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Review COMMONS

The tumor-suppressive long noncoding RNA DRAIC inhibits protein translation and induces autophagy by activating AMPK

Shekhar Saha, Ying Zhang, Briana Wilson, Roger Abounader and Anindya Dutta
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Review timeline

Submission to Review Commons:	7 June 2021
Original submission:	19 August 2021
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Reviewer 1

Evidence, reproducibility and clarity

Saha and colleagues investigated the functions of the long non-coding RNA (lncRNA) DRAIC in malignant glioma. They find that DRAIC expression decreases cell migration/invasion and tumorsphere/colony formation in vitro, and tumor growth in vivo using established cell lines. Mechanistically, DRAIC is known to inhibit NF-kB signaling and the authors demonstrate that DRAIC activates AMPK leading to repression of mTOR, which decreases protein synthesis and increases autophagy. This is a solid study highlighting a potentially interesting pathway of tumor growth and invasion in brain tumors.

Major comments:

- It is unclear whether the presented values (mean +/- SD) in the histograms refer to repeat measurements (in which case n = 1) or independent experiments (n>1). The number of replicate experiments is not stated in the methods or figure legends. This must be included.
- I don't think the immunoblot for p62 in Fig. 5C shows a convincing increase following DRAIC knockout, so the statement on p.8 should be revised.
- On p.8/Fig.5 the authors make a case that increased DRAIC levels increase lysosomal degradation of autophagosome core proteins LC3 II / p62 (resulting in decreased protein levels of both), while simultaneously increasing gene expression of LC3B and p62 (causing increased mRNA levels). The data for DRAIC overexpression fit this logic fairly well (even though I think more work is needed to fully support this claim), but I am finding it difficult to reconcile the DRAIC knockout data with this scenario - here, loss of DRAIC results in increased protein levels to decreased autophagy, but also decreased gene expression. To fully support this argument, rescue experiments would be needed using FoxO3a knockout/overexpression.
- Similarly, the data supporting increased autophagy following DRAIC overexpression (Fig. 5F/G) are a bit weak and lack controls (is the LC3B-GFP overlapping with endogenous LC3B and autophagosomes? Was the transfection efficiency comparable? Is there fusion with lysosomes?). In the absence of stronger data, the authors should temper their claims that DRAIC increases autophagy.
- No information is provided on animal numbers used in this study. How many mice were used per cohort? Were male and female mice used? Authors should follow ARRIVE guidelines in reporting animal experiments. The method for calculating tumor volume needs to be specified.
- Student's T-test is inappropriate for comparisons of more than two groups (i.e. all experiments using DRAIC knockout cells) - for these experiments a Kruskal Wallis test or ANOVA should be used.

Did the authors test for normal distribution of their data? This may affect statistical testing and should be taken into consideration.

Minor comments:

- Authors mention that DRAIC expression is undetectable in immortalized astrocytes and GBM cancer stem cells (Fig. S1). What is the source of these cells and how were they cultured?
- The immunoblot in Fig. 3D could be replaced with a slightly lower exposure to make the difference between WT and DRAIC KO more obvious.
- Some immunoblots in Fig. 3 (panel E, p-S6K and S6K; panel H, actin) are not of the best quality and an effort should be made to replace them.
- Why are different loading controls used in Fig. 3 (a-Tubulin v actin)?
- Compared to other blot images in the same figure (e.g. Fig. 3E), the bands for p-mTOR and mTOR in Fig. 3F look compressed and should be shown appropriately sized.
- The layout of Fig. 4 is somewhat confusing. I would suggest organizing this according to DRAIC overexpression in A172 and U373 cells versus DRAIC knockout in LNCaP cells. Each immunoblot should be clearly labelled with the corresponding cell line, and it should be clearly explained why p-FoxO3a was tested in U251 cells, rather than A172/U373 as in the rest of the figure.
- Labelling of immunoblot in Fig. 5B is confusing and should be improved.
- Changes in GLUT1 expression (Fig. 7A) should be validated on the protein level.

Significance

The authors describe a novel link between the lncRNA DRAIC and AMPK activation through inhibition of NF-kB-mediated regulation of GLUT1. This study extends their previous work on DRAIC inhibition of NF-kB in prostate cancer (Saha et al. Cancer Res 2020). There is one study describing DRAIC effects on growth and invasion in glioma cell lines (Li et al. Eur Rev Med Pharmacol Sci 2020), but the work presented by Saha and colleagues contains stronger experimental data and a more detailed and previously undescribed mechanism.

