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Glucose-based regulation of miR-451/AMPK signaling depends on the OCT1 transcription factor

Khairul I. Ansari[1,*], **Daisuke Ogawa**[1,2,*], **Arun K. Rooj**[1], **Sean E. Lawler**[1], **Anna M. Krichevsky**[3], **Mark D. Johnson**[1], **E. Antonio Chiocca**[1], **Agnieszka Bronisz**[1,‡], and **Jakub Godlewski**[1,‡]

[1]Harvey Cushing Neuro-oncology Laboratories, Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

[2]Department of Neurological Surgery, Kagawa University Hospital, Miki-cho, Kagawa, 761-0793, Japan

[3]Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

SUMMARY

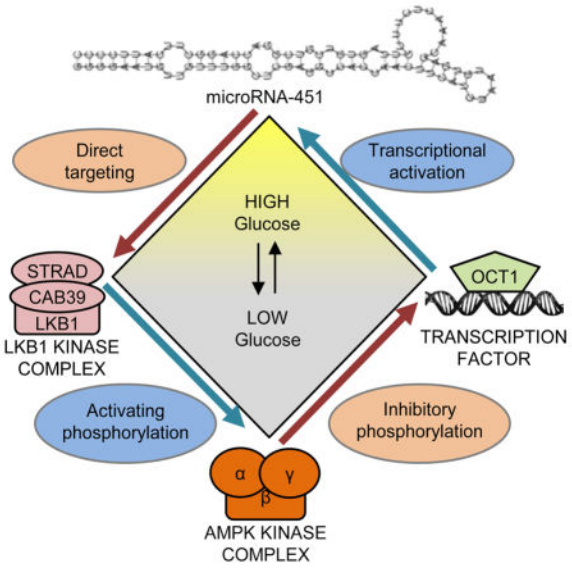
In aggressive, rapidly growing solid tumors such as glioblastoma multiforme (GBM), cancer cells face frequent dynamic changes in their microenvironment including the availability of glucose and other nutrients. These challenges require that tumor cells have the ability to adapt in order to survive periods of nutrient/energy starvation. We have identified a reciprocal negative feedback loop mechanism in which the levels of microRNA-451 (miR-451) are negatively regulated through the phosphorylation and inactivation of its direct transcriptional activator OCT1 by AMPK, which is activated by glucose depletion-induced metabolic stress. Conversely, in a glucose rich environment, unrestrained expression of miR-451 suppresses AMPK pathway activity. These findings uncover miR-451 as a major effector of glucose-regulated AMPK signaling, allowing tumor cell adaptation to variations in nutrient availability in the tumor microenvironment.

Abstract

‡Correspondence: jgodlewski@partners.org, abronisz@partners.org.
*These authors contributed equally to this manuscript.

AUTHOR CONTRIBUTIONS

All authors assisted in drafting and revising the work, approved the final version for publication, and agree to be accountable for all aspects of the work. Individual contributions are as follows: K.I.A. conceived and designed overall work and acquired and analyzed data. D.O. conceived and designed overall work and acquired data. A.K.R. assisted with acquiring data. S.E.L. assisted with writing the manuscript, analysis and interpretation of data. A.M.K. assisted with analysis and interpretation of data. M.D.J. assisted with analysis and interpretation of data. E.A.C. assisted with writing the manuscript, analysis and interpretation of data. A.B. conceived and designed overall work and analyzed and interpreted data. J.G. conceived and designed overall work and analyzed and interpreted data.



INTRODUCTION

GBM is one of the most lethal cancers, with a median survival of 14.6 months with the current standard of care (Stupp et al., 2005). GBM displays a high proliferative index sustained by modifications in blood supply that result in dynamic microenvironmental fluctuations in the availability of oxygen, glucose and other nutrients. This requires that tumor cells can rapidly adapt to survive periods of nutrient starvation. GBM cells are also highly dependent on elevated glucose uptake (Flavahan et al., 2013). Adaptation to metabolic stress in cancer requires transient cellular alterations controlled by changes in transcriptional activity (Dhruv et al., 2013; Horing et al., 2012). Uncovering the molecular circuitry by which alterations in glucose metabolism allow for cancer cell adaptation may provide new insights into cancer pathogenesis (Ward and Thompson, 2012a, b).

AMP-activated kinase (AMPK) is a critical cellular energy sensor. Inadequate energy supply results in a conformational change induced by AMP, allowing activation of AMPK by the LKB1 complex. Once activated, AMPK promotes cell survival by increasing catabolic processes while conserving ATP by switching off anabolic pathways (Hardie et al., 2012).

MicroRNAs are short, non-coding RNA molecules capable of regulating the levels of multiple proteins *via* binding to specific mRNA targets, thereby suppressing their translation and de-regulation of microRNAs has been described in multiple human malignancies (Iorio and Croce, 2012), including GBM (Godlewski et al., 2010b). We previously demonstrated that miR-451 expressed in GBM cells blocks migration and acts as a potent inhibitor of AMPK signaling by targeting components of LKB1 kinase complex as well as numerous downstream effectors. We also demonstrated that miR-451 levels decrease in low glucose, resulting in AMPK activation and increased cell migration (Godlewski et al., 2010a; Godlewski et al., 2010c).

