were used (Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488; A-21206, Thermo Fisher Scientific; Donkey anti-Mouse IgG (H+L) Alexa Fluor 488; A-21202, Thermo Fisher Scientific).

After the last wash of the Duolink PLA manufacturer’s protocol, antibody diluent was mixed with DAPI (final concentration of 0.1 µg/µl) and the cover slips were incubated at room temperature for 45 minutes. The cover slips were washed once with PBS-T and a glass cover was mounted using ProLong Diamond Antifade Mountant. The cover slips were stored at 4°C until fluorescence microscopy.

The microscopy was performed using a LSM 780 Laser Scanning Microscope (Zeiss) equipped with an AxioCam camera and 63x/1.4 objective with immersion oil (Immersion). The microscope was operated using the ZEN 2012 software (Zeiss). The DAPI signal was recorded in one plane to act as a reference for counting the number of cells. The PLA (Alexa 594) signal was recorded as a Z-stack (10 pictures for 10 µm stack). Three images were taken per condition. The images were acquired as .tiff files using the same settings (gain, laser power, pinhole and offset) for the same protein and analysed using the Fiji/ImageJ software. The .tiff files were split into individual channels, the Z-stacks for the PLA signal was projected into a single plane and the brightness was set to the same level in all images of the same channel to enable the comparison of the results. The individual channels were ultimately saved as .tiff files.

To count the PLA signals per cell for each of the different conditions, the CellProfiler Software version 4.1.3 was used. The range of the signal spot size was set to 8 to 20 pixels and the range of the nuclear size (DAPI signal) was set to 100 to 400 pixels. In both instances, the global threshold strategy minimum cross-entropy was used. The threshold smoothing factor was 1.3488 for the PLA signals and 20 for the nuclei. The cells were exposed to high laser power in the 488 channel to retrieve an outline of the cells. Clumped objects were separated by intensity and objects touching the border of the images were discarded. The PLA signal per cell were counted by combining the information of the cellular outline and the DAPI signal. The statistical analysis of these results was performed using Graphpad Prism version 9.

RNA immunoprecipitation
The medium was removed from 15 cm dishes of 80-90% confluent HeLa or mESCs and PBS with a final concentration of 0.1% formaldehyde was added to the cells for 9 minutes (room temperature). The cells were washed twice with excess of PBS and the crosslinking reaction was quenched by adding 125 mM Glycine to the cells for 5 mins (room temperature). Afterwards, the cells were washed 3x with ice-cold PBS for 1-2 mins. The lysis was performed using RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.1% SDS, 0.5% Na-Deoxycholate, Protease Inhibitor). The cells were sonicated with a Bioruptor(Pico) set to ‘High’ for 10 cycles with 30s ON and 30s OFF setting at 4°C. The lysates were centrifuged at 10,000g and 4°C for 15 mins and the protein concentration was measured. For the RNA immunoprecipitation experiment, 300 µg lysate and 3 µg Antibody (ab112994 for HeLa cells or ab155102 for mouse embryonic stem cells, Abcam). The antibody was coupled to 20 µl Protein G magnetic beads for 1 hour at room temperature. 2% of the lysate was used as input material and the lysate was incubated with the antibody-conjugated beads for 1 hour at 4°C while constantly rotating. The IPs were washed once with RIPA lysis buffer, twice with RIPA high salt buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.1% SDS, 0.5% Na-Deoxycholate, Protease Inhibitor), twice with Wash buffer (10 mM Tris-HCl, 0.2% Tween-20, 1 mM EDTA) and once again with RIPA lysis buffer. The samples were eluted with glycine as previously described. 10% of the IP was used for western blotting to assess the immunoprecipitation efficiency. RNase inhibitor and 10 ng total C. elegans RNA (spike-in control) were added to the remainder of the IP samples and the inputs. Subsequently, the samples were digested with pre-warmed (at 37°C for 20 mins) proteinase K mix at 55°C for 30 mins (final concentration: 2 µg/µl enzyme). Acid phenol/chloroform/isoamyl alcohol (pH 6.5) was added to the input and IP samples and they were incubated at 37°C for 5 mins while shaking at 1200 rpm. The samples were then loaded on 2 ml Phase Lock gel Heavy tubes, incubated at 37°C for
5 mins again and then centrifuged at 13,000g for 15 mins at room temperature. The aqueous layer was transferred to 5 ml Eppendorf tubes and the RNA Clean & Concentrator-5 kit was used to purify the RNA, following the manufacturer’s instructions. The RNA eluates were treated with 2 μL Turbo DNase, 2.5 μL 10 x Turbo DNase Buffer in a final 25 μL reaction volume. The mixture was incubated at 37°C for 30 mins. Subsequently, the reaction was stopped by adding 2.4 μL inhibition resin. The reaction mix was incubated for 5 mins at room temperature and then centrifuged at 10,000g for 2 mins. 20 μL of the supernatant was retrieved and all of it was used as input for the SuperScript IV reverse transcription (1x SSIV, 0.5 mM dNTP, 5 mM DTT, 5U/μL Super Script IV Reverse Transcriptase, 2.5 μM random hexamers), following the manufacturer’s instructions. These samples were then used for subsequent qPCR experiments.

