# The Lin28/let-7 Axis Regulates Glucose Metabolism

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The Lin28/let-7 Axis Regulates Glucose Metabolism

Hao Zhu,1,2,3,4 Ng Shyh-Chang,1,2,6,14 Ayellet V. Segre,5,6 Gen Shinoda,1,2 Samar P. Shah,1,2 William S. Einhorn,1,2,4 Ayumu Takeuchi,1,2 Jesse M. Engreitz,7 John P. Hagan,1,2,8,9 Michael G. Kharas,1,2,4 Achia Urbach,1,2 James E. Thornt0n,1,2,8 Robinson Triboulet,1,2,8 Richard I. Gregory,1,2,8 DIAGRAM Consortium,13 MAGIC Investigators,13 David Altshuler,5,6,10 and George Q. Daley1,2,4,8,11,12,*

1Stem Cell Transplantation Program, Stem Cell Program, Division of Pediatric Hematology/Oncology, Children’s Hospital Boston and Dana Farber Cancer Institute, Boston, MA, USA
2Harvard Stem Cell Institute, Boston, MA, USA
3Division of Medical Oncology, Dana Farber Cancer Institute, Boston, MA, USA
4Division of Hematology, Brigham and Women’s Hospital, Boston, MA, USA
5Department of Molecular Biology, Diabetes Unit Department of Medicine, and Center for Human Genetics Research, Massachusetts General Hospital, Boston, MA, USA
6Program in Medical and Population Genetics, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA
7Division of Health Sciences and Technology, MIT, Cambridge, MA, USA
8Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA
9Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University Medical Center, Columbus, OH, USA
10Departments of Genetics and of Medicine, Harvard Medical School, Boston, MA, USA
11Howard Hughes Medical Institute, Boston, MA USA
12Manton Center for Orphan Disease Research, Boston, MA, USA
13Memberships of the consortia are provided in Text S1 available online
14These authors contributed equally to this work
*Correspondence: george.daley@childrens.harvard.edu
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SUMMARY

The let-7 tumor suppressor microRNAs are known for their regulation of oncogenes, while the RNA-binding proteins Lin28a/b promote malignancy by inhibiting let-7 biogenesis. We have uncovered unexpected roles for the Lin28/let-7 pathway in regulating metabolism. When overexpressed in mice, both Lin28a and LIN28B promote an insulin-sensitized state that resists high-fat-diet induced diabetes. Conversely, muscle-specific loss of Lin28a or overexpression of let-7 results in insulin resistance and impaired glucose tolerance. These phenomena occur, in part, through the let-7-mediated repression of multiple components of the insulin-Pi3K-mTOR pathway, including IGF1R, INSR, and IRS2. In addition, the mTOR inhibitor, rapamycin, abrogates Lin28a-mediated insulin sensitivity and enhanced glucose uptake. Moreover, let-7 targets are enriched for genes containing SNPs associated with type 2 diabetes and control of fasting glucose in human genome-wide association studies. These data establish the Lin28/let-7 pathway as a central regulator of mammalian glucose metabolism.

INTRODUCTION

Metabolic disease and malignancy are proposed to share common biological mechanisms. Reprogramming toward glycolytic metabolism can increase a cancer cell’s ability to generate biomass, a phenomenon termed the “Warburg Effect” (Denko, 2008; Engelman et al., 2006; Gao et al., 2009; Guertin and Sabatini, 2007; Laplante and Sabatini, 2009; Vander Heiden et al., 2009; Yun et al., 2009). Likewise, many genes identified in type 2 diabetes (T2D) genome wide association studies (GWAS) are proto-oncogenes or cell cycle regulators (Voight et al., 2010). MicroRNAs (miRNAs) are also emerging as agents of metabolic and malignant regulation in development and disease (Hyun et al., 2009; Peter, 2009). The let-7 miRNA family members act as tumor suppressors by negatively regulating the translation of oncogenes and cell cycle regulators (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Kumar et al., 2008). Widespread expression and redundancy among the well-conserved let-7 miRNAs raise the question of how cancer and embryonic cells are able to suppress this miRNA family to accommodate rapid cell proliferation. In human cancers, loss of heterozygosity, DNA methylation, and transcriptional suppression have been documented as mechanisms to reduce let-7 (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Kumar et al., 2008).
**Lin28a**