In this paper we report that miR-451 transcription in GBM cells is driven by the transcription factor OCT1 (official gene symbol POU2F1), and that AMPK activation by glucose deprivation leads to inactivation of OCT1 *via* direct phosphorylation at serine 335, which leads to inhibition of miR-451 transcription. Inhibition of miR-451 in turn results in sustained AMPK activation, and a robust response to glucose deprivation in GBM cells. Conversely, in the presence of abundant glucose, unrestricted activity of OCT1 drives transcription of miR-451 leading to AMPK inhibition *via* direct targeting of CAB39 – a component of the LKB1 complex (Godlewski et al., 2010c). This study highlights the role of an AMPK/miR-451 reciprocal feedback loop in the adaptation of GBM cells to metabolic stress.

MATERIALS AND METHODS

For standard experimental procedures (cell culture, antibodies, real-time PCR, Western blotting, siRNA transfections, luciferase reporter assays, chromatin immunoprecipitation (ChIP), expression of truncated OCT1 see Supplemental Experimental Procedures.

Oct1-deficient and AMPKβ1/2-deficient cell lines

3T3 *Oct1*$^{-/-}$ mouse fibroblasts and T98G GBM cells stably expressing AMPKβ1/2-specific shRNA lentiviral constructs were obtained from the laboratories of Drs. Dean Tantin (University of Utah) and Biplab Dasgupta (Cincinnati Children's Hospital Medical Center), respectively.

Kinase Assays

Wild type (WT) GST-OCT1 or GST-OCT1-S3353A peptides were dialyzed overnight using HEPES buffer pH 7.0 (62.5 mM Na HEPES, pH 7.0; 62.5 mM NaCl; 62.5 mM NaF; 6.25 mM Na pyrophosphate; 1.25 mM EDTA, 1.25 mM EGTA; 1mM dithiothreitol, 1mM PMSF; 2mM sodium orthovanadate, 10μg/mL aprotinin; 10μg/mL leupeptin and 10μg/mL pepstatin A). For kinase assays, 100 μg GST-OCT1 WT or GST-OCT1-S3353A were incubated in the presence of 100 μg AMPK-IP or 100 ng recombinant AMPK complex containing phospho-AMPKα1, AMPKβ1 and AMPKγ1 in the presence of 0.2mM ATP for 1h at 30 ºC following suppliers' instructions. The phosphorylation of OCT1 was analyzed by Western blotting using anti-phospho-OCT1 (S335) (a gift from Dean Tantin, University of Utah) and OCT1. Anti-GST was used as a loading control.

RESULTS

Glucose regulates miR-451 expression

To examine AMPK/miR-451 signaling in response to glucose deprivation, we measured the levels of primary miR-451 (pri-miR-451) in a panel of GBM cells cultured in 0.3g/L for 16–18h ("low glucose" conditions), which is considered physiologically relevant range (Flavahan et al., 2013). This led to a significant decline in pri-miR-451 levels compared to high glucose (4.5g/L) conditions, in all GBM cell lines. This was also seen in 3T3, but not in HeLa cells with defective AMPK signaling (Lizcano et al., 2004) (Figure 1A). When GBM cells were cultured in high glucose without replenishment, glucose was depleted from

4.5g/L to less than 1.0g/L in 72h and this was associated with a decrease in pri-miR-451 levels (Figure 1B). GBM cells were cultured in three different regimens: continuously high glucose (medium replenished every 12h), continuously low glucose, and low glucose replenished at 24h with a pulse of high glucose medium. In high glucose the expression of pri-miR-451 remained high, while in low glucose it significantly dropped. In the third group, pri-miR-451 increased after the glucose pulse and then levels gradually decreased (Figure 1C). The gradual decrease of pri-miR-451 in cells switched to low glucose, (Figure S1A) was closely mirrored by mature miR-451, suggesting transcriptional rather than post-transcriptional regulation (Figure S1B–D). This effect was specific for miR-451, as levels of other microRNAs were not significantly altered in low glucose (Figure S1E). Interestingly, the introduction of exogenous LKB1 into HeLa cells, in which the expression of pri-miR-451 is comparable to GBM cells (Figure S1F), resulted in rescuing glucose-sensitive response in miR-451 expression concomitant with low glucose induced phosphorylation of AMPKα and OCT1 (Figure S1G). These results indicated that the expression of miR-451 is inherently linked to glucose levels and that its expression is most likely controlled transcriptionally.

The OCT1 transcription factor drives the expression of miR-451

MiR-451 is encoded in a single intergenic locus at Ch.17 q11.2. We screened 10kb upstream of the miR-451 locus for putative transcription factor binding sites using Tfsitescan (Farre et al., 2003). In addition to previously described binding sites GATA-1 and AP-1 (Cheng et al., 2013; Dore et al., 2008) we identified several potential binding sites for OCT1 (Figure 2A, Figure S2A); a transcription factor which genetic knockout promotes adaptation to low glucose (Shakya et al., 2009). We thus hypothesized that OCT1 regulates transcription of miR-451 based on glucose availability. Series of reporter constructs with OCT1 binding sites were engineered (Figure 2A) and co-expression of the clone 3 (C3) and clone 4 (C4) fragments (or both, clone 5 - C5) with exogenous OCT1 led to significant induction of transcriptional activity in 3T3 *Oct1*$^{-/-}$ cells (Figure 2B), while the C1 and/or C2 constructs had no effect. Phosphorylation of OCT1 at S335 blocks its activity by preventing DNA binding (Kang et al., 2011). OCT1 S335A and S335D phospho-site mutants were tested and transcriptional activity of the constitutively active S335A mutant was similar to wild type, while that of the inactive S335D mutant was negligible (Figure 2B, Figure S2B). This indicates that the predicted OCT1 binding sites in the miR-451 promoter are functional.

