JCB

Origin of cytoplasmic GDP-fucose determines its contribution to glycosylation reactions

Paulina Sosicka, Bobby Ng, Lauren Pepi, Asif Shajahan, Maurice Wong, David Scott, Kenjiroo Matsumoto, Zhi-Jie Xia, Carlito Lebrilla, Robert Haltiwanger, Parastoo Azadi, and Hudson Freeze

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June 23, 2022

Re: JCB manuscript #202205038

Dr. Paulina Sosicka
Sanford Burnham Prebys Medical Discovery Institute
Human Genetics Program
10901 N Torrey Pines Rd
La Jolla, CA 92037

Dear Dr. Sosicka,

Thank you for submitting your manuscript entitled "Origin of cytoplasmic nucleotide sugars determines their contribution to glycosylation reactions". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you can see by the reviewers' comments, there was overall high enthusiasm for the paper given that it may be the first example of clear distinction of unique metabolic pools of nucleotide sugar pools used for glycosylation. If you have additional data to demonstrate either spatial or kinetic resolution of the pools, please add that to the paper or comment on the importance of methods to achieve this goal. In addition, it might be useful to have the data analyzed by from a systems perspective; can the pools be defined kinetically in that way? Is it possible that the pools are associated with different organellar subcompartments and if so, how does that impact glycosylation differences observed for different proteins? Imaging methods to discern different pools may not yet be available, but a discussion of these recently published methods should be included. Please also consider the all the reviewers' comments in a suitably revised manuscript. Please include a point-by-point comments and keep in mind that the revised manuscript will be reviewed by the original reviewers and monitoring editor.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jeffrey Esko
Monitoring Editor

Andrea L. Marat
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Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This paper presents a new concept that cellular nucleotide sugars, in particular, GDP-fucose, have multiple cytosolic pools determined by their sources (dietary, de novo synthesis, salvage, etc.), which further define the usage by glycosylation reactions. Radioactive labeling and mass spectrometry analysis are the major techniques used to gain the results, which in general support the proposed new concept. This paper is evaluated to be suitable for publication, with a few remaining concerns:

1. High concentrations of exogenous fucose (e.g., 50 uM) do not increase the overall GDP-Fuc concentration. This finding may be contradictory to the existence of multiple cytosolic pools of GDP-fucose. Otherwise, is there communication between pools of GDP-fucose to regulate the total concentration?

2. I suggest that the authors should also discuss the evidence that does not support the existence of multiple pools of GDP-fucose. I agree that the results presented here are interesting and the proposed mechanism is plausible, but unless different pools of nucleotide are visualized directly or indirectly (see below), the existence of multiple pools of GDP-fucose is still hypothetical. For example, could the organization be not in space, but in time?

3. If different pools of nucleotide sugars and organelles are organized in space (as proposed), would this be visualized? I assume that EM might provide helpful information. In addition, recently, a genetically encoded fluorescent indicator for UDP-GlcNAc has been reported. I understand that it may not be realistic to include all these data in this paper, but the readers may benefit from discussing these opportunities.

4. Figure 1B, adding these three lines typically gives a number less than 50% (e.g., at 0 uM fucose). Does it mean the salvage pathway usually plays a significant role? This seems to be different from what is stated in the paper.

5. Are there any possible reasons to explain why CHO cells are different from other tested cell lines, in terms of the dependence on the salvage pathway?

6. CHO in Fig. 2 versus CHO-Lec13 in Supplementary Fig 1D, when fucose was at 50 uM, Fucose Ex)% is only around 30 in CHO, but ~100 in CHO-Lec13. Is there any reason?