**Tg**

**WT**

**Control**

**B** Lean weight

**C** Fat weight

**D** Lin28a Tg on HFD

**E** Lin28a Tg on HFD GTT

**F** Lin28a Tg on HFD ITT

**H** LIN2BB mRNA expression

**I** let-7 in iLIN2BB induced mice

**J** Fed state glucose after induction

**K** iLIN2BB on normal diet GTT

**L** iLIN2BB on normal diet ITT

**M** iLIN2BB on HFD growth curve

**N** iLIN2BB on HFD GTT

**O** Lin28a muscle specific KO GTT

**P** Lin28a muscle specific KO ITT
**RESULTS**

**Lin28a Tg Mice Are Resistant to Obesity and Diabetes**

We previously described a tetracycline-inducible Lin28a transgenic (Lin28a Tg) mouse that showed leaky constitutive Lin28a expression in the absence of induction (Zhu et al., 2010). In that study, we showed that these mice cleared glucose more efficiently during glucose and insulin tolerance testing (GTT and ITT), classic metabolic tests used for the characterization of whole animal glucose handling. Given that young Lin28a Tg mice exhibited enhanced glucose metabolism, we tested if old Lin28a Tg mice were also resistant to age-induced obesity. Compared to Lin28a Tg mice, wild-type mice fed a normal diet gained significantly more fat mass with age (Figure 1A). Dual Energy X-ray Absorptiometry scans showed increased percentage lean mass and reduced percentage body fat in the Lin28a Tg mouse (Figures 1B and 1C). To rule out behavioral alterations, we measured activity over three days in isolation cages and found no differences in horizontal activity, O2/CO2 exchange, and food/water intake between wild-type and Tg mice (Figures S1A and S1B available online). To determine if these mice were resistant to HFD-induced obesity, we fed mice a diet containing 45% kcals from fat, and observed resistance to obesity in the Lin28a Tg mouse (Figure 1D). Lin28a Tg mice consumed as much high-fat food as their wild-type littermates, ruling out anorexia (data not shown). Furthermore, we inquired if Lin28a Tg mice were resistant to HFD-induced diabetes and found that they had markedly improved glucose tolerance and insulin sensitivity under HFD conditions (Figures 1E and 1F). Lin28a Tg mice also showed resistance to HFD-induced hepatosteatosis (Figure 1G). Taken together, leaky Lin28a expression in the muscle, skin and connective tissues (Zhu et al., 2010) protected against obesity and diabetes in the context of aging and HFD.

**iLIN28B Tg Mice Are Resistant to Diabetes**

Although Lin28a and Lin28b both block let-7 miRNAs, they are differentially regulated, resulting in distinct expression patterns during normal development and malignant transformation (Guo et al., 2006; Viswanathan et al., 2009). Given that LIN28B is overexpressed more frequently than LIN28A in human cancer, we sought to determine if LIN28B exerts a similar effect on glucose metabolism. Thus, we generated a mouse strain carrying an inducible copy of human LIN28B driven by a tetracycline transactivator rtTA placed under the control of the Rosa26 locus (iLIN28B mouse, see Experimental Procedures). After 14 days of treatment with the tetracycline analog doxycycline (dox), high levels of LIN28B were induced and mature let-7’s were
repressed in metabolically important organs (Figures 1H and 1I), resulting in hypoglycemia with an average fasting glucose of <50 mg/dL in induced mice compared to >150 mg/dL in control mice (p < 0.01). To determine the kinetics of this effect, we measured fed state glucose daily and noted falling glucose levels after 5 days (Figure 1J). Glucose and insulin tolerance tests on dox-induced animals on normal diets showed considerable improvements in glucose tolerance and insulin sensitivity (Figures 1K and 1L). When assessing islet β cell hyperactivity, we found that iLIN28B mice produced no more insulin than control littersmates during glucose challenge (data not shown). Under HFD, we found that induced iLIN28B mice were surprisingly resistant to weight gain (Figure 1M) despite a trend toward increased food intake (9.9 versus 4.8 g/mouse/day; p = 0.075). These mice continued to exhibit superior glucose tolerance after 14 days of dox induction under HFD (Figure 1N), when average weights were 34.5 ± 1.05 g for controls and 27.1 ± 0.99 g for iLIN28B mice, demonstrating that HFD had a strong obesogenic and diabetogenic effect on control but not on LIN28B induced animals. Unlike the Lin28a Tg mice, expression was not leaky in the iLIN28B mice (Figure 1H and 3F) and uninduced mice exhibited no growth or glucose phenotypes (Figures S1C and S1D), making this a better model for inducible Lin28 hyperactivation. These data show that both Lin28 homologs have similar effects on glucose metabolism and obesity, suggesting that these effects are mediated through common mRNA or miRNA targets of the Lin28 family.

**Lin28a Is Physiologically Required for Normal Glucose Homeostasis**

We then asked if Lin28a is physiologically required for normal glucose metabolism in one specific adult tissue compartment, skeletal muscle, since previous studies have found low but significant levels of Lin28a expression in the muscle tissues of mice (Yang and Moss, 2003; Zhu et al., 2010). We generated a skeletal muscle-specific knockout of Lin28a (see Experimental Procedures). These muscle-specific knockout mice showed impaired glucose tolerance (Figure 1O) and insulin resistance (Figure 1P) relative to wild-type littermates, demonstrating that Lin28a activity in skeletal muscles is required for normal glucose homeostasis. We analyzed miRNA expression in muscle tissue by qRT-PCR and found no significant difference in let-7 levels during adult (data not shown) or embryonic stages (Figure S1E), suggesting that Lin28a loss of function affects glucose homeostasis either through let-7-independent mRNA binding or through changes in the spatiotemporal distribution of let-7 miRNA. Together, these data show that Lin28 isoforms are important and essential regulators of glucose homeostasis.

**iLet-7 Mice Are Glucose Intolerant**

In addition to their ability to suppress let-7 biogenesis, Lin28a and Lin28b also regulate miRNA targets such as Igf2, HMGMA1, OCT4, histones and cyclins through non-let-7 dependent mechanisms of mRNA binding and enhanced translation (Polesskaya et al., 2007; Xu and Huang, 2009; Xu et al., 2009; Peng et al., 2011). To test if altered let-7 expression might produce the opposite phenotypes of Lin28a/b gain of function, we generated a mouse strain in which let-7g can be induced with dox under the control of the Rosa26 locus (Let-7 mouse, See Experimental Procedures). To ensure that endogenous Lin28 would not block pri- or pre-let-7g biogenesis, we used a chimeric let-7g species called let-7S21L (let-7g Stem, mir-21 Loop), in which the loop region of the precursor miRNA derives from mir-21 and cannot be bound by Lin28, thus allowing for let-7 processing despite Lin28 expression (Piskounova et al., 2008). Global transgene induction from three weeks of age onward increases mature let-7g levels in liver (>50-fold), skin (>20-fold), fat (<4-fold) and muscle (<4-fold) (Figure 2A). This level of let-7 overexpression led to reduced body size and growth rates in induced animals (Figures 2B and 2C). Growth retardation was proportional and not manifested as preferential size reduction in any particular organs (Figure S2A). Similar to the iLIN28B mice, leaky expression was not detected and uninduced male mice exhibited no growth or glucose phenotypes (Figures S2B–S2D).