ChIP assays were used to determine if endogenous OCT1 was recruited to the miR-451 gene promoter in a glucose-dependent manner. Soluble chromatin from U87 cells was isolated after culturing for 18h in low or high glucose and then immunoprecipitated with an OCT1 antibody. There was minor enrichment of OCT1 in the C1 and C2 regions irrespectively of glucose regimen. In contrast, OCT1 was enriched several-fold at the C3 and C4 regions in high glucose, while in low glucose this enrichment was significantly reduced (Figure 2C, D). RNA Polymerase II recruitment to the miR-451 promoter mirrored OCT1 recruitment (Figure 2E).

OCT1 activity inversely correlates with AMPK activation

Knockdown studies were performed to further confirm the role of OCT1 in miR-451expression. OCT1 siRNA transfection reduced pri-miR-451 levels by ~2-fold in GBM cells in high glucose (Figure 3A). Mouse *Oct1*$^{-/-}$ fibroblasts cultured in high glucose were characterized by remarkably low levels of pri-miR-451 when compared to wild type (Figure 3B, S1F). When OCT1 was transiently overexpressed in GBM cells, levels of pri-miR-451 were induced ~4-fold (Figure 3C). Levels of mature miR-451 in OCT1-depleted or overexpressing cells mirrored those of the primary transcript (Figure S2C–E). When either wild type or the constitutively active S335A mutant OCT1 was transiently re-introduced into *Oct1*$^{-/-}$ mouse fibroblasts, miR-451 was significantly induced, while the introduction of the OCT1 S335D mutant construct had no effect. Interestingly, only in cells with re-introduced wild type OCT1 the expression of miR-451 was glucose-dependent, suggesting the potential importance of the S335 residue in this context (Figure 3D, S2F–G).

These results proved glucose-dependent binding of OCT1 to the promoter region of the miR-451, indicating that high glucose led to OCT1 transcriptional activation of miR-451. They also showed the role of S335 phosphorylation in the activity of OCT1. OCT1 and AMPKα in GBM cells are predominantly nuclear (75–90%) irrespective of glucose levels, suggesting that subcellular distribution is not responsible for OCT1 regulation (Figure 3E). The phosphorylation of S335 was significantly increased in low glucose in two GBM cell lines (Figure 3F) and in wild type, but not in *Oct1*$^{-/-}$ mouse fibroblasts, as expected (Figure 3G). Also as expected, in all experiments, the phosphorylation of AMPK at T172 was significantly increased in low glucose (Figure 3F–G). Additionally, low miR-451 in *Oct1*$^{-/-}$ mouse fibroblasts correlated with higher levels of CAB39 protein, resulting in higher levels of LKB1 complex and moderately, but significantly stronger activation of AMPK in low glucose conditions (Figure S2H).

Based on this, and on the fact that S335 in OCT1 has the highest score of all predicted AMPK phosphorylation sites (Xue et al., 2006), we hypothesized that AMPK may be the kinase involved, thereby providing a direct link between AMPK and transcription of miR-451. Transient silencing of AMPK α1 and α2 subunits, both separately and in combination using siRNA led to induction of miR-451 expression in low glucose, and silencing of both subunits led to a moderate additive effect, suggesting a partially redundant function of the two subunits (Figure 3H). We did not observe induction of miR-451 upon AMPK α1/2 knock-down in high glucose, which suggested that only low glucose-activated AMPK can suppress the OCT1/miR-451 axis (Figure S2I). These findings thus showed that OCT1 phosphorylation is glucose-dependent and correlates with AMPK activity, suggesting that AMPK may be responsible for the inactivating phosphorylation of OCT1.

AMPK directly phosphorylates OCT1, thus inhibiting transcription of miR-451

We used AMPK β1/2 double deficient T98 GBM cells (Liu et al., 2014a; Liu et al., 2014b), which when exposed to low glucose, cannot fully activate AMPK (measured by the level of AMPK a1/2 phosphorylation at T172) (Figure 4A). We found that in these cells the phosphorylation of OCT1 at S335 was unaltered in low glucose, in sharp contrast to cells with fully functional AMPK (Figures 4A, 3F–G). Likewise, the expression of pri-miR-451

was only partially suppressed in AMPK β1/2$^{-/-}$ cells while strong suppression occurred in cells with a functional AMPK pathway in low glucose (Figures 4B, 1A, S3A). These results demonstrated that expression of miR-451 inversely correlates with activated AMPK.

