

Molecular glues of the regulatory ChREBP/14-3-3 complex protect beta cells from glucolipotoxicity

Corresponding Author: Dr Donald Scott

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting paper in which Katz and colleagues explore the possibility that trapping the transcription factor ChREBPalpha in the cytosol may restrict activation of carbohydrate response genes and consequently limit beta cell death or dysfunction provoked by glucolipotoxic conditions. The authors have identified 14-3-3 proteins as potential anchors for ChREBPalpha and, in an impressive collaboration involving two separate chemical biology teams, have now generated enhanced versions of their earlier (non cell-permeant) molecular 'glues' capable of stabilizing the above PPI interaction. These compounds are shown to protect both rodent and human beta cells from cell death associated with glucolipotoxicity. Further studies show that the most promising compound, compound 43, enhances cytosolic retention of ChREBPalpha and suppresses the up-regulation of the splice variant and hyper-activated form, ChREBPbeta, under glucolipotoxic conditions. Appropriate controls are performed e.g. to exclude the artefactual loss of ChREBPalpha antibody interaction in the presence of the molecular glue.

As a proof-of-principle, the work is likely to have ramifications beyond the field of glucose (lipid) -regulated genes and diabetes. Nevertheless, I have some suggestions for further improvement, as discussed below.

Major

1. Is interference with the ChREBPalpha-14-3-3 interaction, and reversal of glucolipotoxicity, the sole mechanism of action of the most effective molecular glues developed (30,43,53)? To give just one example, hydrophobic molecules such as this may serve as protonophores, (weakly) uncoupling mitochondria and activating AMP-activating protein kinase (AMPK) to enhanced oxidation of fatty acids. Other potential actions include blockade of calcium channels. These questions could be addressed in several ways, including studies of closely-related structural analogues, measurements of intracellular ATP/ADP ratio, and AMPK activity (or phosphorylation of downstream substrates). Another important and powerful control would be the use of ChREBPalpha null cells.

2. Studies of the effects of the identified molecules on cellular secretory function are completely lacking in the present study, which is a significant weakness. Data need to be presented on glucose (and preferably other agent, such as incretin) - stimulated insulin secretion in vivo and in vitro, preferably complemented by indicators of acute glucose cell signalling in the beta cell (such as intracellular Ca²⁺ dynamics, ATP/ADP increases, mitochondrial membrane potential changes, etc.)

3. Do the compounds described impact apoptosis stimulated by other agents (cytotoxic cytokines, for example?)

4. Is ChREBP phosphorylation by PKA, AMPK etc. likely to influence interaction with 14-3-3s? See <https://pubmed.ncbi.nlm.nih.gov/31275349/>

5. Are 14-3-3 proteins necessarily the best or indeed the only potential anchors for ChREBP? There are several other validated interactors with ChREBP with important roles in the beta cell (e.g. <https://pubmed.ncbi.nlm.nih.gov/22338092/>)

Minor

1. Please include a list of abbreviations

2. Line 56 : imposes or poses?
3. Line 57. As there are multiple 14-3-3 proteins, as later indicated. Please always refer to 14-3-3 proteins in the plural rather than 14-3-3 protein
4. Line 59 – the role of a lowering in beta cell mass in human T2D is debated and unproven; eg see Marselli et al Diabetologia 2014 <https://pubmed.ncbi.nlm.nih.gov/24233056/>. Firm conclusions await measurements of beta cell mass prospectively in the same patient before and then during the course of T2D development
5. Line 119 – by affecting permeation into cells? Meaning unclear. Please clarify.
6. Line 306: calculated free concentration of palmitate?
7. Line 336: beta cell identity. PDX1 + INS1 were upregulated, but please check a few more identity genes e.g. Glut2/slc2a2, Gck as well as 'disallowed' genes, Ldha, Slc16a, Aldh1a3 etc.
8. Line 816: beta cells (plural)

Reviewer #2

(Remarks to the Author)

This study shows an interesting idea of using molecular glues (MGs) and 14-3-3 to target transcriptional factors (TFs). MGs stabilize the interaction between ChREBP α and 14-3-3. The cellular active MGs retain ChREBP α in the cytoplasm to inhibit its TF activity and reduce glucolipotoxicity. The MG mechanisms of several compounds are validated by ternary complex structures, which guide the ligands optimization. Moreover, the specificity of top compounds is shown at biophysical and cellular levels. But there are noted weaknesses that need to be addressed.

