Journal of Cell Science

Identification of motifs and mechanisms for lipid droplet targeting of the lipolytic inhibitors G0S2 and HIG2

Latoya E. Campbell, Aaron M. Anderson, Yongbin Chen, Scott M. Johnson, Cailin E. McMahon and Jun Liu

DOI: 10.1242/jcs.260236

Editor: John Heath

Review timeline

Original submission:	17 May 2022
Editorial decision:	24 June 2022
First revision received:	28 October 2022
Accepted:	15 November 2022

Original submission

First decision letter

MS ID#: JOCES/2022/260236

MS TITLE: Identification of Lipid Droplet Targeting Motifs and Mechanisms for Lipolytic Inhibitors G0S2 and HIG2

AUTHORS: Latoya E Campbell, Aaron M Anderson, Yongbin Chen, Cailin E McMahon, and Jun Liu
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 raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

This manuscript describes identification of LD targeting motifs in ATGL inhibitors G0S2 and HIG2. In G0S2 authors reported that positively charged amino acids within the hairpin structure are important for ATGL independent targeting of G0S2 to LD. Also the presence of ATGL interacting motif YVGL favors ATGL dependent targeting of G0S2 to LD. In HIG2, unlike in G0S2, hairpin structure is missing and HIG2 targeting is ATGL dependent.

Comments for the author

This manuscript describes identification of LD targeting motifs in ATGL inhibitors G0S2 and HIG2.

In G0S2 authors reported that positively charged amino acids within the hairpin structure are important for ATGL independent targeting of G0S2 to LD. Also the presence of ATGL interacting motif YVGL favors ATGL dependent targeting of G0S2 to LD. In HIG2, unlike in G0S2, hairpin structure is missing and HIG2 targeting is ATGL dependent.

Suggestions for revisions:

1) For fig 1 a, it is worth to check if the treatment of forskolin and/ or isoproterenol affects targeting of G0S2 to LD either in the absence or presence of ATGL. Also to check if ATGL activity is the determining factor for targeting of G0S2/HIG2 to LD or not it is worth to do experiment in –OA/+ OA conditions overexpressing catalytically dead (S47A) full length ATGL.
2) For A3 mutant experiments, either fig-4 or Fig 6 it is also helpful to check targeting of A3 mutant with either mutation or deletion of YVGL motif in the same G0S2.
3) It is also helpful to check targeting of either A3 mutant and YVGL mutant targeting to LD in the presence of catalytically dead (S47A) full length ATGL. The same approach also should be tried for HIG2 targeting.
4) Authors reported that a hairpin motif is missing in HIG2 and showed targeting of HIG2 to LD in cells expressing ATGL, two experiments will strengthen this finding a) showing mutation of YVGL like motif and targeting of this mutant HIG2to LD in the presence and absence of ATGL b) also interactions of YVGL like motif mutant HIG2 and ATGL.

Reviewer 2

Advance summary and potential significance to field

Campbell et al study the localization and targeting of the adipose triglyceride lipase inhibitors G0S2 and the related protein HIG2 to the endoplasmic reticulum (ER) and to lipid droplets (LD). G0S2 (and HIG2) are two small proteins with a highly identical hydrophobic domain with 3 (2) predicted alpha-structures (in Suppl. Figures). The authors claim an ATGL-dependent and ATGL-independent mechanism localization for G0S2 (Fig. 1, Fig. 2). ATGL-independent localization of G0S2 to ER and LD is described as dependent on a hairpin-structure (Fig 2 Fig 3), and positively charged residues in the hinge between the two alpha-helices are identified as crucial for sorting G0S2 in the absences of ATGL. ATGL-dependent LD localization is observed for R20A/K22A/K25A -variants of G0S2 (Fig. 6). In this elegant study, they also study the shorter inhibitor HIG2. It lacks the hairpin and is described as ATGL-dependent for full LD targeting (Fig 7). The authors predominantly use immunofluorescence and confocal microscopy studies of selected protein variants (single amino acid exchanges, deletions) in cell studies combined with ‘quantitative’ colocalization analysis. In general the microscopy images are of very high quality and – on a qualitative level – the distribution between LD and ER seems highly plausible. In one experiment, a functional assay for inhibition of TG hydrolase activity is included. A very well thought-of discussion is implemented at the end of this interesting manuscript that will surely advance our knowledge on G0S2 localization and distribution.