Inhibition of AMPK activity by the small molecule inhibitor Compound C (Liu et al., 2014a) resulted in the reduction of OCT1 S335 phosphorylation (Figure S3B) with a concurrent increase in miR-451 levels, regardless of glucose status. This suggests that AMPK activity (confirmed by phosphorylation of its direct target ACC), rather than glucose availability, is the major mechanism of miR-451 suppression (Figure S3C). To determine the role of AMPK in phosphorylation of OCT1 at S335, we used two *in vitro* kinase assays. First, the AMPK complex was immunoprecipitated from low glucose-cultured GBM cells overexpressing Flag-tagged AMPKβ1. Under low glucose, both AMPKa subunits underwent phosphorylation, demonstrating the functionality of the immunoprecipitated complex (Figure S3D–F). Co-incubation of this AMPK complex with the GST-tagged peptide encompassing the S355 OCT1 phosphorylation site resulted in phosphorylation of the wild type but not of the S335A mutant site (Figure 4C). Then the purified recombinant active AMPK complex (SignalChem) was co-incubated with GST-tagged OCT1 peptide, leading to the same result (Figure 4D). This demonstrates that AMPK is sufficient in phosphorylating OCT1 at S335, thus establishing AMPK as the kinase responsible in shutting off OCT1-mediated transcription of miR-451 in response to glucose availability.

We have previously shown that overexpression of miR-451 stimulated cell growth in high glucose (Godlewski et al., 2010c). As shown in this study, in high glucose OCT1 remains unphosphorylated (Figure 3F), which prompted us to test whether the presence and phosphorylation status of OCT1 affects cell growth. We used AMPK-defective GBM cells and an OCT1 overexpression strategy (Figure S4A) and found that overexpression of wild type and phosphodeficient S335A mutant, but not phosphomimetic S335D mutant, increased cell proliferation (Figure S4B). This correlated with the levels of pri-miR-451 in these cells (Figure S4C). This led us to ask whether the expression of miR-451 in glioblastoma cells affects their growth in insufficient glucose. We used a time-dependent glucose depletion model (**see** Figure 1B) and found that cells with dysfunctional AMPK activation by miR-451 overexpression were characterized by faster growth rate as long as glucose remained sufficient (Figure S4D). An *in vivo* flank xenograft study demonstrated moderately, but significantly enhanced growth of miR-451 overexpressing cells (Figure S4E). To test the relevance of the described model to patient-derived samples, we used a large collection of primary glioblastoma neurospheres (Bronisz et al., 2014) for miR-451 profiling (Figure S4F). We selected cells with the strongest and the weakest expression of miR-451. As demonstrated in Figure S4G–H, the dynamics of AMPK/OCT1 phosphorylation upon glucose starvation were dependent on basal levels of miR-451. This suggests that, in patient-derived primary cells, low miR-451 levels allow faster adaptation to low glucose stress by rapid activation of AMPK.

DISCUSSION

We show that there is a transcriptionally regulated microRNA-based loop that links nutrient availability to activation of the major energy sensor of the cell, AMPK. We demonstrated

that: *i)* glucose-dependent fluctuations in the levels of miR-451 are regulated transcriptionally; *ii)* multiple binding sites for the OCT1 are present upstream of miR-451; *iii)* binding of OCT1 to these binding sites promotes transcription of miR-451 and that such binding is mediated by the phosphorylation of OCT1 at S335; and *iv)* that AMPK kinase complex directly phosphorylates OCT1 at S335 in glucose-dependent manner. These results thus show that the AMPK complex/OCT1/miR-451/LKB-1 complex loop provides a nutrient-dependent regulatory mechanism to allow the cell to adapt to changing microenvironmental conditions.

Previously, we reported that the expression of miR-451 is regulated by glucose availability in GBM cells: miR-451 levels were high in a glucose-rich environment and were low in glucose-depletion. This changed the respective phenotypic behaviors of the cell, from proliferative to migratory (Godlewski et al., 2010a; Godlewski et al., 2010c). We and others have shown that miR-451 is a potent inhibitor of the AMPK signaling pathway, by operating at multiple levels. It directly targets CAB39 – a co-activator of the AMPK upstream kinase (Zeqiraj et al., 2009), LKB1, and 14-3-3 ζ – a functional inhibitor of AMPK-phosphorylated Raptor (Gwinn et al., 2008). MiR-451 also diminishes the motility and migratory behavior of GBM cells. These observations led us to postulate the existence of an AMPK/miR-451 reciprocal negative feedback loop, mediated by glucose availability. The findings of this paper demonstrate the existence and the mechanism responsible for this loop.

The levels of pri-miR-451 are inherently linked to glucose levels. Regardless of the glucose regimen used (withdrawal, gradual depletion and surge replenishment), the levels of primary transcript are linked to glucose status. The glucose-deprivation triggered downregulation of miR-451 seems to be transcriptional (not dependent on processing), as the drop in the level of mature miR-451 was always accompanied with a drop in pri-miR-451. The effect is dynamically regulated and fully reversible, as shown by experiments with glucose fluctuation (Figures 1 and S1).