The current study presents a mechanistic advance that increases the understanding of tumor growth and protein synthesis in cancer cells. The data presented in the study are not supported by in vivo experiments (other than suppression of tumor growth by DRAIC overexpression), validation in human tissue and/or primary patient-derived human glioblastoma cells, or even substantial rescue experiments. This limits the influence of the work on the field. I'm also not sure how transferable findings from DRAIC knockout in prostate cancer cell lines are to glioma, although the results are mostly complementary to the data from glioma cell lines. This is particularly relevant to the proposed mechanism of GLUT1 regulation by NF-kB, as the bulk of experimental data in Figures 6 and 7 was generated in prostate cancer cell lines and is only poorly validated in glioma cells. The study results will be most relevant for researchers investigating cell signaling pathways and autophagy in cancer.

Reviewer keywords:

neurooncology, cancer stem cells, signaling pathways in cancer

Reviewer 2

Evidence, reproducibility and clarity

In this manuscript, the authors describe DRAIC as a lncRNA downregulated in prostate cancer. They postulate that DRAIC expression surpasses invasion, migration and growth. Mechanistically, the authors show that DRAIC activates AMPK by suprressing NFkB target

gene TOR and indirectly impacting translation and autophagy. Collectively the observation is interesting and robust. However, I have several technical requests, particularly regarding the mechanistic part of the paper.

- The authors should rescue Ko phenotypes by over expressing DRAIC to consider potential off target effects.
- The blots showing TOR and ULK1 phosphorylation need to be repeated. This is an important part of the paper and I feel that these blots are hard to interpret. p-S6K typically run a bit higher in gels. there may be a technical problem.
- GLUT1-related results are interesting but the authors should provide genetic evidence that the effects are mediated by GLUT1. How do we know that glucose uptake is indeed upregulated upon knockout?

Minor:
The figures need to be updated. FOnts are all different, lots of unaligned graphs, quality of the blots are poor.

Significance

The observation is interesting, but the mechanism is incompletely understood. This is a nice addition to the literature, even without the mechanism.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

Shaha and colleagues present a study demonstrating the tumor suppressive role of DRAIC, a long non-coding RNA transcript, through transmission of the signal from IKK/NF-kB to the AMPK/mTOR pathway via regulation of GLUT1 expression. The inhibition of mTOR by this pathway results in the reduction of protein translation, cellular invasion and activation of autophagy. Several diseases and models as well as multiple genetic and pharmacological manipulations were used to investigate the mechanisms at play. The manuscript is well written and the experiments are well designed. The conclusions are supported by the results. The following major and minor comments should be addressed:

Major comments:
1. In addition to reporting the effect of DRAIC overexpression on tumor volume, the authors should present survival studies with one or more models.
2. Since the authors study metabolic energy sensor pathways, related to glycolysis, it would be important to perform some of the key experiments in physiological level of glucose: e.g., pmTOR, pAMPK, LC3-II expression level in DRAIC overexpressing and deficient cells.
3. In addition to RT-PCR data, GLUT1 protein levels should be investigated in the different DRAIC expressing cells.
4. The effect of DRAIC on GLUT1 expression is also measured in condition of glucose saturation, which does not reflect disease state. The decrease of GLUT1 in response to DRAIC overexpression and the increased GLUT1 level in DRAIC deficient cells should be investigated in physiological levels of glucose.

Minor comments:
1. All the data are generated with established cell lines (e.g., U87) but more clinically relevant models, such as patient-derived primary cells like the ones used in Fig. S1, could be used to replicate some of the key findings.
2. Also please provide further details about the patient-derived cells from Fig. S1.
3. The statistical analysis section states that the number of measurements is indicated however I don't see the sample size of the experiments.

Significance

The study reports a new model of regulation of tumor via long non-coding RNA. This article adds to the growing literature The topic and content of the article is relevant and significant to the field of tumor research but the significance and impact could be enhanced with the use of more physiologically relevant models and conditions as pointed in the major comments.

Author response to reviewers' comments

We are very pleased that the reviewers saw the significance of the paper. It is very exciting to discover that DRAIC lncRNA, through the regulation of NF-kB activity, regulates the AMPKmTOR pathway, and thus represses translation, increases autophagy and thus acts as a tumor suppressor. Based on the reviews, we are certain that this paper will be very well received by the field. The revisions requested have all been done, so the planned revisions, stated below, have already been executed, and so the revised manuscript is also being co-submitted.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Saha and colleagues investigated the functions of the long non-coding RNA (lncRNA) DRAIC in malignant glioma. They find that DRAIC expression decreases cell migration/invasion and tumorsphere/colony formation in vitro, and tumor growth in vivo using established cell lines. Mechanistically, DRAIC is known to inhibit NF-kB signaling and the authors demonstrate that DRAIC activates AMPK leading to repression of mTOR, which decreases protein synthesis and increases autophagy. This is a solid study highlighting a potentially interesting pathway of tumor growth and invasion in brain tumors.