Major points:

1. Line 101 "ChREBP β lacks this N-terminal domain, resulting in a nuclear, constitutively active, hyper-potent TF." If ChREBP β is constitutively active, why does the ChREBP α regulation matter?
 2. Line 102-104 "In healthy conditions, glucose flux leads to dissociation of ChREBP α from 14-3-3 resulting in its translocation to the nucleus and induction of ChREBP β transcription, ultimately leading to glucose-stimulated β -cell proliferation to meet the demand for insulin (Fig. 1b)". ChREBP β is not shown in Fig.1b.
 3. Line 105-108 "However, under prolonged hyperglycemic conditions, a robust positive feedback loop is triggered in which the newly produced ChREBP β binds to its own promoter sites leading to even more ChREBP β synthesis eventually resulting in glucolipotoxicity and β -cell apoptosis (Fig. 1c)."
- Fig.1c shows ChREBP β binds the ChoRE site, which is also the site for ChREBP α binding as shown in Fig.1b. Under prolonged hyperglycemic condition, large amounts of ChREBP β are synthesized and occupy ChoRE. Even when ChREBP α is in the nucleus, it seems hard to act on ChoRE. How could MG help attenuate glucolipotoxicity and protect β -cells?
- "ChREBP β binds to its own promoter sites leading to even more ChREBP β synthesis". If MG-14-3-3 trapped ChREBP α in the cytosol, no ChREBP α will enter nucleus to compete with ChREBP β for the ChoRE-binding. Is it easier for ChREBP β to perform its TF activity and induce β -cell death?
4. In Fig.2c, the affinity of compound#1 to 14-3-3 is $K_{DII} = 161 \mu M$. Where does this number come from? The assay contains two proteins. But this affinity should be measured by titrating the compound to 14-3-3 only. Please also address the similar issue for other figures with K_{DII} (3b-c, S7a, S8).
 5. In Fig.2c, 3b-c, S7a, S8, how is the cooperativity value calculated? Please explain in the manuscript instead of citing a previous paper. It would be convenient if readers can find the explanation in the Figure legends. In addition, what's relationship between cooperativity and the value (e.g., 25x, 138x, 60x...)?
 6. Line 215 "The change in apparent K_D of the ChREBP α /14-3-3 complex, over different concentrations of stabilizer (Fig. S7b)"
- The affinity (K_D) is an intrinsic property among binding partners, which should be independent of ligand or protein concentrations. Here, the apparent K_D changes and likely reflects a mixture of binary and ternary binding events. When the MG concentration is not high enough to saturate 14-3-3, there is a mixture of apo 14-3-3 and the MG-loaded 14-3-3. They show different affinities to ChREBP α .
7. Fig.3d shows compound 43 is selective to ChREBP but not to other 14-3-3 binding proteins. One 14-3-3 isoform (14-3-3 β) is used in the assay. Will 43 enhance interaction between the other 6 isoforms of 14-3-3 and their binding proteins?
 8. Fig.5a. In the control, 14-3-3 can bind ChREBP α in the low glucose condition. In the high glucose 30min condition, why can 43 mediated protein-protein interaction keep ChREBP α in cytoplasm but the basal ChREBP α -14-3-3 binding can't?

9. How does 43 impact the mRNA levels of INS and PDX1 (Fig. 6f-g)? Explanation/discussion is needed.

Minor points:

1. Line 372 "Combined, this high cooperativity and selectivity for the ChREBP α /14-3-3 PPI conferred cellular activity to our lead compound". In the introduction, the authors mentioned previous compounds also enhance the PPI but lack the cellular activity. Here the cellular activity of the lead compound may be due to better cell membrane permeability.

2. Line 403, change "active" to "activity"?

3. page S7 should be "Fig. S7" but not "Fig. S1"

page S10 should be "Fig. S11" but not "Fig. S2"

Reviewer #3

(Remarks to the Author)

This manuscript describes the SAR analysis of a small molecule series that stabilizes the interaction between glucose-responsive transcription factor ChREBP α and the 14-3-3 regulatory scaffold protein in pancreatic β -cells. Stabilization of this interaction prevents the nuclear localization of ChREBP α , an isoform that binds to 14-3-3, in response to high glucose and thus prevents the expression and activation of ChREBP β , a second shorter nuclear-localized isoform lacking 14-3-3 binding domain. When ChREBP β is activated, a feedforward activation loop ultimately leads to glucolipotoxicity and pancreatic cell death in T2D. The report presents a structure-activity relationship investigation for a series of compounds based on a previously identified moderately active compound. Through this work, a set of compounds were identified that demonstrated higher potency and binding selectivity when tested against peptide binding motifs of other 14-3-3 client proteins. Notably, the introduction of a geminal-difluoro group resulted in a slight bend in the small molecule structure leading to the observed stabilization of the complex formation between ChREBP and 14-3-3. Crystal structures for several of the compounds were reported to support these observations and data is presented that shows that some but not all the optimized compounds result in increased complex formation, increased cytoplasmic localization and decreased glucolipotoxicity in cellular assays. While the discovery of this class of molecules was described in an earlier report by several of the co-authors (Sijbesma, et al, Nature Communications, Article number 3954, 2020), the current work represents a considerable advance from those findings. Manuscript is well written and clear and would be of high interest to the broader audience of Nature Communications. Overall this work provides evidence of a significant advance in the ability to use molecular glue technology to modulate a regulatory binding interaction and impact a pathological biological process in a cellular context. Some minor points are noted below:

1. The authors claim that the improvement in cooperativity is not a result of increased binding affinity for 14-3-3, but instead, interactions with ChREBP α based on the analysis of the fluorescence anisotropy assay data. In this experiment, 14-3-3 protein is titrated at a constant compound concentration and this is measured at several compound levels (2D titration). The K_d for binding to 14-3-3 (K_{dII}) and the cooperativity factor α were obtained from a fit of the data using a four-parameter logistics model. Because these values would be correlated in the fit, it is not clear whether the data obtained can definitively support the conclusion stated. Is it possible to get an independent direct measurement of K_{dII} separately? This could be obtained from an experiment such as ITC, Surface Plasmon Resonance, or another direct binding assay. This would reduce the ambiguity of the analysis presented here. The observation of new interactions with the ChREBP α peptide with compound 43 is, however, consistent with the conclusion.

2. The assays for specificity, while compelling, are based wholly on in vitro binding interactions with peptide motifs. The findings would be more impactful and give confidence in translatability if supporting cell-based data was provided. For example, the qPCR assay for mRNA in β -cells or transcriptomics could be used to assess the potential impact on other 14-3-3 client protein functions.

3. As a related question, could there be a secondary effect of sequestration of the available 14-3-3 by ChREBP α binding in high glucose that would have an indirect effect on other 14-3-3 client proteins?

4. Given the stated aspiration to progress this class of molecules as therapeutics, it may be useful to include sequence conservation or alignment of the interacting regions of 14-3-3 and ChREBP α across a set of common preclinical species to understand feasibility of translation for in vivo efficacy and safety studies.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded fully to my suggestions. No further comments.

Reviewer #2

(Remarks to the Author)

My remaining concern is about how the cooperativity value and KDII are determined.

The authors mentioned in the rebuttal letter:

"The cooperativity factor of the ternary complex is determined by fitting, using a thermodynamic equilibrium model (reference#11).

"In addition, this model is also able to determine the KDII value, the affinity of the compound to 14-3-3"

"We think though that our cooperativity model provides for a more standardized and generally accepted analysis of the binding characteristics of molecular glues, also because these are based on multiple titration data, that allow for a more robust analysis."

"Reference#11. Geertjens, N.H., de Vink, P.J., Wezeman, T., Markvoort, A.J. & Brunsveld, L. A general framework for straightforward model construction of multi-component thermodynamic equilibrium systems. bioRxiv, 2021.2011.2018.469126 (2021)."

The authors claim their model is standardized and generally accepted. However, the evidence does not support this assertion. Reference #11 has been cited by fewer than five papers, most of which are from authors associated with this manuscript. After reading reference #11, I remain unconvinced. I would like to compare experimentally determined cooperativity and other affinity values with the model's values to evaluate the model's validity. My intention is to be constructive. If the authors have other examples where experimentally determined values (cooperativity, KDII) match well with the model's values, please provide them.

This concern is shared not only by me (Reviewer #2) but also by Reviewer #3, who raised a similar issue in their first point.

Reviewer #3

(Remarks to the Author)

After reviewing the amendments made by the authors and the responses to my comments as well as those of other reviewers, my primary concerns have been sufficiently addressed and I recommend publication.

Upon review of this revision, I did note one small typographical error in Table S4, where crystallographic statistics are listed. For PDB 8BWH, there appears to be a typo in the field for Rfree, where it looks like the authors have inadvertently duplicated the reflection set (1050(104)) rather list the Rfree value. It would be good to carefully review that table before final publication.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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We thank the reviewers for their positive comments and for their many helpful suggestions. We address each of their comments below in blue. We feel that their comments have been helpful to further strengthen our manuscript and hope that with all these changes made the reviewers and the editorial board now deem the manuscript as suitable for publication in Nature Communications.

Reviewer #1 (Remarks to the Author):

This is an interesting paper in which Katz and colleagues explore the possibility that trapping the transcription factor ChREBPalpha in the cytosol may restrict activation of carbohydrate response genes and consequently limit beta cell death or dysfunction provoked by glucolipotoxic conditions. The authors have identified 14-3-3 proteins as potential anchors for ChREBPalpha and, in an impressive collaboration involving two separate chemical biology teams, have now generated enhanced versions of their earlier (non cell-permeant) molecular 'glues' capable of stabilizing the above PPI interaction. These compounds are shown to protect both rodent and human beta cells from cell death associated with glucolipotoxicity. Further studies show that the most promising compound, compound 43, enhances cytosolic retention of ChREBPalpha and suppresses the up-regulation of the splice variant and hyper-activated form, ChREBPbeta, under glucolipotoxic conditions. Appropriate controls are performed e.g. to exclude the artefactual loss of ChREBPalpha antibody interaction in the presence of the molecular glue.