The analysis of region upstream to the miR-451 locus revealed multiple putative binding sites for OCT1 - a widely expressed transcription factor (Kang et al., 2009). *Oct1*-deficient cells are resistant to glucose deprivation due to reduction of glucose metabolism (Shakya et al., 2009). We have demonstrated dynamic and glucose-dependent binding of OCT1 to its predicted binding sites 6–8 kb upstream of the miR-451 locus, followed by the recruitment of RNA polymerase II. The binding was S335-dependent, as only wild type and constitutively active S335A mutant OCT1 were capable of binding and inducing transcriptional activity, showing that the phosphorylation at S335 is crucial for OCT1 activity (Figures 2 and S2A–B). The abundance of pri-miR-451 depends on the level of OCT1 protein and re-introduction of wild type and constitutively active S335A mutant OCT1 into *Oct1* deficient cells rescued miR-451expression. *Oct1* knockout mice are embryonically lethal due to faulty erythropoiesis (Wang et al., 2004). The latter is of interest, as miR-451 is known as a crucial factor driving erythrocyte maturation in vertebrates (Rasmussen and O'Carroll, 2011). Thus, the exceptionally low levels of miR-451 observed in *Oct1*-deficient cells (Figure 3B, D) provide a plausible explanation for this *in vivo* phenotype. OCT1 becomes inactive in low glucose, suggesting that AMPK may be responsible for its phosphorylation. AMPK knockdown in low glucose resulted in de-

repression of miR-451 expression, while it had no effect in high glucose (Figures 3 and S2C–I). Our results suggest that the AMPK kinase complex phosphorylates OCT1 in low glucose. In AMPK deficient cells OCT1 remained largely de-phosphorylated in low glucose, resulting in the expression of miR-451. However partial reduction of miR-451 in low glucose occurred, most likely due to incomplete AMPK knockdown. We demonstrated by *in vitro* kinase assays that AMPK is directly responsible for OCT1 phosphorylation and that an AMPK inhibitor prevented OCT1 shut down in low glucose (Figure 4 and S3).

OCT1 is dynamically phosphorylated following the exposure of cells to oxidative and genotoxic stress (Kang et al., 2009), which are also known to activate AMPK (Sanli et al., 2014). OCT1 phosphorylated at S335, lacks the ability to bind DNA (Kang et al., 2009). Intriguingly, it was shown that phosphorylation of OCT1 at S335 during M-phase by Nek6 kinase results in its exclusion from mitotic chromatin in LKB/AMPK signaling deficient HeLa cells (Kang et al., 2011). MiR-451 is potent inhibitor of AMPK signaling (Godlewski et al., 2010a), and AMPK-defective cells are characterized by low phospho-OCT1 levels (Figure 4A). On the other hand, miR-451 does not affect M-phase in GBM cells (Godlewski et al., 2010b). Thus the evidence for dynamic phosphorylation of OCT1 upon stress and during cell cycle exists; additionally cell type and kinase availability may modify the phosphorylation of OCT1. It remains to be investigated whether phospho-OCT1 is in fact a mitotic marker in GBM cells in the context of glucose availability.

In GBM cells, high glucose leads to miR-451 activity, the shut-off of AMPK function, and cell proliferation. Conversely, in low glucose AMPK activation occurs, turning on energy-conserving and migratory behavior. Both the energy-conserving metabolic shift and resource-seeking behavioral change require GBM cell to shut down miR-451, while forced expression of miR-451 during stress leads to cytotoxicity (Godlewski et al., 2010c). The downregulation of miR-451 helps GBM cells to escape from metabolically stressful events/locations. MiR-451 thus provides example of a molecule that is not deregulated in GBM, but is instead finely regulated, by promoting or suppressing GBM cell phenotypes based on microenvironmental contexts.

Because AMPK can impede cell growth, it was historically perceived as a *bona fide* tumor suppressor. However recently, a number of studies have emerged that lead to the opposite conclusion, namely that AMPK enables cancer cell survival capabilities under stress (Jeon and Hay, 2012). The AMPK complex endows cancer cells with the ability to survive the stressors, including energy and growth factor deficiency and genomic damage (Sanli et al., 2013). It provides a potent regulatory mechanism by which cancer cells temporarily halt growth upon microenvironmental and therapy-inflicted challenges. Recent studies on the role of AMPK demonstrate increased activity of AMPK in malignant tissue compared with non-malignant tissue *in vivo* and in cancer cells *in vitro* (Li et al., 2012; Park et al., 2009). Activation of AMPK by oncogenic events was demonstrated in rodent models of brain tumors (Jang et al., 2011; Rios et al., 2013). Active AMPK was detected in human GBM and AMPK inhibition, either by small molecule inhibitors or shRNA knockdown, resulted in decreased growth of GBM xenografts (Liu et al., 2014b; Rios et al., 2013). Thus AMPK can be perceived as contextual oncogene enabling cancer cells with behavioral and biochemical flexibility.