Answer: We appreciate Reviewer 1 for the positive feedback of our study

Major Comments:

1) It is unclear whether the presented values (mean +/- SD) in the histograms refer to repeat measurements (in which case n = 1) or independent experiments (n>1). The number of replicate experiments is not stated in the methods or figure legends. This must be included.

Answer: We want to thank Reviewer 1 for pointing out this omission. We have now included this information in the Materials and methods and in figure legends section.

2) I don't think the immunoblot for p62 in Fig. 5C shows a convincing increase following DRAIC knockout, so the statement on p.8 should be revised.

Answer: We have revised the statement to say: Consistent with DRAIC decrease being associated with a decrease in autophagic flux, and despite a decrease in p62 mRNA, the level of P62 protein is increased in three of the DRAIC KO prostate cancer cells (Fig. 5C, KO1, KO2, KO4 compared to WT) and unchanged in the other two.

3) On p.8/Fig.5 the authors make a case that increased DRAIC levels increase lysosomal degradation of autophagosome core proteins LC3 II / p62 (resulting in decreased protein levels of

both), while simultaneously increasing gene expression of LC3B and p62 (causing increased mRNA levels). The data for DRAIC overexpression fit this logic fairly well (even though I think more work is needed to fully support this claim), but I am finding it difficult to reconcile the DRAIC knockout data with this scenario - here, loss of DRAIC results in increased protein levels to decreased autophagy, but also decreased gene expression. To fully support this argument, rescue experiments would be needed using FoxO3a knockout/overexpression.

Answer: Note that the mRNA level is not always correlated with protein expression. This is particularly true for LC3 and p62, whose protein levels are significantly affected by the extent of fusion of autophagosomes and lysosomes and subsequent degradation in autophagolysosomes. Thus, although the mRNA of these genes is decreased in DRAIC KO cells (Fig. 5E), the proteins are increased (Fig. 5C) because of decrease of autophagic flux (and decrease of degradation in the autophagolysosomes).

The overexpression of FoxO3a in the DRAIC KO cells will not restore mRNA levels of LC3 or p62, because we show in Fig. 4H that FoxO3 phosphorylation by AMPK is suppressed by DRAIC KO. This phosphorylation is important for the induction of LC3 or p62 mRNA by FoxO3.

FoxO3 knockout or knockdown in DRAIC OE cells should decrease LC3B or p62 mRNA in Fig. 5D, but it is already known from the Literature that FoxO3a is necessary for inducing LC3B or p62 mRNA. Cell Metab. 2007 Dec;6(6):458-71. doi: 0.1016/j.cmet.2007.11.001.PMID: 18054315.

4) Similarly, the data supporting increased autophagy following DRAIC overexpression (Fig. 5F/G) are a bit weak and lack controls (is the LC3B-GFP overlapping with endogenous LC3B and autophagosomes? Was the transfection efficiency comparable? Is there fusion with lysosomes?). In the absence of stronger data, the authors should temper their claims that DRAIC increases autophagy.

Answer: LC3B of fusion protein LC3B-GFP is known to overlap with the p62 puncta (similar to endogenous LC3B). This result is in Fig. 4A of the citation that we have now added (Proc Natl Acad Sci U S A. 2016 Nov 22;113(47): E7490-E7499. doi: 10.1073/pnas.1615455113. Epub 2016 Oct 17) To support our hypothesis that DRAIC OE induces more autophagy compared to empty vector, we used Bafilomycin A1 in Figure 5B to inhibit the autophagosome and lysosome fusion. We see the accumulation of more LC3B upon treatment with Bafilomycin A1 in the DRAIC OE cells (compared to EV containing U251 cells), consistent with the idea that autophagosome-lysosome fusion is increased by DRAIC OE.

5) No information is provided on animal numbers used in this study. How many mice were used per cohort? Were male and female mice used? Authors should follow ARRIVE guidelines in reporting animal experiments. The method for calculating tumor volume needs to be specified.

Answer: We have included the details about the animal study in methods section and figure legend of our modified manuscript.

6) Student's T-test is inappropriate for comparisons of more than two groups (i.e. all experiments using DRAIC knockout cells) - for these experiments a Kruskal Wallis test or ANOVA should be used. Did the authors test for normal distribution of their data? This may affect statistical testing and should be taken into consideration.