After 5 days of let-7 induction, these iLet-7 mice produced an increase in fed state glucose (Figure 2D). GTT revealed glucose intolerance in mice fed normal (Figure 2E) or HFD (Figure 2F). Surprisingly, ITT failed to detect a difference in insulin sensitivity (Figure 2G). The decreased glucose tolerance in the setting of comparable insulin sensitivity suggested either decreased insulin production from islet β cells in response to glucose, or higher insulin secretion to compensate for peripheral insulin resistance. Thus, we measured insulin production following glucose challenge, and found that iLet-7 mice produced more insulin than controls (Figure 2H). These results demonstrated that broad overexpression of let-7 results in peripheral glucose intolerance and compensatory overproduction of insulin from islet β cells.

To test if let-7 induction could abrogate the glucose uptake phenotype of LIN28B overexpression, we crossed the iLIN28B to the iLet-7 inducible mice. After 10 days of induction, simultaneous induction of LIN28B and let-7g did not result in any differences in glucose tolerance (Figures 2I and 2J), in contrast to LIN28B or let-7g induction alone. Taken together, the opposing effects of Lin28 and let-7 expression on glucose regulation show that Lin28 overexpression influences metabolism in part by suppressing let-7, and that let-7 alone is sufficient to regulate glucose metabolism in vivo.

**Insulin-PI3K-mTOR Signaling Is Activated by Lin28a/b and Suppressed by let-7**

To dissect the molecular mechanism of the effects of Lin28 and let-7 on glucose regulation, we turned to the C2C12 cell culture system. Overexpression of Lin28a in C2C12 myoblasts resulted in protein levels of Lin28a similar to that observed in mouse embryonic stem cells (ESCs) (Figure 3A), and led to robust let-7 suppression (Figure 3B). In C2C12 myotubes differentiated for 3 days, Lin28a promoted Ser473 phosphorylation of Akt and Ser235/236 phosphorylation of GSK3β, suggesting activation of the PI3K-mTOR pathway (Figure S3A). In this setting, Lin28a increased myotube glucose uptake by 50% (Figure 3C). Lin28a-dependent glucose uptake was abrogated by 24hr treatment with the PI3K/mTOR inhibitor LY294002 or the mTOR inhibitor rapamycin (Figure 3C), but not the MAPK/ERK inhibitor PD98059 (Figure S3B), demonstrating that Lin28a-dependent glucose uptake requires the PI3K-mTOR pathway.
To exclude myotube differentiation-dependent phenomena, we tested the effects of Lin28a on PI3K-mTOR signaling in undifferentiated myoblasts under serum-fed, serum-starved, and insulin-stimulated conditions (Figure 3D). In the serum-fed state, we found that Lin28a promoted the activation of PI3K/Akt signaling by increasing Akt phosphorylation at both Ser473 and Thr308, compared to the pBabe control. Furthermore, we found that Lin28a robustly increased the phosphorylation of mTORC1 signaling targets S6 and 4EBP1 in the serum-fed state. Serum-starvation for 18 hr abrogated the phosphorylation of Akt, S6 and 4EBP1, indicating that Lin28a-induced PI3K-mTOR signaling requires exogenous growth factor stimulation. Upon insulin stimulation, Akt phosphorylation increased dramatically and, both phospho-S6 and phospho-4EBP1 levels were

Figure 2. iLet-7 Mice Are Glucose Intolerant
(A) let-7g and let-7a qRT-PCR in tissues of dox induced iLet-7 mice (n = 3) and controls (n = 3).
(B) Reduced size of induced animals.
(C) iLet-7 growth curve for males and females.
(D) Fed state glucose in iLet-7 mice induced for 5 days.
GTTs performed on mice fed with either (E) normal diet or (F) HFD.
(G) ITT on normal diet.
(H) Insulin production during a glucose challenge.
(I) GTT of LIN28B/Let-7 compound heterozygote mice before (blue) and after (red) induction with dox.
(J) Area under the curve (AUC) analysis for this GTT.
Controls for iLet-7 Tg mice carry either the Let-7 or Rosa26-M2rtTa transgene only. The numbers of experimental animals are listed within the charts. Error bars represent SEM. *p < 0.05, **p < 0.01.
increased even further by Lin28a overexpression, suggesting that Lin28a increases the insulin-sensitivity of C2C12 myoblasts. Importantly, we found that rapamycin abrogated the Lin28a-induction of phospho-S6 and phospho-4EBP1 upon insulin stimulation, but did not affect let-7 levels (Figure S3 C) or Lin28a itself (Figure 3D), indicating that the mTOR dependence is occurring downstream of Lin28a.