As a proof-of-principle, the work is likely to have ramifications beyond the field of glucose (lipid) - regulated genes and diabetes. Nevertheless, I have some suggestions for further improvement, as discussed below.

We thank the reviewer for the positive feedback and appreciation of the broad impact of our work. We of course also appreciate the suggestions for further improvement.

1. Is interference with the ChREBPalpha-14-3-3 interaction, and reversal of glucolipotoxicity, the sole mechanism of action of the most effective molecular glues developed (30,43,53)? To give just one example, hydrophobic molecules such as this may serve as protonophores, (weakly) uncoupling mitochondria and activating AMP-activated protein kinase (AMPK) to enhanced oxidation of fatty acids. Other potential actions include blockade of calcium channels. These questions could be addressed in several ways, including studies of closely-related structural analogues, measurements of intracellular ATP/ADP ratio, and AMPK activity (or phosphorylation of downstream substrates). Another important and powerful control would be the use of ChREBPalpha null cells.

We have addressed this important question in a number of ways. One, AICAR, an activator of AMP kinase, did not recapitulate the effects of compound 43 (new Fig 5 c,e). Second, we showed that compound 43 does not uncouple mitochondria, since MitoTracker orange staining did not change upon compound 43 treatment, neither in low (5.5 mM) nor high (20 mM) glucose conditions (new Fig S21b). Third, ATP/ADP ratios did not change in the presence of 43 in 5.5 mM glucose and increased only slightly in 20 mM glucose (new Fig S21c). Fourth, inactive, but structurally similar compounds (12 and 66) did not mimic the actions of 43 (new Fig 4). Five, 43 was not able to block the actions of ChREBPbeta overexpression (new Fig S18), consistent with our

interpretation that ChREBP α (and its release from 14-3-3) is necessary to induce the run-away production of ChREBP β and drive apoptosis. These complementary experiments thus all support our view that the stabilized ChREBP-14-3-3 interaction is the molecular basis of the observed phenotypic effects.

2. Studies of the effects of the identified molecules on cellular secretory function are completely lacking in the present study, which is a significant weakness. Data need to be presented on glucose (and preferably other agent, such as incretin) -stimulated insulin secretion in vivo and in vitro, preferably complemented by indicators of acute glucose cell signalling in the beta cell (such as intracellular Ca²⁺ dynamics, ATP/ADP increases, mitochondrial membrane potential changes, etc.)

We have added glucose-stimulated insulin secretion and found that **43** treatment does not significantly reduce the secretion index, in response to glucose or KCl, and in fact increases the secretion index after beta cells are cultured overnight in high glucose (new **Fig 4f**).

In addition, **43** does not appreciably change mitochondrial activity as determined by MitoTracker orange and ATP/ADP levels as mentioned above and shown in new **Fig S21b,c**. Furthermore, we imaged intracellular Ca⁺⁺ as 5.5 mM glucose was increased to 20 mM glucose and found no difference in Ca⁺⁺ signals (new **Fig S21a**).

3. Do the compounds described impact apoptosis stimulated by other agents (cytotoxic cytokines, for example?)

This is a very interesting question - one that we have asked ourselves. We very recently found that ChREBP β and probably also ChREBP α levels increase dramatically after cytokine exposure and enhanced ER stress combined with high glucose levels. In addition, we found that the molecular glues protect β -cells from these stresses just as well as from glucolipotoxicity. This suggests that ChREBP is a contextual stress responsive-transcription factor, rather than just a carbohydrate-sensing transcription factor. We hope the reviewer understands that we believe this is conceptually novel, but outside the scope the present manuscript, and the topic of a near future manuscript.

4. Is ChREBP phosphorylation by PKA, AMPK etc. likely to influence interaction with 14-3-3s? See <https://pubmed.ncbi.nlm.nih.gov/31275349/>

PKA phosphorylates residue Ser196 of ChREBP and this phosphorylation has been reported to contribute to 14-3-3 binding of ChREBP¹. Multivalent binding by client proteins to the bivalent 14-3-3 platform has been described for a number of 14-3-3 binding partners²⁻⁴. Our molecular glues are specifically designed for the α 2 helix of ChREBP (residues 117-137) and 14-3-3, which is phosphorylation-independent (see new **Fig S22**). As such, a ChREBP phosphorylation event would most probably be beneficial for the strengthening of the interaction between the two proteins, in synergy with the mode of action of the molecular glues. As for AMPK, we tested if AICAR recapitulated the effects of **43** and found that, in agreement with Uyeda and colleagues, AMPK does not affect ChREBP/14:3:3 interaction (**Fig 4, ¹**).