Comments for the author

Major comments:

1) all Microscopy images need scale bars, indicate the origin of the enlarged parts

2) Where all the sequences derived from mouse or human? Should be stated clearly in the material and methods section and figure legends. This is important, since the different organisms have different numbers of positively charged residues in the hinge region.

3) For Fig 3, many of the G0S2 constructs are inactive in inhibiting lipid degradation, esp. delta 27-42, delta 15-26. Therefore, no LDs are expected and seen (visually, no LD stain was used). Along this line, G0S2 could not localize to non-existing LDs. Please comment or show that LD would be there to which G0S2 variants can localize (similar as done for Fig 4 and Fig5).

4) Figure 6A: G0S2 is reported as highly potent inhibitor of ATGL. Can you comment on the relatively low reduction of TG-hydrolytic activity observed in the used assay when performed with cellular lysates? Assuming overexpression of ATGL, is all the residual activity coming from other TG lipases? Please indicate that absolute values of TG hydrolase activity that is being measured at least in the figure legend (not only 100%). Does G0S2 derive from the in vitro translation system? What tag is on it? Why is the immunoblot signal of G0S2-mutant R20A/K22A/K25A lower compared to WT, what antibody was used? Please use the R20A/K22A/K25A nomenclature. Are these ATGL -/- Hela Cells as used for Fig 7A

5) Figure 6B: The authors should include the ATGL-deltaLBD variant. This would be a really good and important control to distinguish between cause and effect of co-localization for both, ATGL and G0S2. Just relying on the literature might be too vague, especially since this is a very prominent point for the arguments of the paper 'Intriguingly, while individually they were both deficient in LD localization' ….(and it also contributes and complements significantly to the nice experiments on Hig2 in Fig. 7.)

6) The positively charged hinge-region seems to be very important for LD-localizations shown by the comparison of G0S2 and HIG2. It would be really nice to know, if the 'hinge region' by its own could rescue the LD-localization of HIG2 in a "G0S2-hinge/HIG2 hybrid", or if the contribution of the first helix is also essential.

Minor aspects:

1) Please include more details on the quantitative bioimaging. Which algorithm was used, what mask was used, lenses (pixel densities), and other parameters should be included also to ensure the readers that statistics have been fully met.

2) Why where different LD-stains used? E.g. in Fig.1 vs Fig. 2?

3) Please use the R20A/K22A/K25A nomenclature throughout the manuscript as nicely done I Fig. 4 to improve readability of the figures at a first glance (not A(sup)3, not 'TripleA').

4) Could you give close-ups of the beautiful images in Fig. 4A and 4C ?

5) Fig 5: Immunoprecipitation. Can you give more experimental details? Are the tags N- oder C-terminal? Include the Tags on Panel 5B for ATGL. FLAG-GoS2 is misspelled, should be 'zero' rather than the letter 'O' When Myc-ATGL and G0S2-FLAG are co-expressed is the level of G0S2-FLAG expression so little, or do the antibodies work better for MYC-ATGL detection? ATGL is a ca 500 amino acid protein, does it degrade? Can you include the full WB?

7) Supplemental Figures 1 and 2: The color codes of the sequences should be adapted to the Alpha-Fold colors or vice versa. The bluish colors in both panels with different meanings are confusing. If not the entire figures, at least Panel A should be brought to the main manuscript including a brief indication of the used constructs. The hinge-residues in Suppl.1B should be indicated with residue numbers for clarity.

8) Fig 7A: is on OA treatment the same as or comparable to 15hrs in Figure 2?

9) Fig 7A/7B: Can you comment on the size of LD in Fig 7A vs 7B? Atgl -/- seems to have smaller LDs compared to the cells co-expressing ATGL. These seems counter-intuitive.

First revision

Author response to reviewers' comments

We thank the editor and the reviewers for the most helpful comments and suggestions. We have addressed each point with additional data or additional explanation, and have made modifications to the manuscript accordingly.

Reviewer 1:

1) For fig 1 a, it is worth to check if the treatment of forskolin and/or isoproterenol affects targeting of G0S2 to LD either in the absence or presence of ATGL. Also to check if ATGL activity is the determining factor for targeting of G0S2/HIG2 to LD or not it is worth to do experiment in - OA/+ OA conditions overexpressing catalytically dead (S47A) full length ATGL.

In primary hepatocytes, forskolin did not appear to change the LD localization of G0S2 (data not shown). As revealed by co-staining of G0S2 and the S47A mutant of ATGL (new Fig. 1C), the LD targeting of G0S2 by ATGL seems to be independent of ATGL activity. Same was observed with the LD localization of HIG2 when ATGL/S47A mutant was coexpressed (new Fig. 7C).