To test if the effects of Lin28a on insulin-PI3K-mTOR signaling are let-7-dependent, we transfected either mature let-7f duplex or a negative control miRNA into both Lin28a-overexpressing and pBabe control myoblasts (Figure S3D and Figure 3E). Because mature let-7 duplexes cannot be bound and inhibited by Lin28a protein, this experiment tests if PI3K-mTOR activation is occurring downstream of let-7. Transfection with control miRNA did not affect Lin28a-induction of the phosphorylation of Akt, S6, or 4EBP1 in serum-starved myoblasts upon insulin stimulation. Transfection with let-7f, however, attenuated the Lin28a-induction of phospho-Akt (Ser473), and abrogated the increase in S6 and 4EBP1 phosphorylation upon insulin stimulation in Lin28a-overexpressing myoblasts (Figure 3E). In pBabe control myoblasts, let-7 duplex still suppressed S6 and 4EBP1 phosphorylation in the serum-fed state, serum-starved, and

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**Figure 3. Insulin-PI3K-mTOR Signaling Is Activated by Lin28a/b and Suppressed by let-7**

(A) Western blot analysis of Lin28a protein expression in C2C12 myoblasts infected with control pBabe or Lin28a overexpression vector, and mouse ESCs, with tubulin as the loading control.

(B) Quantitative PCR for let-7 isoforms in C2C12 myoblasts, normalized to sno142, after Lin28a overexpression.

(C) 2-deoxy-D-[3H] glucose uptake assay on 3-day-differentiated C2C12 myotubes with and without Lin28a overexpression, treated with DMSO, the PI3K inhibitor LY294002, and the mTOR inhibitor rapamycin for 24 hr.

(D) Western blot analysis of the effects of Lin28a overexpression on PI3K-mTOR signaling in C2C12 myoblasts, under serum-fed (fed), 18 hr serum starved (SS) or insulin-stimulated (Ins) conditions. Insulin stimulation was performed in serum-starved myoblasts with 10 μg/mL insulin for 5 min. Prior to insulin stimulation, serum-starved myoblasts were treated with either DMSO or 20 ng/mL rapamycin for 1 hr.

(E) Western blot analysis of the effects of let-7f or control miRNA on PI3K-mTOR signaling in C2C12 myoblasts under serum-fed (fed), 18 hr serum starved (SS) or insulin-stimulated (Ins) conditions.

(F) Western blot analysis of the effects of LIN28B induction by dox on PI3K-mTOR signaling in quadriceps muscles in vivo (n = 3 iLIN28B Tg mice and 3 LIN28B Tg only mice).

(G) Insr and p-4EBP1 protein levels in wild-type and Lin28a muscle-specific knockout adults. Error bars represent SEM. *p < 0.05, **p < 0.01.
insulin-stimulated conditions, relative to total S6 and 4EBP1 protein. The suppression of mTOR signaling by let-7 even in the absence of Lin28a implies that let-7 can act independently downstream of Lin28a. Together with data indicating that let-7 abrogates Lin28a-specific induction of p-Akt, p-S6 and p-4EBP1 upon insulin stimulation, this demonstrates that the effects of Lin28a on PI3K-mTOR signaling are at least in part due to let-7 and that Lin28 and let-7 exert opposing effects on PI3K-mTOR signaling.

To test if these effects of Lin28 on insulin-PI3K-mTOR signaling are also relevant in vivo, we examined the quadriceps muscles of LIN28B mice and found that dox-induction led to increases in the phosphorylation of Akt (S473), S6 and 4EBP1, the targets of PI3K-mTOR signaling (Figure 3F). Furthermore, the insulin-like growth factor 1 receptor (Igf1r) and the Insulin receptor (Insr) proteins were upregulated in the muscles upon LIN28B induction, reinforcing the fact that Lin28a/b drives insulin-PI3K-mTOR signaling in C2C12 myoblasts and within mouse tissues. On the other hand, similar analysis of the Lin28a muscle-specific knockout mice revealed reduced Insr and p-4EBP1 expression (Figure 3G), demonstrating that Lin28a is both necessary and sufficient to influence glucose metabolism through the regulation of insulin-PI3K-mTOR signaling in vivo.

**Lin28a/b and let-7 Regulate Genes in the Insulin-PI3K-mTOR Pathway**

On the RNA level, Lin28a overexpression in C2C12 myoblasts leads to an increase in mRNA levels of multiple genes in the insulin-PI3K-mTOR signaling pathway (Figure S4A). Although both Lin28a suppression of let-7 and direct Lin28a binding to mRNAs could increase mRNA stability and thus increase mRNA levels, it is possible that these increases do not reflect direct interactions. To find direct targets, we performed a bio-informatic screen using the TargetScan 5.1 algorithm (Grimson et al., 2007), and found that 16 genes in the insulin-PI3K-mTOR pathway contained evolutionarily conserved let-7 binding sites in their respective 3’UTRs (Figures 4A and 4B). Next, we performed 3’UTR luciferase reporter assays to determine if these genes were bona fide and direct targets of let-7. To do this, we generated luciferase reporters with twelve human 3’UTR fragments containing conserved let-7 sites. Luciferase reporter expression in human HEK293T cells after transfection of mature let-7 duplex normalized to negative control miRNA. Shown also are mutations in the seed sequence of the let-7 binding sites for INSR, IGF1R and IRS2. Western blot analysis of Lin28a, Irs2, and tubulin in C2C12 myoblasts with and without Lin28a overexpression.