In GBMs and other solid tumors, glucose metabolism is elevated; yet rapid tumor growth can cause localized nutrient deprivation. Thus, a potent and rapid response to glucose deficiency could be beneficial to cell survival. Here we were able to demonstrate that primary GBM cells with low levels of miR-451 are capable of rapid and dynamic activation of AMPK in response to glucose deprivation which may lead to enhanced cell survival in the presence of a stressor. Upon sensing dwindling glucose availability, the AMPK complex becomes active and, by direct phosphorylation of OCT1 at S335, prevents its DNA binding/activity resulting in rapidly declining levels of miR-451 (Figure 4E). This, in addition to execution of energy-saving and less glucose-dependent metabolic shift, allows effective implementation of pro-migratory behavior of the cells seeking for more favorable environment (Godlewski et al., 2010c). Our and other researchers' evidence (recently reviewed by (Vartanian et al., 2014)) strongly suggests that the concept of GBM cell plasticity at the metabolic level should be added to the multifaceted landscape of this deadly disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Expression of miR-451 is transcriptionally repressed in low glucose conditions.
- OCT1 directly activates the transcription of miR-451.
- Activated AMPK inhibits the action of OCT1 by direct phosphorylation at serine 335.
- A miR-451/AMPK feedback loop allows GBM cells to adapt to metabolic stress.

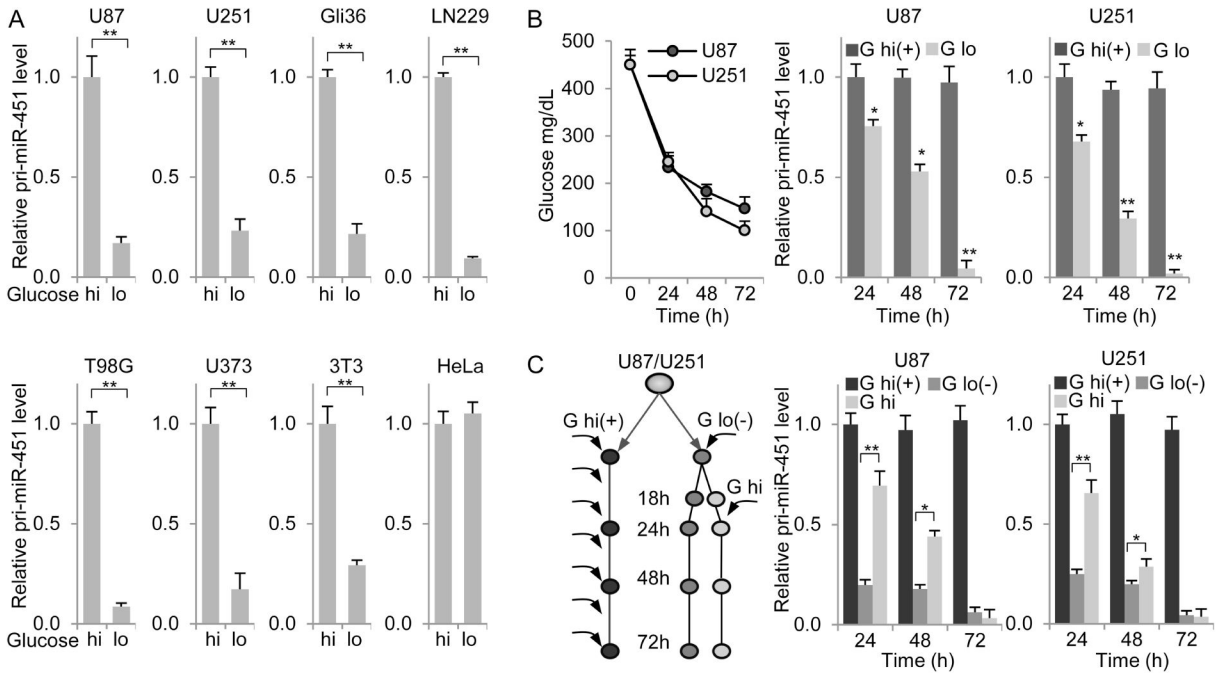

Figure 1. Glucose regulates the levels of pri-miR-451

(**A**) qRT-PCR analysis of pri-miR-451 expression after 18h in low glucose. See also Figure S1A-B. *p<0.05, **p<0.01

(**B**) Glucose depletion by proliferating cells (left panel). qRT-PCR analysis of pri-miR-451 expression in glucose-depleted media (right panels). See also Figure S1C. *p<0.05, **p<0.01

(**C**) Glucose regimens used in the experiment (left panel). qRT-PCR analysis of pri-miR-451 expression in different glucose regimens (right panels). See also Figure S1D. *p<0.05, **p<0.01

