nature portfolio

Peer Review File

Engineered Depalmitoylases Enable Selective Manipulation of Protein Localization and Function

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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)
The authors have developed a set of new methods to artificially induce the removal of palmitates from proteins, thus altering their localization and/or function. The basic concept is that by splitting the depalmitoylase ABHD17C into two parts (an N-terminal membrane-targeting domain and a C-terminal catalytic domain) a variety of inducible localization systems can be used to accomplish three key goals: (1) depalmitoylation of proteins from specific subcellular locations, (2) targeted depalmitoylation of individual protein complexes, and (3) inducible depalmitoylation in response to cell signaling events.

Using these approaches, the authors were able to induce changes in the localization of a variety of putative substrates. They were able to target specific proteins, and in many cases were able to demonstrate phenotypic effects. I found the overall concept to be very clever, and I appreciated the use of quantitative readouts for many experiments (membrane/cytosol localization ratios, action potentials, whole cell voltage clamp recordings). The ERK biosensor feedback loop system was particularly creative. They also made the intriguing observation that the catalytic domains of ABHD17B and ABHD17C altered neuronal action potential firing rate in opposite ways, even if the underlying mechanism is unclear. The field currently lacks specific approaches to remove palmitate from proteins, and the widely used palmitoylation inhibitor 2BP has many off-target effects and is not suitable for functional studies. Therefore, these methods are likely to be of wide interest, and have the potential to uncover important biological insights.

Despite this, my enthusiasm for the work, as presented, was dampened by a few key issues:
- protein palmitoylation was only examined for one example, and in most cases only changes in localization were studied and assumed to reflect depalmitoylation, which was unsatisfying. It would also be helpful to show the changes were blocked using a catalytically inactive mutant, or reversed using an ABHD17-specific inhibitor.
-the lack of effect of some enzyme chimeras (eg. ER-targeted forms of the enzymes) is not interpretable, since these chimeras may be non-functional. Similarly, no conclusions can be drawn about differences in the activity of different ABHD17A/B/C chimeras without controls.
-there was no indication that the heterologous targeting system preserved the substrate selectivity of endogenous enzymes thus I was not convinced this system could be used to study substrate specificity as suggested.

Specific comments:

1. The relocalization of the FacePalm constructs to the appropriate cellular locations after addition of rapamycin should be shown. This is particularly important for the targeting to different subcellular organelles in Fig3, and it might explain the patchy substrate distribution in Fig1f. Comparing the time course of FacePalm vs substrate relocalization with the kinetics of palmitate removal (using pulse-chase click methods) would also be desirable.

2. Effects on protein palmitoylation and not just localization should be shown. Furthermore, not all palmitate modifications are dynamic, many are believed to be stable. Does FacePalm remove only those modifications that are normally dynamic or does it remove even stable palmitates from a given protein? Does FacePalm also depalmitoylate and inactivate endogenous APTs such as APT2?

3. It was not demonstrated that the FacePalm system faithfully reproduces enzyme specificity. This could be shown using STREX BK channels as a substrate. Yokoi et al (2016) also showed that ABHD17C is less active than ABHD17A/B on

PSD95 when expressed in HEK cells. The same paper also showed that targeting the catalytic domain of ABHD17B to the cell surface with a PM localization signal only partially restores activity, which highlights the concern that not all chimeras described in the paper will be active.

4. I found the organelle relocalization experiments in Fig3 to be poorly controlled. The ER-targeted FacePalm may not have an effect simply because the enzyme is non-functional. This section would be more convincing if ER-localized FacePalm was shown to act specifically on ER-localized palmitoylated substrates. The organelle targeting determinants were not described in the paper, but it appears from the references that Lyn was used to target the PM. If so this is problematic, because Lyn is palmitoylated and Fig2 shows that it is at least partially mislocalized by FacePalm recruitment. It is not explained how the PM-localized substrate is mislocalized by early or late endosome-targeted FacePalm. Is this because the substrate is recycled through endocytic compartments, or because FacePalm targeting is non-specific?