(E) Western blot analysis of Lin28B, total IRS2, INSR and TUBULIN in HEK293T cells with either let-7 transfection or shRNA knockdown of LIN28B. Error bars represent SEM. *p < 0.05, **p < 0.01.

**Figure 4. Lin28a/b and let-7 Regulate Genes in the Insulin-PI3K-mTOR Pathway**

(A) Shown are the numbers of conserved let-7 binding sites within 3’UTRs found using the TargetScan algorithm.

(B) Putative let-7 binding sites in 16 genes of the insulin-PI3K-mTOR pathway and in Lin28a/b.

(C) 3’UTR luciferase reporter assays performed to determine functional let-7 binding sites. Bar graphs show relative luciferase reporter expression in human HEK293T cells after transfection of mature let-7 duplex normalized to negative control miRNA. Shown also are mutations in the seed sequence of the let-7 binding sites for INSR, IGF1R and IRS2.

(D) Western blot analysis of Lin28a, Irs2, and tubulin in C2C12 myoblasts with and without Lin28a overexpression.

(E) Western blot analysis of LIN28B, total IRS2, INSR and TUBULIN in HEK293T cells with either let-7 transfection or shRNA knockdown of LIN28B. Error bars represent SEM. *p < 0.05, **p < 0.01.
regulatory mechanisms hold in both mouse and human cells, and in the setting of both LIN28B gain and loss of function (Figure 4E). This establishes a direct mechanism for let-7’s repression and Lin28’s derepression of multiple components in the insulin-PI3K-mTOR signaling cascade.

Previously, Lin28a has been shown to enhance Igf2 translation independently of let-7 (Poleskaya et al., 2007), offering an alternative mechanism by which Lin28a might activate the insulin-PI3K-mTOR pathway. To determine the relative contribution of this mechanism, we performed in vitro and in vivo loss of function experiments. Following siRNA knockdown of Igf2 in C2C12 (the efficacy of knockdown is shown in Figure S4B), we found only minimal changes in S6 and 4EBP1 phosphorylation (Figure S4C). In these C2C12 myotubes, glucose uptake was unaffected by Igf2 knockdown, but significantly decreased by let-7a (Figure S4D). In addition, we crossed the Lin28a Tg mice with Igf2 knockout mice and found that the absence of Igf2 did not abrogate enhanced glucose uptake, insulin sensitivity, or the anti-obesity effect mediated by Lin28a (Figure S4E–H). Taken together, these data indicate that the metabolic phenotypes we have observed are not solely due to the ability of Lin28a/b to promote translation of Igf2 mRNA, but do not rule out the possibility that Lin28a/b might modulate other mRNAs in the insulin-PI3K-mTOR signaling pathway.

**mTOR Mediates Lin28a’s Enhancement of Growth and Glucose Metabolism In Vivo**

Given that Lin28a activates the insulin-PI3K-mTOR pathway both in vitro and in vivo, we asked whether the metabolic effects of Lin28a in vivo could be abrogated by pharmacological inhibition of the mTOR pathway. To do this, we injected Lin28a Tg and wild-type littermates with rapamycin 3 times per week beginning when mice were 18 days old. Rapamycin abrogated the growth enhancement in Lin28a Tg mice at doses that had minimal growth suppressive effects on wild-type mice (Figures 5A and 5B), suggesting that Lin28a promotes growth in an mTOR-dependent manner. Selective suppression of Lin28a-driven growth was observed using several parameters: weight (Figures 5B and 5C), crown-rump length (Figure 5D), and tail width (Figure 5E). We also tested if the enhanced glucose uptake phenotype in vivo was likewise dependent on mTOR. Indeed, glucose tolerance testing showed that short-term rapamycin reversed the enhanced glucose uptake effect of Lin28a (Figures 5F and 5G) and reduced the insulin-sensitivity of Lin28a Tg mice to wild-type levels (Figures 5H and 5I). These data indicate that the glucose uptake, insulin sensitivity, and animal growth phenotypes of Lin28a overexpression in vivo are dependent on mTOR signaling.

**let-7 Target Genes Are Associated with Type 2 Diabetes in Human GWAS**

Finally, we sought to assess the relevance of the Lin28/let-7 pathway to human disease and metabolism, using human genetic studies of T2D and fasting glucose levels. Because the Lin28/let-7 pathway has not been previously implicated in T2D, we first asked whether any of the genes that lie in T2D association regions identified in T2D GWAS and meta-analyses (Voight et al., 2010) are known or predicted let-7 targets. We used TargetScan 5.1 to computationally predict let-7 targets (Grimson et al., 2007), and found that 14 predicted let-7 target genes lie in linkage disequilibrium to 39 validated common variant associations with T2D, including IGFB2BP2, HMGAA2, KCNJ11 and DUSP9 (strength of T2D association signals p < 4 x 10^{-3}) (Table 1). Of the computationally predicted let-7 targets associated with T2D, IGFB2BP1/2/3 and Hmga2 have been verified as let-7 targets in several studies (Boyerinas et al., 2008; Mayr et al., 2007). To validate the connection between Lin28 and GWAS candidate genes, we analyzed the expression of Igf2bp and Hmga family members in C2C12 cells with and without Lin28a overexpression, and observed increases in Igf2bp1, Igf2bp2, and Hmga2 mRNA following Lin28a overexpression (Figure 6A). To ensure that this was not a C2C12- or muscle-specific phenomenon, we confirmed the upregulation of these genes in 3T3 cells following human LIN28A or LIN28B overexpression on the mRNA (Figure 6B) and the protein level for the Igf2bp family (Figure 6C). We also observed increased expression of Igf2bp2 and Igf2bp3 (Figure 6D) in Lin28a Tg muscle, confirming this link in vivo.