RT-qPCR and qPCR
RNA was isolated using the Direct-zol RNA Miniprep kit, as recommended by the manufacturer. 1.0 μg total RNA was used to synthesize cDNA using the Maxima First Strand cDNA Synthesis Kit for quantitative reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR was performed using the SYBR-Green qPCR Master mix on a QuantStudio 6 Flex Real-Time-PCR-System. Gene expression values were normalized to 3 samples using ACTB and are shown as a relative fold change to the value of control samples. All experiments were performed in biological triplicates and error bars indicate ± standard deviation as assayed by the ΔΔCt method. Statistically significant differences were detected using two-way ANOVA with multiple comparisons correction (Tukey). All RT-qPCR primers are listed in Table S1.

RNAseq library preparation
RNA was isolated using the Direct-zol RNA Miniprep kit, as recommended by the manufacturer. 1.0 μg total RNA was used as input for the library preparation. The TruSeq Stranded mRNA kit was used for the library preparation. The samples were multiplexed and sequenced on a HiSeq2000.

RNAseq analysis and statistical analysis
Reads were trimmed using Cutadapt (Martin, 2011) (v1.16), mapped to the human genome (GRCh38.p3) with STAR (Dobin et al., 2013) (v2.5.0.a) and summarized with featureCounts (Liao et al., 2014) (v1.6.4). Resulting counts were pre-filtered to retain genes with more than 4 counts in at least 3 samples. DESeq2 (Love et al., 2014) (v1.26) using local dispersion fit and likelihood ratio test with IHW (Ignatiadis et al., 2016) for multiple hypothesis correction was used to determine significantly enriched RNAs in ENO1 knock-down versus control samples (adjusted p-value <0.1; log2 fold change >0.5).

Sucrose density centrifugation and fractionation
For the preparation of lysates for the testing of RNA dependency through ultracentrifugation, previously published protocols were used as a basis (Caudron-Herger et al., 2019; Höck et al., 2007). HeLa cells were cultured on a 15 cm dish and lysed in 300 μl lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 0.5% NP-40, 2 mM EDTA, 1 mM NaF, 0.5 mM DTT, protease inhibitor). A pre-clearing step was performed by centrifugation at 10,000g for 10 minutes at 4°C. The lysates were then treated with a combination of RNase I, RNase T1/A or no RNase was added at 37°C for 15 minutes. For the fractionation, gradients from 5% (w/v) to 25% (w/v) sucrose in 150 mM KCl, 25 mM Tris-HCl (pH 7.4) and 2 mM EDTA were prepared using a gradient maker (settings: 1:52, angle 81.5, speed: 15). Lysates were separated by centrifugation at 30,000 rpm and 4°C in an SW40 rotor for 18 hours. The lysate fractions were collected by hand through careful pipetting from the top (16 fractions were collected of approximately 600 μl). For the protein precipitation, 150 μl of 100% Trichloroacetic acid (TCA) was added and left on ice for 30 minutes. The individual fractions were centrifuged at full speed and 4°C for 20 minutes. The TCA supernatant was carefully removed, and the pellets were washed once with 1 ml cold acetone (stored at −20°C). The samples were vortexed, and an additional centrifugation step was performed at full speed and 4°C for 30 minutes. The supernatant was again carefully removed, and the pellet was air-dried. Finally, the pellets were taken up in 1× loading buffer containing benzamidine and used for SDS-PAGE and immunoblotting.