Minor comments:

Fig 1G: label the X-axis. Also explain in the legend that “1-3” represents the cells shown in 1F.
Fig1H: explain what HA stands for in the legend
Fig2: why is the x-axis for all graphs marked with a bar labeled 30mins? I would expect the time point to be indicated by a single point, and not a range
Fig3: explain which sequences were used for targeting
Fig4G: why does the control become less cytosolic over time?

Reviewer #2

(Remarks to the Author)
The manuscript by Jayaraman et al. developed a rapamycin-activated depalmitoylase ABHD17, which is composed of two regions: (1) FKBP rapamycin binding domain (FRP)-fused N-terminal region of ABHD17 and (2) FK506 binding protein (FKBP)-fused C-terminal catalytic region of ABHD17. The authors showed that the addition of rapamycin could reconstitute the ABHD17 depalmitoylase activity (named FacePalm) toward several substrates, including Cavβ2A, Kchip2, Lyn, NRas and PSD95. FacePalm was modified to specifically target the various organelles (ER, golgi, plasma membrane and early/late endosomes) and specific protein complexes. Furthermore, the authors applied FacePalm to make the negative feedback loop that activates the depalmitoylase toward NRas upon NRas -induced Erk activation, leading to the downregulation of the NRas signaling. Finally, the authors expressed Facepalm of ABHD17a, 17b and 17c in cultured neurons and showed their different effects on neuronal activity. Although there are several interesting things in this paper, there are also some significant shortcomings that temper this reviewer’s enthusiasm for the paper. Addressing the following points would strengthen this paper.

Major comments
1. The FKBP/FRB/rapamycin system has been widely employed to probe molecular events like protein kinase activity and inositol phosphatase activity. Although FacePalm the authors developed seems useful, the principle of this tool itself is not new. To increase the novelty, further innovations are necessary, such as reversibility of the manipulation.

2. In Fig. 2d-f and 4b, f, the re-localization of target proteins from the plasma membrane to cytosol is not apparent upon rapamycin treatment. The authors should quantify the intensity of target proteins at the plasma membrane in addition to the cytosol. In fact, in Fig. 4b and 4f, the fluorescence intensity at plasma membrane seems unchanged upon rapamycin treatment. In addition, the authors should show the biochemical data using the Acyl-RAC or ABE method as in the Fig. 1h.

3. In Fig. 3, the authors target FacePalm to specific organelles and showed the different effect on Cavβ2A localization. Because most of Cav 2A is localized at the plasma membrane, their experiments are not suitable to show the subcellular specific depalmitoylase activity of FacePalm. The authors should investigate the effect of organelle specific FacePalm on the ER, Golgi, endosome-localized palmitoylated proteins. In addition, the FacePalm localization after treatment with rapamycin should be presented.

4. The authors showed that FacePalm-17A, -17B and -17C commonly reduce the puncta density of PSD-95 in neurons. In contrast, FacePalm-17A, -17B and -17C showed the different effect on neuronal activity. Although several possibilities are discussed, the molecular mechanism remains unclear. The authors should show the molecular mechanism of these different effects, such as identification of specific substrates for ABH17 isoforms.

Minor comments
1.For time-lapse experiments, a protein synthesis inhibitor (e.g., cycloheximde) should be added to neglect the effect of newly synthesized proteins.
2. line 141; Extended Data Fig.1e should be 1d.
line 147; Extended Data Fig.1f should be 1e.

Reviewer #3

(Remarks to the Author)
By adapting a depalmitoylase in a chemically inducible dimerization tool, Jayaraman et al. develop a chemical genetics technique to induce depalmitoylation of various proteins simultaneously or individually. Implementation of such a technique

in cultured neurons led to alteration of their electrical properties. While this is claimed to be the first achievement of depalmitoylation that is both inducible and substrate-specific, the technological advance does not seemingly stand out among a large number of similar strategies employed for different enzymes (Ref #65, as well as PMID 20353181, 29590011, 22584056, 23299847). The manuscript is in general written well with justification of significance and approaches, along with helpful visual aids in the main figures. However, some of the key control experiments are missing, while there are statements that do not appear to be well supported by the provided dataset.