We next asked whether there is a more widespread connection between T2D susceptibility and let-7 targets, in addition to the targets in validated T2D association regions (p < 5 x 10^{-8}). To address this, we applied a computational method called MAGENTA (Meta-Analysis Gene-set Enrichment of variAN Associations) (Segrè et al., 2010) to GWAS meta-analyses of T2D and fasting glucose blood levels, and tested whether the distributions of disease or trait associations in predefined let-7 target gene sets are skewed toward highly ranked associations (including ones not yet reaching a level of genome-wide significance) compared to matched gene sets randomly sampled from the genome (Table 1). We tested three types of let-7 target definitions with increasing levels of target validation, from in silico predicted let-7 targets using TargetScan 5.1 (Grimson et al., 2007) to experimentally defined targets. For the latter, we used (i) a set of genes with at least one let-7 site in their 3’ UTR and whose mRNA was downregulated by let-7b overexpression in primary human fibroblasts (Legesse-Miller et al., 2009), and (ii) a set of genes whose protein levels were most strongly downregulated by let-7b overexpression in HeLa cells (Selbach et al., 2008). We first tested the let-7 target sets against the latest T2D meta-analysis of eight GWAS (called DIAGRAM+) (Voight et al., 2010), and found significant enrichment (Table 1). The enrichment rose from 1.05-fold for the broadest definition of let-7 targets predicted using TargetScan (~1800 genes; p = 0.036) to 1.92-fold for the experimentally validated target set based on protein level changes in response to let-7 overexpression (~100 genes; p = 1 x 10^{-6}). In the latter case, an excess of about 20 genes regulated by let-7 at the protein level are predicted to contain novel SNP associations with T2D. Notably IGFB2BP2, which is a canonical let-7 target that lies in a validated T2D association locus, was found in all types of let-7 target definitions in Table 1. Furthermore, the genes driving the T2D enrichment signals for the different let-7 target sets include both functionally redundant homologs of T2D-associated genes, such as IGFB2BP1 (IGFB2BP2), HMGAA1 (HMGAA2), DUSP12 and DUSP16 (DUSP9), and genes in the insulin-PI3K-mTOR pathway, including IRS2, INSR, AKT2 and TSC1 (best local SNP association p = 10^{-4} to 4x10^{-3}).
We next tested for enrichment of let-7 target gene associations with fasting glucose levels, using data from the MAGIC (Meta-Analysis of Glucose and Insulin-related traits Consortium) study of fasting glucose levels (Dupuis et al., 2010). We observed an over-representation of multiple genes modestly associated with fasting glucose at different levels of significance for the different let-7 target gene sets (Table 1). The strongest enrichment was found in the genes downregulated at the mRNA level by let-7, an enrichment of 1.20 fold over expectation ($p = 1 \times 10^{-7}$). Taken together, our human genetic results support the hypothesis that genes regulated by let-7 influence human metabolic disease and glucose metabolism.

Recently it has also become clear that Lin28a/b has important let-7-independent roles in RNA metabolism, as evidenced by numerous direct mRNA targets whose translation is enhanced by LIN28A (Peng et al., 2011). Using GSEA, we found that this list of direct mRNA targets is also significantly enriched for glucose, insulin and diabetes-related genes (Table S1). Thus, Lin28a/b may regulate metabolism through direct mRNA-binding as well as let-7 targets.

**DISCUSSION**

**Lin28 and let-7 Are Mutually Antagonistic Regulators of Growth and Metabolism**

Our work defines a new mechanism of RNA-mediated metabolic regulation. In mice, Lin28a and LIN28B overexpression results in insulin sensitivity, enhanced glucose tolerance, and resistance...
to diabetes. Our analysis of \textit{iLet-7} Tg mice shows that \textit{let-7} upregulation is also sufficient to inhibit normal glucose metabolism, supporting the idea that gain of \textit{Lin28a/b} exerts effects on whole animal glucose metabolism at least in part through \textit{let-7} suppression. Previously, we showed that transgenic overexpression of \textit{Lin28a} causes enhanced growth and delayed puberty, phenotypes that mimicked human traits linked to genetic variation in the \textit{Lin28/let-7} pathway in GWAS (Zhu et al., 2010). The statistical enrichment for genes associated with T2D and fasting glucose among \textit{let-7} targets using the MAGENTA algorithm. The TargetScan algorithm was used to define the “All human \textit{let-7} targets” and the “Conserved \textit{let-7} targets” gene sets (http://www.targetscan.org/). mRNA downregulation following \textit{let-7} overexpression (OE) was measured in primary human fibroblasts (Legesse-Miller et al., 2009), and protein downregulation following \textit{let-7} OE was measured in HeLa cells (Selbach et al., 2008). The enrichment cutoff used is the 75th percentile of all gene association scores in the genome. The enrichment fold is the ratio between the observed and expected number of genes above the enrichment cutoff. Genes linked to validated GWAS SNPs (39 SNPs for T2D and 14 SNPs for fasting glucose) were ordered according to the number of target gene sets they appear in and then alphabetically. *The following genes were removed from the analysis: (i) genes absent from the full human gene list used in the analysis, (ii) genes that had no SNPs within 110 kb upstream or 40 kb downstream to their most extreme transcript boundaries, or (iii) to correct for potential inflation of enrichment due to physical proximity of \textit{let-7} target genes along the genome, subsets of proximal genes assigned the same best local SNP were collapsed to one gene and assigned the score of the most significant gene p-value in that subset. *gene sets that pass a Bonferroni corrected cutoff (p < 0.004).
et al., 2010). Given that Lin28a/b is downregulated in most tissues after embryogenesis, while let-7 increases in adult tissues, lingering questions from our earlier report were first, whether let-7 was sufficient to influence organismal growth, and second, what function does let-7 have in adult physiology? Our observation that the iLin28a, iLIN28B, and iLet-7 Tg gain of function mice, as well as muscle-specific Lin28a loss of function mice manifest complementary phenotypes supports the notion that Lin28a/b and let-7 are both regulators of growth and developmental maturation. We propose that different developmental time-points demand distinct metabolic needs, and that global regulators such as Lin28 temporally coordinate growth with metabolism. The dynamic relationship between Lin28, let-7 and metabolic states during major growth milestones in mammals is reminiscent of the heterochronic mutant phenotypes originally defined in C. elegans (Ambros and Horvitz, 1984; Moss et al., 1997; Boehm and Slack, 2005), and suggests that metabolism, like differentiation, is temporally controlled.