Answer: We have now modified our statistical calculation and included in the statistical analysis section in our modified manuscript.

Minor Comments:

7) Authors mention that DRAIC expression is undetectable in immortalized astrocytes and GBM cancer stem cells (Fig. S1). What is the source of these cells and how were they cultured?

Answer: The immortalized astrocytes and GBM stem cells and their culture conditions is now described.

8) The immunoblot in Fig. 3D could be replaced with a slightly lower exposure to make the difference between WT and DRAIC KO more obvious.

Answer: We have now replaced the immunoblot with lower exposure.

9) Some immunoblots in Fig. 3 (panel E, p-S6K and S6K; panel H, actin) are not of the best quality and an effort should be made to replace them.
Answer: We have now replaced the immunoblot p-S6K as reviewer mentioned.

10) Why are different loading controls used in Fig. 3 (a-Tubulin v actin)?

Answer: We use multiple loading control to make sure that we are not underestimating or overestimating changes in the experimental protein because of unexpected changes in the loading controls.

11) Compared to other blot images in the same figure (e.g. Fig. 3E), the bands for p-mTOR and mTOR in Fig. 3F look compressed and should be shown appropriately sized.

Answer: We have modified the Figure as reviewer suggested.

12) The layout of Fig. 4 is somewhat confusing. I would suggest organizing this according to DRAIC overexpression in A172 and U373 cells versus DRAIC knockout in LNCaP cells. Each immunoblot should be clearly labelled with the corresponding cell line, and it should be clearly explained why p-FoxO3a was tested in U251 cells, rather than A172/U373 as in the rest of the figure.

Answer: We thank the reviewer for the constructive criticism. We have labeled all the cell lines in the Figure as reviewer suggested. We have now systematically alternated the prostate cancer cells (for KO) and the GBM cells (for OE), as we looked at each relevant marker. We have now included the western blot for p-FoxO3a from another glioblastoma cell line U373.
Please find the modified Figure 4K for p-FoxO3a.

13) Labelling of immunoblot in Fig. 5B is confusing and should be improved.

Answer: We have modified the Fig. 5B to make the label clearer.

14) Changes in GLUT1 expression (Fig. 7A) should be validated on the protein level.

Answer: We have included the immunoblot for GLUT1 from DRAIC KO cells in Figure 7B. GLUT1 protein is increased upon DRAIC KO.

Reviewer #1 (Significance (Required):

The authors describe a novel link between the lncRNA DRAIC and AMPK activation through inhibition of NF-kB-mediated regulation of GLUT1. This study extends their previous work on DRAIC inhibition of NF-kB in prostate cancer (Saha et al. Cancer Res 2020). There is one study describing DRAIC effects on growth and invasion in glioma cell lines (Li et al. Eur Rev Med Pharmacol Sci 2020), but the work presented by Saha and colleagues contains stronger experimental data and a more detailed and previously undescribed mechanism.

The current study presents a mechanistic advance that increases the understanding of tumor growth and protein synthesis in cancer cells. The data presented in the study are not supported by in vivo experiments (other than suppression of tumor growth by DRAIC overexpression), validation in human tissue and/or primary patient-derived human glioblastoma cells, or even substantial rescue experiments. This limits the influence of the work on the field. I'm also not sure how transferable findings from DRAIC knockout in prostate cancer cell lines are to glioma, although the results are mostly complementary to the data from glioma cell lines. This is particularly relevant to the proposed mechanism of GLUT1 regulation by NF-kB, as the bulk of experimental data in Figures 6 and 7 was generated in prostate cancer cell lines and is only poorly validated in glioma cells. The study results will be most relevant for researchers investigating cell signaling pathways and autophagy in cancer.

Answer: We like to thank reviewer for the positive comments on our study. The DRAIC KO experiments of Fig. 6 and 7 cannot be done in glioma cells, because as we show in Supp. Fig. S1, there are no glioma cells or GSC that express DRAIC to levels comparable to LnCaP. We have shown that GLUT1 mRNA decreases in the glioma cells when DRAIC is overexpressed (Supp. Fig. S4. We also show in Fig. 7G that AMP levels increase when DRAIC is overexpressed in glioma cells.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this manuscript, the authors describe DRAIC as a lncRNA downregulated in prostate cancer. They postulate that DRAIC expression surpasses invasion, migration and growth.
Mechanistically, the authors show that DRAIC activates AMPK by suppressing NFkB target gene TOR and indirectly impacting translation and autophagy. Collectively the observation is interesting and robust. However, I have several technical requests, particularly regarding the mechanistic part of the paper.