Protein Purification
The ENO1 wildtype and mutant DNA sequence were cloned into the pETM-22 plasmid (generously provided by the Protein Expression and Purification core facility at EMBL) using the NcoI and XhoI site. Through the expression of the ENO1 variants, the proteins are tagged with the protein Thioredoxin to enable a clear distinction between the cleaved and uncleaved protein, which can be cleaved off using the HRV 3C Protease and removed using a reverse Ni-NTA column.

For the protein purification, the ENO1 variants were expressed by transforming the plasmid into E.coli BL21(DE3) CodonPlus-RIL competent cells and plated on Kanamycin-containing LB plates. One colony was picked and used for protein expression in a 400 ml flask. Once the OD600 was reached, the culture was cooled down to 18°C and IPTG was added to reach a final concentration of 1 mM. The bacteria were pelleted and lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM b-2ME, 5% glycerol, 40 mM imidazole, 0.01% NP40), supplemented with RNase A, Turbo DNase and Protease Inhibitor to the lysis buffer. For efficient lysis, the lysates were processed with a microfluidizer. The His-tagged thioredoxin was used for the purification of ENO1 using a HisTrap HP column on an Äkta go protein purification system. The protein was eluted with increasing concentrations of imidazole and the protein-containing fractions were verified on a Coomassie gel. The solubility tag was cleaved by HRV 3C protease during

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Measurements were performed at 298 K, using 0.2 mM ENO1 in NMR buffer, supplemented with 5% D2O for the deuterium lock. This strength, corresponding to a proton Larmor frequency of 800 MHz, equipped with a cryogenic triple resonance gradient probe head.

Mouse embryonic stem cells were exposed to specific or unspecific RNAs through nucleofection. Afterwards, the cells were prepared, TEMED) native gel was poured for the separation of the free radioactive probe and the formed complex of ENO1 and RNA. The reaction mix was added.

P3 Primary Cell 4D-Nucleofector X Kit, programme CG-104, 1 million cells). The cells were then cultured for an additional 90 minutes according to the manufacturer’s guidelines (HeLa cells: SE Cell Line 4D-Nucleofector X Kit, programme CN-114, 1 million cells; mESCs: P3 Primary Cell 4D-Nucleofector X Kit, programme CG-104, 1 million cells). The synthesized specific and unspecific RNAs were nucleofected into HeLa cells or mESCs using the Nucleofector 4D system according to the manufacturer’s instructions.

NOR data acquisition and titration

1H, 15N-HSQC spectra of deuterated ENO1 were recorded using an Avance III Bruker NMR spectrometer, which operates at a field strength, corresponding to a proton Larmor frequency of 800 MHz, equipped with a cryogenic triple resonance gradient probe head. Measurements were performed at 298 K, using 0.2 mM ENO1 in NMR buffer, supplemented with 5% D2O for the deuterium lock. This sample was titrated with increasing concentrations of 18-mer synthetic RNA (IBA) (0.02, 0.04, 0.05, 0.16, 0.24 and 0.4 mM) and at each titration step another 1H, 15N-HSQC was recorded. Data were processed and analysed using NMRPipe and Sparky.

Nucleofection of target and control RNAs

The synthesized specific and unspecific RNAs were nucleofected into HeLa cells or mESCs using the Nucleofector 4D system according to the manufacturer’s guidelines (HeLa cells: SE Cell Line 4D-Nucleofector X Kit, programme CN-114, 1 million cells; mESCs: P3 Primary Cell 4D-Nucleofector X Kit, programme CG-104, 1 million cells). The cells were then cultured for an additional 90 minutes in DMEM, free of phenol red and FBS, to allow the collection of lactate-containing medium.