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Reviewer #2 (Comments to the Authors (Required)):

The authors have a very clear and interesting hypothesis that they set out to prove, namely that the metabolic origin of fucose determines its use. In addition, this leads to distinct and multiple GDP-fucose pools within the cell. The authors report the results of several different experiments to address the contributions of exogenous, salvaged and de novo sources of fucose to global N-glycosylation and that of individual glycoproteins. All these experiments lead to the support of their overall starting hypothesis that GDP-fucose exist in different pools within the cell. Certain experiments are performed in multiple different cell lines to add further evidence for their hypothesis.
This is a clearly written paper where the authors repeatedly prove their hypothesis using essentially the same experiment on differing targets. The introduction is clearly written with enough information for a non-specialist in the field. The premise for this study is that it is generally thought there is a homogenous pool of sugars within the cell, which is used for all cellular processes, where as they disagree and believe there are different pools.
This is an interesting idea, and I was wondering whether there is any other experimental evidence in the literature to support or indeed disagree with these ideas, as only one paper is cited which states there is only one pool. I feel the lack of additional insights from the literature makes the study a little hard to put into the wider context. Also while I feel that it is an interesting concept, I was a little disappointed that no experiments were performed to try and understand how these separate pools are achieved or how they are maintained or ultimately how each pool makes a specific contribution to glycosylation processes. I would prefer to see these factors addressed somewhere in the paper as these more mechanistic insights are I feel of particular value and interest for the field.
Could the authors also comment on the following:
• Why was fucose used as a model monosaccharide and how relevant are the conclusions to other sugars. On this note I think the title should be altered to acknowledge that only one sugar is studied.
• Why was a low concentration of mannose used (50 uM), in the plasma it is found between 50-100uM.
• Fig 2A shows that there is a difference in cell types please can you discuss why this may be. Are the enzymes upregulated in these cells for example, understanding this may shed light into the mechanism of the utilisation of different pools.

1st Revision - Authors' Response to Reviewers: July 14, 2022

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The reviewer raises some great questions that naturally stem from our exciting results. Indeed, we too have been interested in what further insights could be obtained from considering these data could be analyzed from a systems perspective. Thus, in collaboration with Dr. Nathan Lewis from UCSD as an intended follow-on study, we have begun to take a systems biology approach to study the kinetics of the formulation of GDP-fucose pools. Our preliminary analysis proves that our data cannot be explained with a single, homogenous pool of GDP-fucose, and that there are indeed multiple pools. However, to fully parameterize, refine, and test the model, and then follow up the model analyses with proper experiments, it will take a substantial amount of time, and would best be a full study on its own.

Suitable paragraph was added in the discussion:

“Directly visualizing multiple intracellular GDP-fucose pools in living cells that show real time responses to metabolic perturbations would certainly strengthen our claims. We have considered various indirect approaches, such as designing highly specific GDP-fucose sensors similar to that described for UDP-GlcNAc (Li et al, 2021) that show spatial responses to alterations of exogenous or salvaged fucose. Similar metabolic perturbations might also affect the distribution/association of their biosynthetic enzymes visualized by Förster resonance energy transfer (FRET) or proximity ligation analysis. This may indicate whether spatial and/or kinetic factors predominate.”

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1. High concentrations of exogenous fucose (e.g., 50 uM) do not increase the overall GDP-Fuc concentration. This finding may be contradictory to the existence of multiple cytosolic pools of GDP-fucose. Otherwise, is there communication between pools of GDP-fucose to regulate the total concentration?

We agree that there must be some communication/sharing within the pools and added this paragraph to the discussion.

"Since the overall size of GDP-fucose pool remains constant, the (presumably) separate pools must be in communication. While this may be driven by changes in subcellular distribution of the biosynthetic enzymes, clearly, feedback inhibition of GMDS by GDP-fucose must be the mechanism."

2. I suggest that the authors should also discuss the evidence that does not support the existence of multiple pools of GDP-fucose. I agree that the results presented here are interesting and the proposed mechanism is plausible, but unless different pools of nucleotide are visualized directly or indirectly (see below), the existence of multiple pools of GDP-fucose is still hypothetical. For example, could the organization be not in space, but in time?