**Lin28a/b and let-7 Influence Glucose Metabolism through the Insulin-PI3K-mTOR Pathway**

We have shown that Lin28a/b and let-7 regulates insulin-PI3K-mTOR signaling, a highly conserved pathway that regulates growth and glucose metabolism throughout evolution. PI3K/Akt signaling is known to promote Glut4 translocation to upregulate glucose uptake, while mTOR signaling can promote glucose uptake and glycolysis by changing gene expression independently of Glut4 translocation (Brugarolas et al., 2003; Buller et al., 2008; Duvel et al., 2010). Previous studies have shown that Lin28a directly promotes Igf2 (Polesskaya et al., 2007) and HMGA1 translation (Peng et al., 2011), and that let-7 suppresses IGF1R translation in hepatocellular carcinoma cells (Wang et al., 2010). Consistent with these findings, our results define a model whereby Lin28a/b and let-7 coordinately regulate the insulin-PI3K-mTOR pathway at multiple points (Figure 6E), a concept that is consistent with the hypotheses that miRNAs and RNA binding proteins regulate signaling pathways by tuning the production of a broad array of proteins rather than switching single components on or off (Kennell et al., 2008; Hatley et al., 2010; Small and Olson, 2011). Coordinated regulation is important because negative feedback loops exist within the insulin-PI3K-mTOR pathway. Loss-of-function and pharmacological inhibition studies have shown that the mTOR target S6K1, for instance, inhibits and desensitizes insulin-PI3K signaling by phosphorylating IRS1 protein and suppressing IRS1 gene transcription (Harrington et al., 2004; Shah et al., 2004; Tremblay et al., 2007; Um et al., 2004). Conversely, TSC1-2 promotes insulin-PI3K signaling by suppressing mTOR signaling (Harrington et al., 2004; Shah et al., 2004). Although the effects of let-7 and Lin28a/b on the expression of individual genes are modest, simultaneous regulation of multiple components such as IGF2, IGF1R, INS1, IRS2, PIK3IP1, AKT2, TSC1, RICTOR in the

**Figure 6. let-7 Target Genes Are Associated with Type 2 Diabetes Mellitus and a Model of the Lin28/let-7 Pathway in Glucose Metabolism**

mRNA expression of Igf2bp and Hmga family members in (A) C2C12 with and without Lin28a overexpression and in (B) 3T3 cells with and without LIN28A or LIN28B overexpression. (C) Western blot of NIH 3T3 cells with Lin28a overexpression showing Lin28a and Igf2bp1/2/3 protein levels (n = 3 biological replicates). (D) Igf2bp2 and Igf2bp3 mRNA in Lin28a Tg muscle. (E) Model of Lin28/let-7 pathway in glucose metabolism. Error bars represent SEM. *p < 0.05, **p < 0.01.
insulin–PI3K–mTOR signaling pathway could explain how this RNA processing pathway coordinates regulation of insulin sensitivity and glucose metabolism by effectively bypassing these negative feedback loops.

Whereas our work has implicated let-7 as a regulator of insulin–PI3K–mTOR signaling, we do not exclude a parallel role for direct mRNA targets of Lin28a/b in glucose metabolism, a hypothesis supported by the recent findings that HMGA1 is translationally regulated by Lin28a and mutated in 5%–10% of T2D patients (Peng et al., 2011; Chiefari et al., 2011). Such non-let-7 functions are also suggested by the fact that muscle-specific loss of Lin28a results in glucose derangement without significant let-7 changes. Nevertheless, it remains likely that during other developmental stages or in other tissues, let-7 suppression by Lin28a or Lin28b is required for normal glucose homeostasis. The effects of the Lin28/let-7 pathway on glucose metabolism in murine models, together with our observation that genes regulated by let-7 are associated with T2D risk in humans, indicates important functional roles for both Lin28a/b and let-7 in human metabolism.