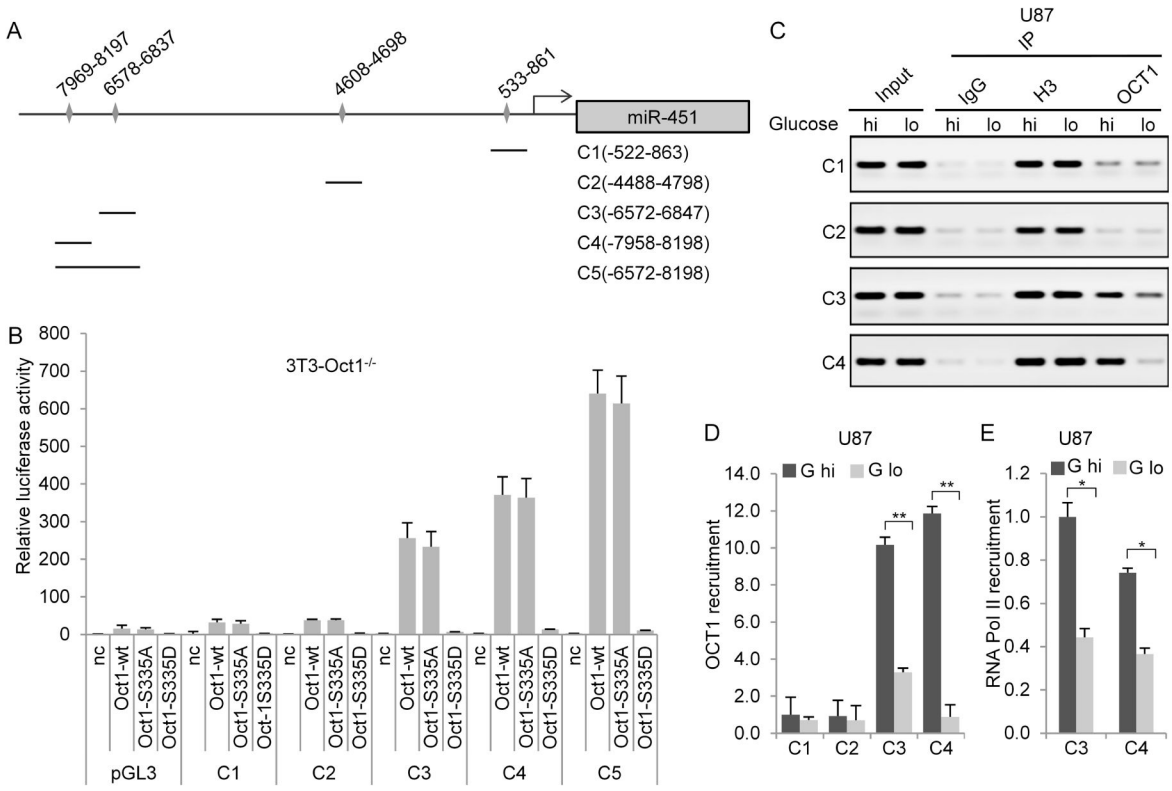

Figure 2. OCT1 is a positive transcriptional modulator of miR-451

(**A**) A schematic representation of OCT1 binding sites within the putative promoter of miR-451 and cloned fragments (C1-C5). Numbering is relative to the transcription start site. See also Figure S2A.

(**B**) The effect of OCT1 on the expression of miR-451. Luciferase assay in 3T3 *Oct1*$^{-/-}$ cells co-transfected with wild type and mutant (S335A and S335D) OCT1 constructs and luciferase construct containing different putative OCT1 binding sites. See also Figure S2B.

(**C–E**) Glucose deprivation decreases the recruitment of OCT1 to the promoter of miR-451. ChIP analysis in low glucose conditions in miR-451 promoter regions (**C**) was validated by qRT-PCR of OCT1 (**D**) and RNA Pol II (**E**). *p<0.05, **p<0.01

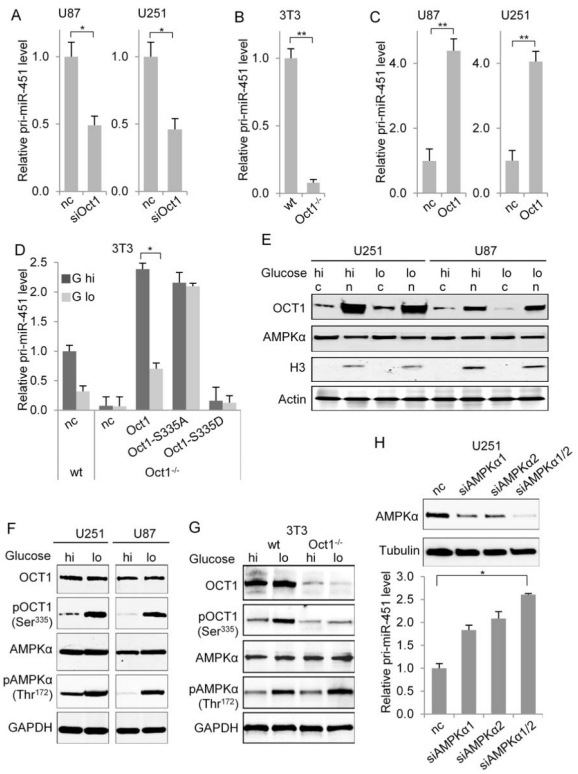

Figure 3. OCT1 plays critical roles in miR-451 expression

(**A–B**) qRT-PCR analysis of pri-miR-451 expression upon OCT1 knockdown (**A**) and in Oct1-deficient cells (**B**). See also Figure S3A–B. *p<0.05, **p<0.01

(**C**) qRT-PCR analysis of pri-miR-451 expression upon OCT1 overexpression. See also Figure S3C. *p<0.05, **p<0.01

(**D**) qRT-PCR analysis of pri-miR-451 expression in 3T3 *Oct1*$^{-/-}$ cells transfected with wild type and mutant OCT1 vectors and cultured in high and low glucose. See also Figure S3D–E. *p<0.05, **p<0.01

(**E**) The predominant nuclear localization of OCT1 in GBM cells is independent of glucose levels. Immunoblotting of cytoplasmic (c) and nuclear (n) cellular fractions of GBM cells cultured in high and low glucose.