Answer: We appreciate the positive feedback. We have addressed the reviewer's concerns in our modified manuscript.

Major Comments:

1) The authors should rescue Ko phenotypes by over expressing DRAIC to consider potential off target effects.

Answer: DRAIC OE alone is sufficient to have exactly the opposite effect as DRAIC KO in protein translation (Fig. 3C-F), so DRAIC OE will rescue the effect of DRAIC KO. We make a similar argument for all the phenotypes, including mTOR, S6K and ULK1(S757) phosphorylation (Fig. 3G-J), AMPK and FoxO3a phosphorylation (rearranged now as Fig. 4B-C; J-L), autophagic flux (Fig. 5B, C) and effects on LC3B and p62 mRNAs (Fig. 5D, E). The same is true for our published phenotypes of DRAIC KO on invasion, migration and NF-kB activity (Saha, Cancer Research, 2020)

2) The blots showing TOR and ULK1 phosphorylation need to be repeated. This is an important part of the paper and I feel that these blots are hard to interpret. p-S6K typically run a bit higher in gels. there may be a technical problem.

Answer: We are not sure which specific blots the reviewer is referring to, and it is possible that the blots the other reviewers pointed to are the ones under question. We have changed those blots so that the results are clear.

3) GLUT1-related results are interesting, but the authors should provide genetic evidence that the effects are mediated by GLUT1. How do we know that glucose uptake is indeed upregulated upon knockout?

Answer: In Fig. 7 C-F we show that the effects of DRAIC KO on invasion, protein translation, AMP levels and AMPK activity are reversed by the GLUT1 inhibitor Bay-876. This is a cleaner result than using siRNA to knockdown GLUT1. siRNAs can have off-target activity and sometimes cannot decrease a protein sufficiently below the threshold necessary to see reversal of action.

Minor Comments:

4) The figures need to be updated. Fonts are all different, lots of unaligned graphs, quality of the blots are poor.

Answer: We have updated the Figures and changed fonts as reviewer mentioned.

Reviewer #2 (Significance (Required):

The observation is interesting, but the mechanism is incompletely understood. This is a nice addition to the literature, even without the mechanism.

Answer: We want to thank the reviewer for the constructive criticism.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

Shaha and colleagues present a study demonstrating the tumor suppressive role of DRAIC, a long non-coding RNA transcript, through transmission of the signal from IKK/NF-kB to the AMPK/mTOR pathway via regulation of GLUT1 expression. The inhibition of mTOR by this pathway results in the reduction of protein translation, cellular invasion and activation of autophagy. Several diseases and models as well as multiple genetic and pharmacological manipulations were used to investigate the mechanisms at play. The manuscript is well written and the experiments are well designed. The conclusions are supported by the results. The following major and minor comments should be addressed:

Answer: We appreciate the reviewer for the positive comments on our study.

Major Comments:

1) In addition to reporting the effect of DRAIC overexpression on tumor volume, the authors should present survival studies with one or more models.

Answer: We thought of doing the survival study in our glioblastoma model but unfortunately, the tumor growth is very rapid (exceeding the size permitted by our IACUC in 2-3 weeks). The animal ethics welfare committee did not allow us to keep the mice for a longer time to perform the survival study.

2) Since the authors study metabolic energy sensor pathways, related to glycolysis, it would be important to perform some of the key experiments in physiological level of glucose: e.g., pmTOR, pAMPK, LC3-II expression level in DRAIC overexpressing and deficient cells.

Answer: The concentration of glucose in plasma is 1G/L, while that of the RPMI medium is 2G/L. We do not think we are too far from the physiological levels of glucose.

3) In addition to RT-PCR data, GLUT1 protein levels should be investigated in the different DRAIC expressing cells.

Answer: We have incorporated the GLUT1 protein expression data from DRAIC KO cells in Figure 7B and DRAIC overexpressing cells in supplementary Figure 4G-H. The blots from the same gels were split into different panels, the loading control GAPDH remains same in Figure 4K and Supplementary Figure S4H.

4) The effect of DRAIC on GLUT1 expression is also measured in condition of glucose saturation, which does not reflect disease state. The decrease of GLUT1 in response to DRAIC overexpression and the increased GLUT1 level in DRAIC deficient cells should be investigated in physiological levels of glucose.

Answer: Same as above. We are near physiological levels of glucose.

Minor Comments:

5) All the data are generated with established cell lines (e.g., U87) but more clinically relevant models, such as patient-derived primary cells like the ones used in Fig. S1, could be used to replicate some of the key findings.