5. Are 14-3-3 proteins necessarily the best or indeed the only potential anchors for ChREBP? There are several other validated interactors with ChREBP with important roles in the beta cell (e.g. <https://pubmed.ncbi.nlm.nih.gov/22338092/>)

This is an important question. Sorcin is a cytoplasmic anchor for ChREBP and lipid droplets may also sequester ChREBP in the cytoplasm^{5,6}. These anchors may be very important under specific physiological situations. That the molecular glues enhancing ChREBP:14-3-3 interaction are so effective at protecting β -cells from glucolipotoxicity suggests that increasing this interaction is sufficient to suppress nuclear entry and transcription activity, but does not in any way exclude the potential important physiological roles played by other anchors. This idea was added to the Discussion.

Minor

1. Please include a list of abbreviations

We have added a list of abbreviations.

2. Line 56 : imposes or poses?

Thank you – changed to imposes.

3. Line 57. As there are multiple 14-3-3 proteins, as later indicated. Please always refer to 14-3-3 proteinS in the plural rather than 14-3-3 protein

Thank you. This has been done.

4. Line 59 – the role of a lowering in beta cell mass in human T2D is debated and unproven; eg see Marselli et al Diabetologia 2014 <https://pubmed.ncbi.nlm.nih.gov/24233056/>. Firm conclusions await measurements of beta cell mass prospectively in the same patient before and then during the course of T2D development

We agree this is an important point and have changed our description to “functional β -cell mass”.

5. Line 119 – by affecting permeation into cells? Meaning unclear. Please clarify.

This was clarified by saying “increasing its permeation into cells”.

6. Line 306: calculated free concentration of palmitate?

We did not calculate the free palmitate concentration but rather used a diluted BSA:palmitate solution in a 6:1 ratio (5 mM) as purchased by Cayman (Cat# 29558); this was clarified in the Methods.

7. Line 336: beta cell identity. PDX1 + INS1 were upregulated, but please check a few more identity genes e.g. Glut2/slc2a2, Gck as well as 'disallowed' genes, Ldha, Slc16a, Aldh1a3 etc.

We added LDHA, ALDH1a3, GCK, and SLC2a2 to Fig S20.

8. Line 816: beta cells (plural)

Thank you. This was fixed.

Reviewer #2 (Remarks to the Author):

This study shows an interesting idea of using molecular glues (MGs) and 14-3-3 to target transcriptional factors (TFs). MGs stabilize the interaction between ChREBP α and 14-3-3. The cellular active MGs retain ChREBP α in the cytoplasm to inhibit its TF activity and reduce glucolipotoxicity. The MG mechanisms of several compounds are validated by ternary complex structures, which guide the ligands optimization. Moreover, the specificity of top compounds is shown at biophysical and cellular levels. But there are noted weaknesses that need to be addressed.

We thank the reviewer for this positive feedback on our study and for the suggestions to improve the manuscript.

Major points:

1. Line 101 "ChREBP β lacks this N-terminal domain, resulting in a nuclear, constitutively active, hyper-potent TF." If ChREBP β is constitutively active, why does the ChREBP α regulation matter?

This is an important question. We added a section to Results to address this as follows: "ChREBP β is a constitutively active transcription factor that promotes its own production by binding to its own promoter^{7,8}. This raises questions of how ChREBP β production gets turned off and what is its relationship to ChREBP α once it begins the positive-feedback mechanism. To address this, we analyzed the two isoforms' half-lives. ChREBP β , under high glucose concentrations, exhibits substantial instability, with a half-life as short as 12 minutes. In contrast, ChREBP α displays increased stability, with its half-life extending beyond 30 minutes (Fig. S2). These findings suggest that without continuous priming by ChREBP α activity, ChREBP β production would be self-limiting, and provides a mechanistic explanation of how the positive-feedback production of ChREBP β can be turned off when glucose levels decrease, or when ChREBP α is prevented from entering the nucleus when glucose levels are high." Thus, the molecular glues block both the initiation of the feed-forward production of ChREBP β and the maintenance of the production of ChREBP β .

2. Line 102-104 “In healthy conditions, glucose flux leads to dissociation of ChREBP α from 14-3-3 resulting in its translocation to the nucleus and induction of ChREBP β transcription, ultimately leading to glucose-stimulated β -cell proliferation to meet the demand for insulin (Fig. 1b)”. ChREBP β is not shown in Fig.1b.

We have added ChREBP β to Fig 1b.

3. Line 105-108 “However, under prolonged hyperglycemic conditions, a robust positive feedback loop is triggered in which the newly produced ChREBP β binds to its own promoter sites leading to even more ChREBP β synthesis eventually resulting in glucolipotoxicity and β -cell apoptosis (Fig. 1c).”

Fig.1c shows ChREBP β binds the ChoRE site, which is also the site for ChREBP α binding as shown in Fig.1b. Under prolonged hyperglycemic condition, large amounts of ChREBP β are synthesized and occupy ChoRE. Even when ChREBP α is in the nucleus, it seems hard to act on ChoRE. How could MG help attenuate glucolipotoxicity and protect β -cells?

“ChREBP β binds to its own promoter sites leading to even more ChREBP β synthesis”. If MG-14-3-3 trapped ChREBP α in the cytosol, no ChREBP α will enter nucleus to compete with ChREBP β for the ChoRE-binding. Is it easier for ChREBP β to perform its TF activity and induce β -cell death?