Without direct visual evidence, multiple GDP-fucose pools must still be considered hypothetical. However, our data is not compatible with a single, homogenous pool of GDP-fucose that provides fucose to three different glycosylation pathways and at least five glycosyltrasferases located in two different compartments, ER and Golgi. Considering a temporal, rather than spatial separation of the pools, we agree that time can be a contribution factor to the pools separation, however with technical limitations that don't allow us to visualize the pools directly, we cannot measure time contribution.

3. If different pools of nucleotide sugars and organelles are organized in space (as proposed), would this be visualized? I assume that EM might provide helpful information. In addition, recently, a genetically

encoded fluorescent indicator for UDP-GlcNAc has been reported. I understand that it may not be realistic to include all these data in this paper, but the readers may benefit from discussing these opportunities.

Suitable paragraph was added in the discussion:

"Directly visualizing multiple intracellular GDP-fucose pools in living cells that show real time responses to metabolic perturbations would certainly strengthen our claims. We have considered various indirect approaches, such as designing highly specific GDP-fucose sensors similar to that described for UDP-GlcNAc (Li et al, 2021) that show spatial responses to alterations of exogenous or salvaged fucose. Similar metabolic perturbations might also affect the distribution/association of their biosynthetic enzymes visualized by Förster resonance energy transfer (FRET) or proximity ligation analysis. This may indicate whether spatial and/or kinetic factors predominate."

4. Figure 1B, adding these three lines typically gives a number less than 50% (e.g., at 0 uM fucose). Does it mean the salvage pathway usually plays a significant role? This seems to be different from what is stated in the paper.

We cannot distinguish salvage vs. exogenous fucose contributions for cell-associated glycans (Figure 1); it is only possible for secreted, newly synthesized glycans using cells preloaded with fucosylated glycans (Figure 2). Based on these results, we believe that salvaged fucose makes a major contribution to cell-associated glycans, but it cannot directly measure it.

5. Are there any possible reasons to explain why CHO cells are different from other tested cell lines, in terms of the dependence on the salvage pathway?

Suitable paragraph was added in the discussion:

"To our knowledge, our study is the first to define and quantify the contributions of exogenous vs. salvaged monosaccharides, so there are no reliable precedents. Huh7 and HepG2 are professional secretory cells, sending a substantial portion of newly synthesized glycoproteins into the plasma. By comparison, CHO cells secrete much less glycoprotein and are likely engineered to preferentially recycle fucose."

6. CHO in Fig. 2 versus CHO-Lec13 in Supplementary Fig 1D, when fucose was at 50 uM, Fucose Ex)% is only around 30 in CHO, but ~100 in CHO-Lec13. Is there any reason?

CHO-Lec13, like HCT116, are GMDS mutants that lack the de novo pathway. Analysis of MS data from these cells using 50uM Fucose was defined 100%. CHO cells (Figure 2) analyzed newly-synthesized, secreted glycoproteins allowing analysis of the contribution of all sources.

7. The incorporation preference of GlcNAz could be explained by the preferences of different transferases to the azide analog.

Suitable sentence was added in the discussion:

"The other possible explanation is that different GlcNAc transferases may have various preference towards UDP-GlcNAc and UDP-GlcNAz."

Reviewer #2 (Comments to the Authors (Required)):

The authors have a very clear and interesting hypothesis that they set out to prove, namely that the metabolic origin of fucose determines its use. In addition, this leads to distinct and multiple GDP-fucose pools within the cell. The authors report the results of several different experiments to address the contributions of exogenous, salvaged and de novo sources of fucose to global N-glycosylation and that of individual glycoproteins. All these experiments lead to the support of their overall starting hypothesis that GDP-fucose exist in different pools within the cell. Certain experiments are performed in multiple different cell lines to add further evidence for their hypothesis.
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Based on our literature search there are many studies on biosynthesis of nucleotides and lipids that compare the contribution of the de novo biosynthesis to exogenous precursors, but they never simultaneously address all biosynthetic routes e.g., Moitra et al., 2021, Nieto et al., 2008, Wu et al., 2020, Zhang et al., 2019. To our knowledge there are only few studies addressing the contribution of different biosynthetic routs to nucleotide sugar biosynthesis i.e., Yurchenco and Atkinson, 1975; Yurchenco and Atkinson, 1977, Ichikawa et al., 2014 and Radenkovic et al., 2019. We ensured that all this literature is cited. Following sentences were added in the discussion:

“In contrast, for nucleotide sugars there is only a limited number of studies addressing this problem. In addition to the studies from 1970s’ on contributions of glucose, mannose and exogenous fucose into GDP-fucose (Yurchenco and Atkinson, 1975; Yurchenco and Atkinson, 1977), it has been demonstrated that exogenous mannose and exogenous galactose can overcome de novo produced GDP-mannose and UDP-galactose respectively, when provided in sufficient concentration (Ichikawa et al., 2014; Radenkovic et al., 2019).”

In addition, a suitable paragraph was added in the discussion:

“Directly visualizing multiple intracellular GDP-fucose pools in living cells that show real time responses to metabolic perturbations would certainly strengthen our claims. We have considered various indirect approaches, such as designing highly specific GDP-fucose sensors similar to that described for UDP-GlcNAc (Li et al, 2021) that show spatial responses to alterations of exogenous or salvaged fucose. Similar metabolic perturbations might also affect the distribution/association of their biosynthetic enzymes visualized by Förster resonance energy transfer (FRET) or proximity ligation analysis. This may indicate whether spatial and/or kinetic factors predominate.”

Could the authors also comment on the following:
• Why was fuc
ose used as a model monosaccharide and how relevant are the conclusions to other sugars. On this note I think the title should be altered to acknowledge that only one sugar is studied.

We decided to use fucose as a model monosaccharide because exogenous fucose is only converted to GDP-fucose. There is no evidence that fucose-1-P can be converted to any other substrate than GDP-fucose and GDP-fucose is only utilized for fucosylation and does not get converted to any other nucleotide sugar. Therefore, fucose metabolism is much easier to analyze comparing to other monosaccharides.

The title was modified as suggested.

• Why was a low concentration of mannose used (50 uM), in the plasma it is found between 50-100uM.

Physiological concentration of mannose in healthy mammals varies between 45 and 60uM (Alton et al. 1997, Soyama et al. 1984, Akazawa et al. 1986, Pitkanen et al. 1994). In our previous studies (Sharma and Freeze 2011, Ichikawa et al. 2014) we always used 50uM mannose as it is very close to an average mannose concentration 52.5uM. That is the reason we used 50uM mannose in this study as well.

• Fig 2A shows that there is a difference in cell types please can you discuss why this may be. Are the enzymes upregulated in these cells for example, understanding this may shed light into the mechanism of the utilisation of different pools.

Suitable paragraph was added in the discussion:

“To our knowledge, our study is the first to define and quantify the contributions of exogenous vs. salvaged monosaccharides, so there are no reliable precedents. Huh7 and HepG2 are professional secretory cells, sending a substantial portion of newly synthesized glycoproteins into the plasma. By comparison, CHO cells secrete much less glycoprotein and are likely engineered to preferentially recycle fucose.”

August 9, 2022

RE: JCB Manuscript #202205038R

Dr. Paulina Sosicka
Sanford Burnham Prebys Medical Discovery Institute
Human Genetics Program
10901 N Torrey Pines Rd
La Jolla, CA 92037

Dear Dr. Sosicka:

Thank you for submitting your revised manuscript entitled "Origin of cytoplasmic GDP-fucose determines its contribution to glycosylation reactions". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
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h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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Jeffrey Esko
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The authors have addressed all of my concerns. The current manuscript is suitable for publication.

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My concerns with the manuscript have been addressed to a certain degree and I have no further comments.