**let-7 Targets Are Relevant to Disparate Human Diseases: Cancer and T2D**

Metabolic reprogramming in malignancy is thought to promote a tumor’s ability to produce biomass and tolerate stress in the face of uncertain nutrient supplies (Vander Heiden et al., 2009). During their rapid growth phase early in development, embryos may utilize similar programs to maintain a growth-permissive metabolism. Dissecting the genetic underpinnings of embryonic metabolism would likely provide important insights into the nutrient uptake programs that are co-opted in cancer. While loss of function studies in the early embryo would help define the metabolic roles of oncofetal genes in their physiologic context, classical in vivo metabolic assays are difficult to perform in embryos. Lin28a and Lin28b are oncofetal genes, and thus highly expressed in early embryogenesis and then silenced in most adult tissues, but reactivated in cancer (Yang and Moss, 2003; Viswanathan et al., 2009). Cancer cells may utilize the embryonic function of Lin28a/b to drive a metabolic shift toward increased glucose uptake and glycolysis—a phenomenon termed the “Warburg effect.” Previously, we showed that Lin28a expression promotes glycolytic metabolism in muscle in vivo and in C2C12 myoblasts in vitro (Zhu et al., 2010). Though we cannot yet readily determine the metabolic effects of shutting off Lin28a/b within the embryo, we have dissected the potent effects of reactivating and inactivating this oncofetal program in adults. Conversely, in normal adult tissues that do not express high levels of Lin28a or Lin28b, one might ask if a role for the highly abundant let-7 is to lock cells into the metabolism of terminally differentiated cells to prevent aberrant reactivation of embryonic metabolic programs. Further studies are required to understand how this pathway may link mechanisms of tumorigenesis and diabetogenesis.

Our report implicates Lin28a/b and let-7 as important modulators of glucose metabolism through interactions with the insulin–PI3K–mTOR pathway and T2D-associated genes identified in GWAS. Although it is likely that additional mechanisms and feedback loops exist, our data suggests a model whereby Lin28a/b and let-7 coordinate the GWAS identified genes and the insulin–PI3K–mTOR pathway to regulate glucose metabolism (Figure 6E). It also suggests that enhancing Lin28 function or abrogating let-7 may be therapeutically promising for diseases like obesity and diabetes. Likewise, results from this work might shed light on the physiology of aging and, specifically, how the accumulation of let-7 in aging tissues may contribute to the systemic insulin resistance that accompanies aging.

**EXPERIMENTAL PROCEDURES**

**Mice**

All animal procedures were based on animal care guidelines approved by the Institutional Animal Care and Use Committee. Mouse lines used in this study are described in the Extended Experimental Procedures and Figure S5.

**Indirect Calorimetry**

The apparatus used was a set of 16 Oxymax® Metabolic Activity Monitoring chambers (Columbus Instruments; Columbus, OH, USA). Each chamber consisted of a self-contained unit capable of providing continuous measurements of an individual mouse’s total activity and feeding behavior. Monitoring occurred over a 3-day period. Each subject was placed into an individual chamber on day 1, with free access to food and water during the course of the experiment. Subjects were maintained under a normal 12:12 hr light:dark cycle. All measurements were sampled periodically (at approximately 12 min intervals) and automatically recorded via the OXymax Windows V3.22 software. Activity measures over the final 24 hr period were parceled into 2-h bins and these were used to express diurnal activity levels.

**Quantitative RT–PCR**

Performed with standard methods, which are described in detail in the Extended Experimental Procedures.

**Histology**

Tissue samples were fixed in 10% buffered formalin or Bouin’s solution and embedded in paraffin.

**Glucose and Insulin Tolerance Tests**

Overnight-fasted mice were given i.p. glucose (2 mg/g body weight). For insulin tolerance test, 5 hr fasted mice were given 0.75 U insulin/kg body weight by i.p. injection (Humulin). Blood glucose was determined with a Lifescan One Touch glucometer. Insulin levels were measured by ELISA (Crystal Chem).

**Cloning**

Murine Lin28a and human LIN28B cDNA was subcloned into pBabe.Puro and pMSCV.Neo retroviral vectors. LIN28B and Control shRNA in lentiviral plasmids were purchased from Sigma-Aldrich and previously reported in Viswanathan et al., 2009. UTR cloning for luciferase reporters is described in Table S2.

**Cell Culture, Viral Production, and Transfection**

Performed using standard methods as described in the Extended Experimental Procedures.

**Glucose Uptake Assay**

In vitro glucose uptake assays were performed as described in Berti and Gammeltoft, 1999.

**Drug Treatments**

Rapamycin was injected i.p. 3 times a week for mouse experiments. For cell culture, C2C12 myotubes differentiated for 3 days were incubated with inhibitors for 1 day prior to glucose uptake assays. See Extended Experimental Procedures for further details.
Western Blot Assay
Performed using standard methods. Detailed methods and reagents used are described in the Extended Experimental Procedures.

Luciferase Reporter Assay
10 ng of each construct was co-transfected with 10 nM miRNA duplexes into HEK293T cells in a 96-well plate using lipofectamin-2000 (Invitrogen). After 48 hr, the cell extract was obtained; firefly and Renilla luciferase activities were measured with the Promega Dual-Luciferase® reporter system.

MAGENTA Analysis
See Results, Table 1 Legend, and Extended Experimental Procedures for detailed methods.

Statistical Analysis
Data is presented as mean ± SEM, and Student’s t test (two-tailed distribution, two-sample unequal variance) was used to calculate p values. Statistical significance is displayed as p < 0.05 (one asterisk) or p < 0.01 (two asterisks). The tests were performed using Microsoft Excel where the test type is always set to two-sample equal variance.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, a list of DIAGRAM and MAGIC consorsia members with affiliations, and five figures and can be found with this article online at doi:10.1016/j.cell.2011.08.033.

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