Please see answer to Question 1. In short, blocking ChREBP α blocks both the induction and the maintenance of the feed-forward production of the hyperactive ChREBP β .

4. In Fig.2c, the affinity of compound#1 to 14-3-3 is $K_{DII} = 161 \mu\text{M}$. Where does this number come from? The assay contains two proteins. But this affinity should be measured by titrating the compound to 14-3-3 only. Please also address the similar issue for other figures with K_{DII} (3b-c, S7a, S8).

5. In Fig.2c, 3b-c, S7a, S8, how is the cooperativity value calculated? Please explain in the manuscript instead of citing a previous paper. It would be convenient if readers can find the explanation in the Figure legends. In addition, what's relationship between cooperativity and the value (e.g., 25x, 138x, 60x...)?

6. Line 215 “The change in apparent K_D of the ChREBP α /14-3-3 complex, over different concentrations of stabilizer (Fig. S7b)”

The affinity (K_D) is an intrinsic property among binding partners, which should be independent of ligand or protein concentrations. Here, the apparent K_D changes and likely reflects a mixture of binary and ternary binding events. When the MG concentration is not high enough to saturate 14-3-3, there is a mixture of apo 14-3-3 and the MG-loaded 14-3-3. They show different affinities to ChREBP α .

(Questions 4-6 Reviewer 2, and Question 1 of Reviewer 3)

We combine our answers to these three questions and suggestions here, as this allows for a more concise response.

Cooperativity is a well-known phenomenon in the field of molecular glues where the binding of the molecular glue enhances the binding affinity between two proteins, and vice versa, resulting in a more stable ternary complex^{9, 10}. The cooperativity factor (α), in combination with the K_{DII} , allows for an objective metric to express the potency of molecular glues for PPI stabilization independent of the concentration of the protein components (apparent K_D or EC_{50} values are typically dependent on the relative concentrations of the components in the system, which makes that such values are not fully objective).

The cooperativity factor of the ternary complex is determined by fitting, using a thermodynamic equilibrium model¹¹. We have added this statement to the figure legends describing the cooperativity factor. In addition, this model is also able to determine the K_{DII} value, the affinity of the compound to 14-3-3; the typical low affinity of small molecular glues for the individual proteins limits their direct experimental determination with classical biophysical techniques. Based on the equilibrium equations of the system, Fluorescence Anisotropy titration data and the known K_D (affinity 14-3-3/ChREBP), the model can fit the cooperativity factor and K_{DII} value simultaneously, using the 2D-titrations. New Figure S5 is added to further clarify the underlying thermodynamic equilibrium model. (It should be noted that the approach to determine cooperativity and affinity of molecular glues via 2D-titrations and fitting based on a thermodynamic equilibrium model are not new to this publication but has been published previously by us and others and is currently state-of-the-art in the molecular glue community.)

To support the K_{DII} value calculated by the model, ITC experiments were attempted to directly measure the K_{DII} . However, as with other molecular glues, this did not work because the individual interaction to only one of the proteins from the interaction pair is too weak to be measured. As an alternative, competition-based FA assays were performed, in which the compound is titrated to constant concentrations of 14-3-3 and a FITC-labelled client peptide. Since the peptide and compound are binding in the same 14-3-3 binding pocket, the compound can potentially compete out the FITC-labelled peptide. While the K_i cannot directly be fitted due to the lack of a bottom plateau (because of the low affinity of the compounds), we could approximate this value by fitting with a mathematical model. For this, the bottom plateau was given as input (approximately 35 based on EC0 anisotropy level), which resulted in a K_i value of ~0.5 mM for compound **53**. These experiments thus support the high μ M affinity of the compound to 14-3-3 found by the cooperativity model. We think though that our cooperativity model provides for a more standardized and generally accepted analysis of the binding characteristics of molecular glues, also because these are based on multiple titration data, that allow for a more robust analysis.

7. Fig.3d shows compound **43** is selective to ChREBP but not to other 14-3-3 binding proteins. One 14-3-3 isoform (14-3-3 β) is used in the assay. Will **43** enhance interaction between the other 6 isoforms of 14-3-3 and their binding proteins?

Compound **43** enhances the interaction of ChREBP with all 7 isoforms of 14-3-3, as shown in new **Fig S11**. The shift in ChREBP/14-3-3 binding affinity upon the addition of compound **43** differs somewhat between isoforms, as typically observed also for other 14-3-3/client protein stabilization examples^{4, 12}. In addition, we now also measured the selectivity of compound **43** against 5 client proteins of 14-3-3 for a selection of 3 of the 14-3-3 isoforms. Gratifyingly (and expected based on stabilization studies with other types of molecular glues), **43** only stabilized

the 14-3-3/ChREBP interaction and was fully selective against all the other 14-3-3-based interactions with all 3 isoforms (new **Fig S12**).