Answer: As we showed in Fig. S1 that DRAIC is not expressed in glioma stem cells, and so knockout experiments are not possible. We believe that the knockout experiments are the most relevant to this paper because they do not run the risk of artefacts from overexpression of an RNA far beyond physiological levels.

6) Also please provide further details about the patient-derived cells from Fig. S1.

Answer: We have mentioned the details of the cell lines in our modified manuscript.

7) The statistical analysis section states that the number of measurements is indicated however I don't see the sample size of the experiments.

Answer: We have now incorporated the number the experiments in our modified text.

Reviewer #3 (Significance (Required):

The study reports a new model of regulation of tumor via long non-coding RNA. This article adds to the growing literature The topic and content of the article is relevant and significant to the field of tumor research but the significance and impact could be enhanced with the use of more physiologically relevant models and conditions as pointed in the major comments.

Answer: We want to thank the reviewer for the positive feedback on our study.

3. Description of the revisions that have already been incorporated in the transferred manuscript

All the revisions described in the revision plan above have been carried out and included in the manuscript.

4. Description of analyses that authors prefer not to carry out

1) On p.8/Fig.5 the authors make a case that increased DRAIC levels increase lysosomal degradation of autophagosome core proteins LC3 II / p62 (resulting in decreased protein levels of both), while simultaneously increasing gene expression of LC3B and p62 (causing increased mRNA levels). The data for DRAIC overexpression fit this logic fairly well (even though I think more work is needed to fully support this claim), but I am finding it difficult to reconcile the DRAIC knockout data with this scenario - here, loss of DRAIC results in increased protein levels to decreased autophagy, but also decreased gene expression. To fully support this argument, rescue experiments would be needed using FoxO3a knockout/overexpression.

Answer: Note that the mRNA level is not always correlated with protein expression. This is particularly true for LC3 and p62, whose protein levels are significantly affected by the extent of fusion of autophagosomes and lysosomes and subsequent degradation in autophagolysosomes. Thus, although the mRNA of these genes is decreased in DRAIC KO cells (Fig. 5E), the LC3 and p62 proteins are increased (Fig. 5C) because of decrease of autophagic flux (and decrease of degradation in the autophagolysosomes).

The overexpression of FoxO3a in the DRAIC KO cells will not restore mRNA levels of LC3 or p62, because we show in Fig. 4H that FoxO3 phosphorylation by AMPK is suppressed by DRAIC KO. This phosphorylation is important for the induction of LC3 or p62 mRNA by FoxO3.

FoxO3 knockout or knockdown in DRAIC OE cells should decrease LC3B or p62 mRNA in Fig. 5D, but it is already known from the Literature that FoxO3a is necessary for inducing LC3B or p62 mRNA. Cell Metab. 2007 Dec;6(6):458-71. doi: 10.1016/j.cmet.2007.11.001.PMID: 18054315.

2) Similarly, the data supporting increased autophagy following DRAIC overexpression (Fig. 5F/G) are a bit weak and lack controls (is the LC3B-GFP overlapping with endogenous LC3B and autophagosomes? Was the transfection efficiency comparable? Is there fusion with lysosomes?). In the absence of stronger data, the authors should temper their claims that DRAIC increases

autophagy.

Answer: LC3B of fusion protein LC3B-GFP overlaps with endogenous p62 puncta, and thus endogenous LC3B []. The experiments in Fig. 5F/G were done with stably transfected derivatives. The important thing here is that the diffuse staining seen in EV panels, becomes punctate when DRAIC is overexpressed.

To support our hypothesis that DRAIC OE induces more autophagy compared to empty vector, we used Bafilomycin A1 in Figure 5B to inhibit the autophagosome and lysosome fusion. We see the accumulation of more LC3B upon treatment with Bafilomycin A1 in the DRAIC OE cells (compared to EV containing U251 cells), consistent with the idea that autophagosome-lysosome fusion is increased by DRAIC OE.

Based on the above facts, we believe that the data is strong enough to say that DRAIC increases autophagic flux. We do not have an alternate explanation, but if we missed an alternate hypothesis that the reviewer has spotted, we will incorporate that in the manuscript to temper our claim.

3) Why are different loading controls used in Fig. 3 (a-Tubulin vactin)?

Answer: We use multiple loading control to make sure that we are not underestimating or overestimating changes in the experimental protein because of unexpected changes in the loading controls.

4) The authors should rescue Ko phenotypes by over expressing DRAIC to consider potential off target effects.