(**F**) OCT1 is phosphorylated at S335 in a glucose-dependent manner in GBM cells. Immunoblotting of GBM cells cultured in high and low glucose.

(**G**) OCT1 is phosphorylated at S335 in glucose-dependent manner in mouse fibroblasts; phosphorylation of AMPK by low glucose conditions is not impaired in *Oct1*$^{-/-}$ cells. Immunoblotting of 3T3 cells cultured in high and low glucose.

(**H**) Knockdown of AMPK a1, a2 and a1/a2 increases the expression of miR-451 in low glucose environment. Immunoblotting of GBM cells cultured in low glucose (upper panels). qRT-PCR analysis of pri-miR-451 expression upon AMPK knockdown (bottom). See also Figure S3G. *p<0.05, **p<0.01

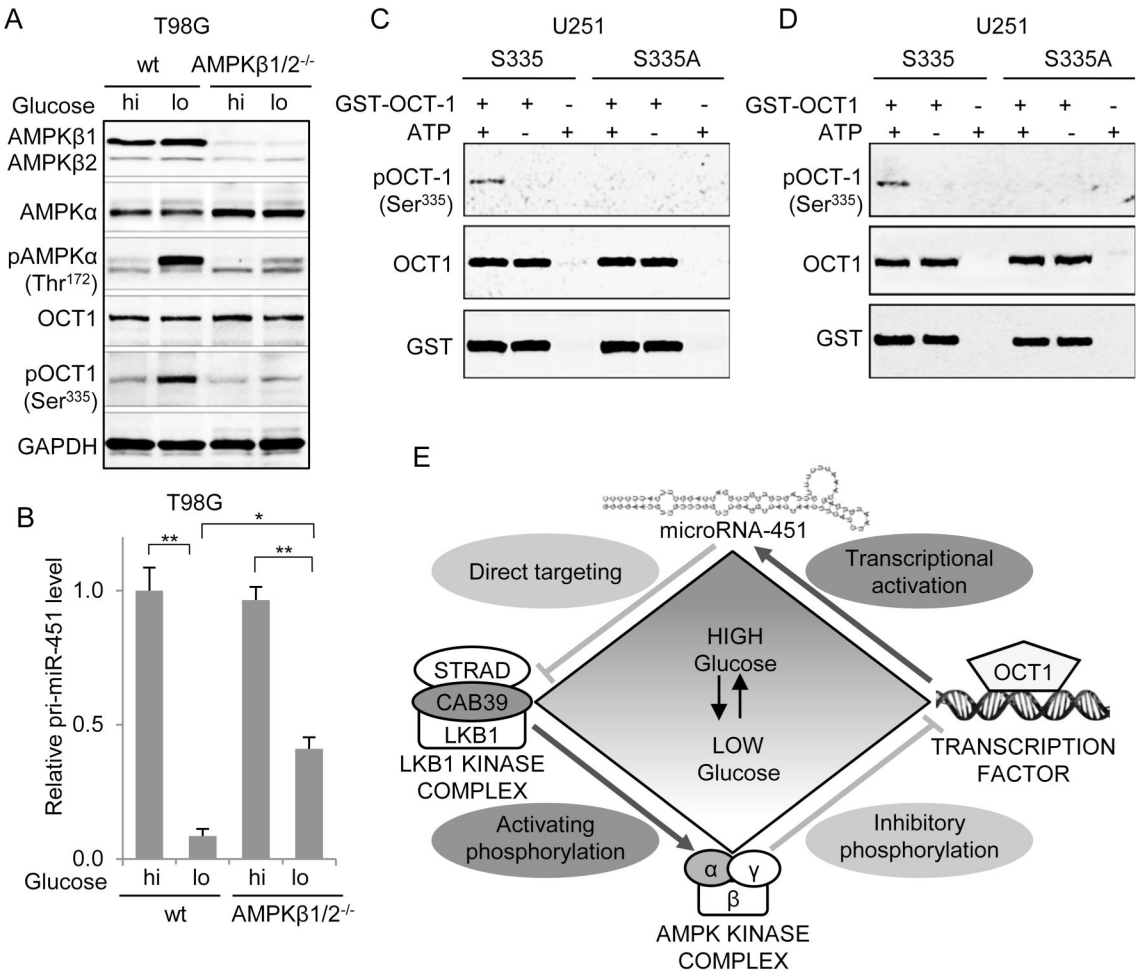

Figure 4. Role of AMPK in phosphorylation of OCT1 and transcription of miR-451

(**A**) Phosphorylation of OCT1 at S335 is impaired in AMPKβ1/2-deficient GBM cells. Immunoblotting of AMPKβ1/2-deficient GBM cells cultured in high and low glucose.

(**B**) qRT-PCR analysis of pri-miR-451 expression. See also Figure S4A. *p<0.05, **p<0.01

(**C–D**) AMPK directly phosphorylates OCT1 at S335. Kinase assays were performed with AMPK complex immuno-precipitated from U251 cells expressing Flag-AMPK in low glucose conditions (**C**), or recombinant, active AMPK complex containing AMPKa1, β1, and 1 (**D**), and GST-OCT1 peptide fragment containing S335 or S335A. See also Figure S4D-F.

(**E**) A proposed miR-451/AMPK regulatory loop in a fluctuating glucose microenvironment.