8. Fig.5a. In the control, 14-3-3 can bind ChREBP α in the low glucose condition. In the high glucose 30min condition, why can 43 mediated protein-protein interaction keep ChREBP α in cytoplasm but the basal ChREBP α -14-3-3 binding can't?

As stated in the text, ChREBP α dissociates from 14-3-3 in response to increased glucose metabolism and enters the nucleus. In our previous paper⁸ we showed with different methodological approaches that in high glucose conditions, already after 2 h, the majority of ChREBP α returns to the cytoplasm, though some ChREBP α continues to prime the production of ChREBP β as long as the glucose signal remains. **43** increases the affinity of ChREBP α to 14-3-3. This higher affinity afforded by the ternary complex via **43** keeps ChREBP α bound to 14-3-3 and in the cytoplasm. We have tried to explain this better in the text.

9. How does 43 impact the mRNA levels of INS and PDX1 (Fig. 6f-g)? Explanation/discussion is needed.

Glucolipotoxicity results in oxidative stress that causes the degradation and dysregulation of transcription factors that are necessary to maintain the functional identity of mature β -cells. This leads to a de-differentiation that is manifested by decreased insulin and Pdx1 mRNA levels. The exact mechanisms are incompletely understood, but we have evidence that unrestrained expression of ChREBP β contributes to this general phenotype⁸. **43** prevents the upregulation of ChREBP β and thus preserves the expression of Pdx1 and insulin. This is now explained in more detail in the discussion.

Minor points:

1. Line 372 "Combined, this high cooperativity and selectivity for the ChREBP α /14-3-3 PPI conferred cellular activity to our lead compound". In the introduction, the authors mentioned previous compounds also enhance the PPI but lack the cellular activity. Here the cellular activity of the lead compound may be due to better cell membrane permeability.

Thank you, we added "possibly due to increased membrane permeability."

2. Line 403, change "active" to "activity"?

Thank you. This was changed.

3. page S7 should be "Fig. S7" but not "Fig. S1"

page S10 should be "Fig. S11" but not "Fig. S2"

These errors have been fixed.

Reviewer #3 (Remarks to the Author):

This manuscript describes the SAR analysis of a small molecule series that stabilizes the interaction between glucose-responsive transcription factor ChREBP α and the 14-3-3 regulatory scaffold protein in pancreatic β -cells. Stabilization of this interaction prevents the nuclear localization of ChREBP α , an isoform that binds to 14-3-3, in response to high glucose and thus prevents the expression and activation of ChREBP β , a second shorter nuclear-localized isoform lacking 14-3-3 binding domain. When ChREBP β is activated, a feedforward activation loop ultimately leads to glucolipotoxicity and pancreatic cell death in T2D. The report presents a structure-activity relationship investigation for a series of compounds based on a previously identified moderately active compound. Through this work, a set of compounds were identified that demonstrated higher potency and binding selectivity when tested against peptide binding motifs of other 14-3-3 client proteins. Notably, the introduction of a geminal-difluoro group resulted in a slight bend in the small molecule structure leading to the observed stabilization of the complex formation between ChREBP and 14-3-3. Crystal structures for several of the compounds were reported to support these observations and data is presented that shows that some but not all the optimized compounds result in increased complex formation, increased cytoplasmic localization and decreased glucolipotoxicity in cellular assays. While the discovery of this class of molecules was described in an earlier report by several of the co-authors (Sijbesma, et al, Nature Communications, Article number 3954, 2020), the current work represents a considerable advance from those findings. Manuscript is well written and clear and would be of high interest to the broader audience of Nature Communications. Overall this work provides evidence of a significant advance in the ability to use molecular glue technology to modulate a regulatory binding interaction and impact a pathological biological process in a cellular context. Some minor points are noted below:

[We thank the reviewer for this positive feedback on our study and for points noted.](#)

1. The authors claim that the improvement in cooperativity is not a result of increased binding affinity for 14-3-3, but instead, interactions with ChREBP α based on the analysis of the fluorescence anisotropy assay data. In this experiment, 14-3-3 protein is titrated at a constant compound concentration and this is measured at several compound levels (2D titration). The K_d for binding to 14-3-3 (K_{dII}) and the cooperativity factor α were obtained from a fit of the data using a four-parameter logistics model. Because these values would be correlated in the fit, it is not clear whether the data obtained can definitively support the conclusion stated. Is it possible to get an independent direct measurement of K_{dII} separately? This could be obtained from an experiment such as ITC, Surface Plasmon Resonance, or another direct binding assay. This would reduce the ambiguity of the analysis presented here. The observation of new interactions with the ChREBP α peptide with compound 43 is, however, consistent with the conclusion.