Answer: DRAIC OE alone is sufficient to have exactly the opposite effect as DRAIC KO in protein translation (Fig. 3C-F), so DRAIC OE will rescue the effect of DRAIC KO. We make a similar argument for all the phenotypes, including mTOR, S6K and ULK1(S757) phosphorylation (Fig. 3G-J), AMPK and FoxO3a phosphorylation (rearranged Fig. 4B-C; J-L), autophagic flux (Fig. 5B, C) and effects on LC3B and p62 mRNAs (Fig. 5D, E). The same is true for our published phenotypes of DRAIC KO on invasion, migration and NF-kB activity (Saha, Cancer Research, 2020)

5) The blots showing TOR and ULK1 phosphorylation need to be repeated. This is an important part of the paper and I feel that these blots are hard to interpret. p-S6K typically run a bit higher in gels. there may be a technical problem.

Answer: We are not sure which specific blots Reviewer #2 is referring to, and it is possible that the blots the other reviewers pointed to are the ones under question. We have changed those blots so that the results are clear.

6) GLUT1-related results are interesting, but the authors should provide genetic evidence that the effects are mediated by GLUT1. How do we know that glucose uptake is indeed upregulated upon knockout?

Answer: In Fig. 7 C-F we show that the effects of DRAIC KO on invasion, protein translation, AMP levels and AMPK activity are reversed by the GLUT1 inhibitor Bay-876. This is a cleaner result than using siRNA to knockdown GLUT1. siRNAs can have off-target activity and sometimes cannot decrease a protein sufficiently below the threshold necessary to see reversal of action.

7) In addition to reporting the effect of DRAIC overexpression on tumor volume, the authors should present survival studies with one or more models.

Answer: We thought of doing the survival study in our glioblastoma model but unfortunately, the tumor growth is very rapid (exceeding the size permitted by our IACUC in 2-3 weeks). The animal ethics welfare committee did not allow us to keep the mice for a longer time to perform the survival study.

8) Since the authors study metabolic energy sensor pathways, related to glycolysis, it would be important to perform some of the key experiments in physiological level of glucose: e.g., pmTOR,

pAMPK, LC3-II expression level in DRAIC overexpressing and deficient cells.

Answer: The concentration of glucose in plasma is 1G/L, while that of the RPMI medium is 2G/L. We do not think we are too far from the physiological levels of glucose.

Minor Comments:

9) All the data are generated with established cell lines (e.g., U87) but more clinically relevant models, such as patient-derived primary cells like the ones used in Fig. S1, could be used to replicate some of the key findings.

Answer: As we showed in Fig. S1 that DRAIC is not expressed in glioma stem cells, and so knockout experiments are not possible. We believe that the knockout experiments are the most relevant to this paper because they do not run the risk of artefacts from overexpression of an RNA far beyond physiological levels.

Original submission

First decision letter

MS ID#: JOCES/2021/259306

MS TITLE: A tumor suppressive long noncoding RNA, DRAIC, inhibits protein translation and induces autophagy by activating AMPK

AUTHORS: Shekhar Saha, Ying Zhang, Briana Wilson, Roger Abounader, and Anindya Dutta
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports. Note that I realise that you have already addressed the points of Reviewer 1 in your revised version. Reviewer 2 is mostly satisfied with your revisions, but raises a point that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on your ability to do these minor revisions.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Shaha and colleagues present a study demonstrating the tumor suppressive role of DRAIC, a long non-coding RNA transcript, through transmission of the signal from IKK/NF-kB to the AMPK/mTOR pathway via regulation of GLUT1 expression. The inhibition of mTOR by this pathway results in the reduction of protein translation, cellular invasion and activation of autophagy. Several diseases and models as well as multiple genetic and pharmacological manipulations were used to investigate the mechanisms at play. The manuscript is well written and the experiments are well designed. The conclusions are supported by the results. Some major and minor comments should be addressed as described in the following section.

Significance The study reports a new model of regulation of tumor via long non-coding RNA. This article adds to the growing literature The topic and content of the article is relevant and significant to the field of tumor research but the significance and impact could be enhanced with the use of more physiologically relevant models and conditions as pointed in the major comments.

Comments for the author

Major comments:
1. In addition to reporting the effect of DRAIC overexpression on tumor volume, the authors should present survival studies with one or more models.
2. Since the authors study metabolic energy sensor pathways, related to glycolysis, it would be important to perform some of the key experiments in physiological level of glucose: e.g., pmTOR, pAMPK, LC3-II expression level in DRAIC overexpressing and deficient cells.
3. In addition to RT-PCR data, GLUT1 protein levels should be investigated in the different DRAIC expressing cells.
4. The effect of DRAIC on GLUT1 expression is also measured in condition of glucose saturation, which does not reflect disease state. The decrease of GLUT1 in response to DRAIC overexpression and the increased GLUT1 level in DRAIC deficient cells should be investigated in physiological levels of glucose.