2) For A3 mutant experiments, either fig-4 or Fig 6 it is also helpful to check targeting of A3 mutant with either mutation or deletion of YVGL motif in the same G0S2.

We have included the data on targeting of G0S2/R20A/K22A/K25A/Δ27-30 in the new Fig. 6B, which shows that deletion of the ATGL-interacting YVGL motif in the G0S2/R20A/K22A/K25A mutant abolished its LD recruitment by ATGL.

3) It is also helpful to check targeting of either A3 mutant and YVGL mutant targeting to LD in the presence of catalytically dead (S47A) full length ATGL. The same approach also should be tried for HIG2 targeting.

We have included new data in Fig. 1C and Fig. 7C to show that the S47A mutation does not affect the ability of ATGL to recruit G0S2 to LDs.

4) Authors reported that a hairpin motif is missing in HIG2 and showed targeting of HIG2 to LD in cells expressing ATGL, two experiments will strengthen this finding a) showing mutation of YVGL like motif and targeting of this mutant HIG2to LD in the presence and absence of ATGL b) also interactions of YVGL like motif mutant HIG2 and ATGL.

In our previous paper, deletion of LYVGL motif in HIG2 led to a complete loss of ATGL interaction (Fig. 2F in PMC5739538) and inhibition (Fig. 3A-3E in PMC5739538). As a result, the LDs were degraded by ATGL in the presence of HIG2 ΔLYVGL (Fig. 3G in PMC5739538).

Reviewer 2:

Major points:

1) All Microscopy images need scale bars, indicate the origin of the enlarged parts.

Scale bars have now been added, and the origin of the enlarged areas marked.

2) Where all the sequences derived from mouse or human? Should be stated clearly in the material and methods section and figure legends. This is important, since the different organisms have different numbers of positively charged residues in the hinge region.

All the sequences are derived from mouse proteins, which are now stated specifically in the Methods and figure legends.

3) For Fig 3, many of the G0S2 constructs are inactive in inhibiting lipid degradation, esp. delta 27-42, delta 15-26. Therefore, no LDs are expected and seen (visually, no LD stain was used). Along this line, G0S2 could not localize to non-existing LDs. Please comment or show that LD would be there to which G0S2 variants can localize (similar as done for Fig 4 and Fig5).

We have included BODIPY staining in the new Fig. S2. to reveal the LDs. The experiment was performed in ATGL$^{-/-}$ HeLa cells, which accumulate LDs upon OA treatment.

4) Figure 6A: G0S2 is reported as highly potent inhibitor of ATGL. Can you comment on the relatively low reduction of TG-hydrolytic activity observed in the used assay when performed with cellular lysates? Assuming overexpression of ATGL, is all the residual activity coming from other TG lipases? Please indicate that absolute values of TG hydrolase activity that is being measured at least in the figure legend (not only 100%). Does G0S2 derive from the in vitro translation system? What tag is on it? Why is the immunoblot signal of G0S2-mutant R20A/K22A/K25A lower compared to WT, what antibody was used? Please use the R20A/K22A/K25A nomenclature. Are these ATGL -/- Hela Cells as used for Fig 7A.

This specific experiment was conducted by mixing equal amounts of in vitro translated ATGL with lysates of ATGL$^{-/-}$ HeLa cells expressing vector alone or FLAG-tagged G0S2. The absolute amount of ATGL protein produced from the Promega in vitro transcription/translation system was not quantifiable, making it impossible to calculate the absolute values of TG hydrolase activity in this specific instance. However, as a qualitative rather than a quantitative assay, we think it serves the purpose of demonstrating the difference in the inhibitory activity between WT and R20A/K22A/K25A mutant of G0S2, especially when they were expressed at similar levels.

The reason why the R20A/K22A/K25A mutant band is lower than the WT protein, we suspect, is that the reduced amount of positive charges renders the mutant migrate faster on SDS-PAGE.

We have now replaced all “TripleA” with “R20A/K22A/K25A” as suggested.

5) Figure 6B: The authors should include the ATGL-deltaLBD variant. This would be a really good and important control to distinguish between cause and effect of co-localization for both, ATGL and G0S2. Just relying on the literature might be too vague, especially since this is a very prominent point for the arguments of the paper ‘Intriguingly, while individually they were both deficient in LD localization’ ….(and it also contributes and complements significantly to the nice experiments on Hig2 in Fig. 7.)

Good point. We have now included ATGL-ΔLBD alone in Fig. 6C as suggested.