[Please see answer to reviewer 2, questions 4-6 above.](#)

2. The assays for specificity, while compelling, are based wholly on in vitro binding interactions with peptide motifs. The findings would be more impactful and give confidence in translatability if

supporting cell-based data was provided. For example, the qPCR assay for mRNA in β -cells or transcriptomics could be used to assess the potential impact on other 14-3-3 client protein functions.

We performed qPCR on the mRNAs of several client proteins and found no changes with **43** (new **Fig S20**). Importantly we also found by a proximity ligation assay using a pan-14-3-3 antibody and an NFATC1 antibody that **43** did not block the dissociation of these two proteins in response to glucose under identical conditions where **43** blocked the dissociation of ChREBP α and 14-3-3 (new **Fig S19**). In addition, we also performed more fluorescence anisotropy experiments using several additional isoforms of 14-3-3, showing a similar degree of specificity of binding between ChREBP α and 14-3-3, but not affecting the binding of other client proteins (new **Figs S11, S12**).

3. As a related question, could there be a secondary effect of sequestration of the available 14-3-3 by ChREBP α binding in high glucose that would have an indirect effect on other 14-3-3 client proteins?

This could purely theoretically be possible, but given the very high cellular concentration of 14-3-3 (high micromolar) as compared to the low concentration of a transcription factor like ChREBP α , this is very unlikely. Additionally, based on the evidence we've gathered, there seems to be no immediate effect on client protein interaction with 14-3-3 due to **43** (see answer to previous question).

4. Given the stated aspiration to progress this class of molecules as therapeutics, it may be useful to include sequence conservation or alignment of the interacting regions of 14-3-3 and ChREBP α across a set of common preclinical species to understand feasibility of translation for in vivo efficacy and safety studies.

Thank you for this suggestion. We have now done this and added this as new **Fig S22**.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

References for our response

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12. Wu, Q. *et al.* Discovery of 14-3-3 PPI Stabilizers by Extension of an Amidine-Substituted Thiophene Fragment. *Chembiochem* **25**, e202300636 (2024).

Response to reviewers' comments

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded fully to my suggestions. No further comments.

Thank you!

Reviewer #2 (Remarks to the Author):

My remaining concern is about how the cooperativity value and KDII are determined.

The authors mentioned in the rebuttal letter:

"The cooperativity factor of the ternary complex is determined by fitting, using a thermodynamic equilibrium model (reference#11).

"In addition, this model is also able to determine the KDII value, the affinity of the compound to 14-3-3"

"We think though that our cooperativity model provides for a more standardized and generally accepted analysis of the binding characteristics of molecular glues, also because these are based on multiple titration data, that allow for a more robust analysis."

"Reference#11. Geertjens, N.H., de Vink, P.J., Wezeman, T., Markvoort, A.J. & Brunsveld, L. A general framework for straightforward model construction of multi-component thermodynamic equilibrium systems. *bioRxiv*, 2021.2011. 2018.469126 (2021)."

The authors claim their model is standardized and generally accepted. However, the evidence does not support this assertion. Reference #11 has been cited by fewer than five papers, most of which are from authors associated with this manuscript. After reading reference #11, I remain unconvinced. I would like to compare experimentally determined cooperativity and other affinity values with the model's values to evaluate the model's validity. My intention is to be constructive. If the authors have other examples where experimentally determined values (cooperativity, KDII) match well with the model's values, please provide them.

This concern is shared not only by me (Reviewer #2) but also by Reviewer #3, who raised a similar issue in their first point.

Reviewer 2 requests to individually determine the parameters that we obtain via fitting the multicomponent binding assay. However, the actual reason that we use a cooperativity fitting model is that it is not experimentally possible to determine the cooperativity factor and binary affinity of the stabilizer individually. The paper that we cite here, which the reviewer criticizes has only 5 citations, reports on the fundamental mathematical description of the model and a framework that reduces time and expertise requirements for using the model. There may have been a miscommunication on our part as the reviewer cites "reference #11" – a 2019 *bioRxiv* version of the now peer-reviewed manuscript that was published in 2023 in the Royal Society of Chemical Biology *RSC Chem. Biol.*, 2023,4, 252. This is now reference 32 in the revised manuscript. The principles behind the model to solve the ternary body problem are generally accepted in the field (1-5). These citations (1-5) can be found in the citations of the

2023 manuscript. These derive from citations therein to older work, for example on three-component cooperative binding in the GPCR field, for which this concept was first established.

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Reviewer #3 (Remarks to the Author):

After reviewing the amendments made by the authors and the responses to my comments as well as those of other reviewers, my primary concerns have been sufficiently addressed and I recommend publication.

Thank you!

Upon review of this revision, I did note one small typographical error in Table S4, where crystallographic statistics are listed. For PDB 8BWH, there appears to be a typo in the field for Rfree, where it looks like the authors have inadvertently duplicated the reflection set (1050(104)) rather list the Rfree value. It would be good to carefully review that table before final publication.

Thank you for pointing out this error, it is fixed.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to

provide appropriate recognition for Early Career Researchers who co-review manuscripts.