Minor comments:
1. All the data are generated with established cell lines (e.g., U87) but more clinically relevant models, such as patient-derived primary cells like the ones used in Fig. S1, could be used to replicate some of the key findings.
2. Also please provide further details about the patient-derived cells from Fig. S1.
3. The statistical analysis section states that the number of measurements is indicated however I don't see the sample size of the experiments.

Reviewer 2

Advance summary and potential significance to field

In their manuscript 'A tumor suppressive long noncoding RNA, DRAIC, inhibits protein translation and induces autophagy by activating AMPK', the authors investigate the role of DRAIC in brain cancer. DRAIC expression is associated with reduced cell migration, invasion, clonogenicity and glioma growth in vivo. DRAIC regulates expression of GLUT1 through NFkB. Loss of DRAIC promotes growth of tumor cells through increased expression of GLUT1 leading to decreased phosphorylation of AMPK and subsequently increased protein synthesis and decreased autophagy. The study provides a significant and novel contribution to the community, as it finds a novel link between DRAIC and AMPK.

Comments for the author

In this revision the authors have addressed my comments on the previous version of the manuscript.
I only have one additional minor comment:

Across all figures, immunoblots and micrographs should be consistently labelled with the cell line shown for added clarity. While this information is present in most panels, it is missing in some.

Reviewer 3

Advance summary and potential significance to field

Overall identification of a role for DRAIC in translation and signaling is interesting and appropriate addition to JCS.

Comments for the author

I am overall ok with the manuscript but most of the blots in the manuscript are either overexposed (6A, 6F, 5G (big smear), 5J (a running issue), 5K (air bubble), 5D (overexposed), 3E...Some of these are making the interpretation highly difficult. That being said this is an editorial decision.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
Shah and colleagues present a study demonstrating the tumor suppressive role of DRAIC, a long non-coding RNA transcript, through transmission of the signal from IKK/NF-kB to the AMPK/mTOR pathway via regulation of GLUT1 expression. The inhibition of mTOR by this pathway results in the reduction of protein translation, cellular invasion and activation of autophagy. Several diseases and models as well as multiple genetic and pharmacological manipulations were used to investigate the mechanisms at play. The manuscript is well written and the experiments are well designed. The conclusions are supported by the results. Some major and minor comments should be addressed as described in the following section.

Reviewer 1

Answer: As the editor points out, we have already addressed the points raised by Reviewer 1 in the revised version of the manuscript that was under review.

Reviewer 2 Advance Summary and Potential Significance to Field:
… The study provides a significant and novel contribution to the community, as it finds a novel link between DRAIC and AMPK.

Reviewer 2 Comments for the Author:
In this revision the authors have addressed my comments on the previous version of the manuscript. I only have one additional minor comment:
Across all figures, immunoblots and micrographs should be consistently labelled with the cell line shown for added clarity. While this information is present in most panels, it is missing in some.

Answer: We thank Reviewer 2 and have now labeled all the Figures with the respective cell lines used in our study.

Reviewer 3 Advance Summary and Potential Significance to Field:
Overall identification of a role for DRAIC in translation and signaling is interesting and appropriate addition to JCS.

Reviewer 3 Comments for the Author:
I am overall ok with the manuscript but most of the blots in the manuscript are either overexposed (6A, 6F, 5G (big smear), 5J (a running issue), 5K (air bubble), 5D (overexposed), 3E..Some of these are making the interpretation highly difficult. That being said this is an editorial decision.

Answer: We appreciate the editor for not taking up this issue, because the criticisms do not make sense. Fig. 6A, 6F: the puromycin pulse labeling experiment is expected to give a smeared lane because of global peptide labeling with puromycin. Fig. 5G: the result is that the diffuse immunofluorescence (smear?) in the EV panel becomes punctate immunofluorescence in the DRAIC overexpressing cells. Fig. 5J: no such panel. Fig. 5K: no such panel. Also did not see a gel with an air bubble. We had one in the previous version, but that had been replaced in the revised paper in blot 3E. Fig. 5D: is a bar graph that cannot be overexposed. Fig. 3E: do not see any problem.

Second decision letter

MS ID#: JOCES/2021/259306

MS TITLE: A tumor suppressive long noncoding RNA, DRAIC, inhibits protein translation and induces autophagy by activating AMPK

AUTHORS: Shekhar Saha, Ying Zhang, Briana Wilson, Roger Abounader, and Anindya Dutta
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.