6) The positively charged hinge-region seems to be very important for LD-localizations shown by the comparison of G0S2 and HIG2. It would be really nice to know, if the ‘hinge region’ by its own could rescue the LD-localization of HIG2 in a “G0S2-hinge/HIG2 hybrid”, or if the contribution of the first helix is also essential.

Thanks for this excellent suggestion. We have included new data in the new Fig.7C to show that addition of the first helix and hinge sequence of G0S2 to the N-terminus of HIG2 was able to fully drive the hybrid protein to LDs that are detached from ER. In comparison, addition of the G0S2 hinge sequence alone yielded a fusion protein that partially localizes to the ER-attached LDs. The data support the concept that a complete hairpin is required for sorting the protein from ER onto the LD surface.

Minor aspects:

1) Please include more details on the quantitative bioimaging. Which algorithm was used, what mask was used, lenses (pixel densities), and other parameters should be included also to ensure the readers that statistics have been fully met.

We have included more details on the image quantification in the Methods.

2) Why where different LD-stains used? E.g. in Fig.1 vs Fig. 2?

We used green BODIPY for LDs in Fig. 1 to accommodate ATGL staining with a Alexa633 secondary.

3) Please use the R20A/K22A/K25A nomenclature throughout the manuscript as nicely done I Fig. 4 to improve readability of the figures at a first glance (not A(sup)3, not 'TripleA').

Done as suggested. Thanks.

4) Could you give close-ups of the beautiful images in Fig. 4A and 4C ?

We have now provided close-up images in Fig. 4A and 4C.

5) Fig 5: Immunoprecipitation. Can you give more experimental details? Are the tags N- or C-terminal? Include the Tags on Panel 5B for ATGL. FLAG-GoS2 is misspelled, should be 'zero' rather than the letter 'O' When Myc-ATGL and G0S2-FLAG are co-expressed is the level of G0S2-FLAG expression so little, or do the antibodies work better for MYC-ATGL detection? ATGL is a ca 500 amino acid protein, does it degrade? Can you include the full WB?

The detailed procedure for immunoprecipitation is described in the "Transient transfection and protein analysis" section of the Methods. We have now included more details on the orientation of the epitope tags as well as corrected the misspelling. The Western signals of G0S2-FLAG and Myc-ATGL are not directly comparable as different primary antibodies were used at different dilution ratios. The reason why G0S2-FLAG looks faint in lysates is because lysates and IP samples were run side by side, and we highly diluted FLAG antibody for Western so to avoid oversaturation of G0S2-FLAG signal in IPs.

7) Supplemental Figures 1 and 2: The color codes of the sequences should be adapted to the Alpha-Fold colors or vice versa. The bluish colors in both panels with different meanings are confusing. If not the entire figures, at least Panel A should be brought to the main manuscript including a brief indication of the used constructs. The hinge-residues in Suppl.1B should be indicated with residue numbers for clarity.

In AlphaFold, the default colors indicate model confidence. To avoid confusion and as suggested, we have moved the sequence alignment in old Fig. S1A to the new Fig. 3A, which now precedes the deletion analysis in the new Fig. 3B.

8) Fig 7A: is on OA treatment the same as or comparable to 15hrs in Figure 2?

Yes. We have now changed "O.N." in Fig. 7A to "15 hrs" to be consistent.

9)Fig 7A/7B: Can you comment on the size of LD in Fig 7A vs 7B? Atgl -/- seems to have smaller LDs compared to the cells co-expressing ATGL. These seems counter-intuitive.

Yes, we consistently observed larger LDs when HIG2 was co-expressed with ATGL, suggesting a gain of function by the ATGL/HIG2 complex in addition to ATGL inhibition. Currently the underlying mechanisms are still under investigation.

Second decision letter

MS ID#: JOCES/2022/260236

MS TITLE: Identification of Lipid Droplet Targeting Motifs and Mechanisms for Lipolytic Inhibitors G0S2 and HIG2

AUTHORS: Latoya E Campbell, Aaron M Anderson, Yongbin Chen, Scott M Johnson, Cailin E McMahon, and Jun Liu
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

Authors of this revised manuscript satisfied quarries I raised during my first review. Therefore, I feel this revised manuscript should be accepted for publication in JCS.

Comments for the author

Authors of this revised manuscript satisfied quarries I raised during my first review. Therefore, I feel this revised manuscript should be accepted for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

Wonderful paper, congratulations to the team of authors!

Comments for the author

All points have been addressed in the revision.