13C-glucose tracing experiment and GC-mass spectrometry

Mouse embryonic stem cells were exposed to specific or unspecific RNAs through nucleofection. Afterwards, the cells were transferred to 5 ml Eppendorf tubes containing 5 ml warm DMEM free of phenol red and FBS containing D-Glucose (U-13C6). The cells were exposed to the labelling medium for 60 minutes and then they were collected by centrifugation at 500g (4°C) for 3 minutes.
The cells were re-suspended in ice-cold PBS and the centrifugation step was repeated. The PBS was removed, and the cell pellet was re-suspended in 5 ml of fresh ice-cold PBS. The cells were transferred into a 2 ml Eppendorf tube, and centrifuged again at 500g for 3 minutes. Finally, the PBS was removed and 500 µl of very cold HPLC-grade methanol was added, and the suspension was mixed well. The samples were mixed and kept on dry ice until they were stored at -80°C. On the day of the extraction, all tubes were placed on ice, vortexed and 5 µl of adonitol (50 µg/ml) was added. The samples were incubated at 72°C for 15 minutes in a water bath. 500 µl MilliQ H2O was added to the extract. The samples were vortexed and placed at -80°C for one hour. After the incubation, the samples were thawed on ice, thoroughly vortexed and centrifuged at 15,000g at 4°C for 12 minutes. The supernatants were transferred to high recovery vials and vacuum was applied for drying the samples (Genevac EZ-2 Plus evaporator; program, hplc fraction; temperature, 30°C). The dried polar metabolites were derivatized with 50 µl of 20 mg/mL methoxyamine hydrochloride solution in pyridine for 90 minutes at 37°C, followed by reaction with 100 µl N-methyl-N-(trimethylsilyl)trifluoroacetamide for 10 hours at room temperature, as justified in Kanani and Klapa (2007). GC-MS analysis was performed using a Shimadzu TQ8040 GC-(triple quadrupole) MS system (Shimadzu Corp.) equipped with a 30 m x 0.25 mm x 0.25 µm ZB-50 capillary column (Phenomenex, 7HG-G004-11). One µl of sample was injected in split mode (split ratio 1:5) at 250°C using helium as a carrier gas with a flow rate of 1 mL/minute. GC oven temperature was held at 100°C for 4 minutes followed by an increase to 320°C with a rate of 10°C/minute, and a final constant temperature period at 320°C for 11 minutes. The interface and the ion source were held at 280°C and 230°C, respectively. The detector was operated both in scanning mode recording in the range of 50-600 m/z, as well as in MRM mode for specified metabolites. For peak annotation, the GCMSolution software was utilized. The metabolite identification was based on an in-house database with analytical standards utilized to define the retention time, the mass spectrum, and the quantifying ion fragment for each specified metabolite. The ratio of the different mass isotopologues for each metabolite was determined by integrating the area under the curve (AUC) of the quantifying ion fragments followed by correction for the presence of natural abundant isotopes with the Isotope Correction Toolbox (ICT) (Jungreuthmayer et al., 2016). All peak integrations were manually checked.

**Lactate accumulation measurement**

For the determination of lactate levels in the supernatant of HeLa and mESCs, the supernatant was collected 30, 60 and 90 minutes after the replacement with DMEM, free of phenol red and FBS. The lactate colorimetric/fluorometric assay kit for the determination of lactate levels was utilized according to the manufacturer’s instructions.

**Lactate accumulation and oxygen consumption of pluripotent and differentiated cells**

mESC were differentiated in 15 cm dishes for seven days in the absence of LIF or maintained for two days in the pluripotent state. Seeding of cells was done asynchronously so that pluripotent and differentiated cells could be processed in parallel on the same day. For determining oxygen consumption, cells were washed with PBS, trypsinized, pelleted by centrifugation (5 minutes at 230g) and resuspended in mESC medium (+ or − LIF as appropriate). Live cell number was counted in a TC20 automated cell counter using trypan blue staining. Immediately afterwards, the oxygen consumption rate of 300 µl of cell suspension containing ~one million living cells was determined in an Oxytherm System at 37°C using the Oxygraph Plus data acquisition software. For determining lactate secretion, the medium of the 15 cm dishes with pluripotent/differentiated cells was exchanged with 11 ml of mESC medium without serum and without phenol red. After 30 min of incubation, an aliquot of the conditioned medium was taken and stored at −80°C. Cells were lysed in RIPA buffer and total protein quantified using the Protein Assay Dye Reagent Concentrate. Lactate concentration was measured in 3 µl of conditioned medium with the Lactate Assay kit following manufacturer’s instructions. Lactate measurements were normalized by the protein amount on the dish.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis of results was performed using two-tailed Student’s t-test (parametric), or analysis of variance (ANOVA), followed by Sidak’s or Tukey’s multiple comparison tests, as stated in the figure legends. All the analyses were done using GraphPad Prism, version 8.4.2. Statistical significance is represented in all figures, as follows: p-value of <0.0001: ****, p-value of 0.0001 to 0.001: ***, p-value of 0.001 to 0.01: **, p-value of 0.01 to 0.05: * and p-value of ≥0.05: not significant.