Major criticism:
Experimental design and physiological relevance: Despite the clear goal set up in the Introduction (lines 91-96), the corresponding functional assays shown in Fig. 6 do not associate with critical experiments. First, the depalmitoylation conducted is non-specific (why not use the target specific nanobody design here?). Second, molecular characterization is not adequate, thus leaving key questions unaddressed: what molecules are depalmitoylated and which ones are responsible for the phenotypes? Are the phenotypes really caused by depalmitoylation? Some of these questions can be readily assessed by rescuing palmitoylation on proteins of interest, and/or repeating the experiments with a control construct where the catalytic activity of a given depalmitoylase is defective. The same argument holds true for the data shown in Fig. 3. Alteration in the output responses could be due to indirect effects. In addition, the study is conducted only for overexpressed protein substrates. If so, it is imperative to demonstrate that the tool can be applied to “endogenous” proteins.

Miscellaneous comments:
1. Introduction is too thorough for a research paper. A more focused Introduction (only describing information centrally related to the study) would be helpful for readers to engage with the study. Similarly, Discussion can also be more concise than now by removing similar statements used elsewhere.
2. Too many “thus”; line 173 is representative.
3. “high precision” (line 110); it is not clear in which parameter (space, time, target specificity?)
4. Refs #57, 58 do not appear to match the statement (line 125).
5. Line 284 claims that this tool “provides a potential therapeutic strategy” for cancers, which sounds like an overstatement, as if one were to introduce a gene into cancer cells, it would be more straightforward to introduce a non-mutated form of a gene that is mutated (such as N-Ras WT vs. N-Ras Q61K).
6. Similarly to #5, the use of the term “exquisite” in line 315 is not well justified, because there seems to be no dataset that helps judge how “exquisite” the present tool is over existing ones.
7. Figure legends may need to be more instructive. For example, what is “HA” in Fig. 1h, and “control” in Fig. 1l?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this resubmitted manuscript, the authors have added several new experiments and made significant revisions. I was satisfied by the new controls showing that palmitoylation of several different substrates was reduced in a FacePalm activity-dependent manner, and that the FacePalm constructs were recruited to the targeted organelles. The new reversible optogenetic system and the specific targeting of PSD95 in neurons extend the scope of the study. Overall, these changes strengthen the manuscript and address my concerns.

I have only a few minor comments that can be addressed primarily by edits to the figure legends:
-molecular weight markers are rarely labeled on the figures and instead are described in the legends or not at all. The molecular weight markers should be consistently labeled on the figure itself.
-scale bars are shown on the figures but the size of the bar is not mentioned in the legends.
-the value of “n” is not always supplied in all legends, especially in supplementary data
-legends should be checked for typos (eg. “trials” not “trails”)
-the sequences used to direct constructs to different organelles are mentioned only in the methods. I would prefer that the identity of the targeting sequences also be mentioned in the main text or in the figure legend to make this information easier to find.

Reviewer #2

(Remarks to the Author)
The authors tried to address my comments and incorporated some new data into the revised manuscript. Especially, the addition of “a reversible optogenetic system” to ABHD17 manipulation increases the novelty of this manuscript (figure 3). To validate this new opto-probe, the authors should add the biochemical data (e.g., acyl-Rac) in figure 3 (at time: 0, 60 min, 180). Also, the local, reversible effect of opto-ABHD17 stimulation on PSD-95 clusters should be examined (figure 7). These data strengthen the paper.

Regarding differential effects of FacePalm-17A, -17B, and -17C on neuronal excitability in figure 7, the authors did not add

any data to show/support the molecular mechanism, and just mentioned that this would be beyond the scope of this manuscript. This reviewer was disappointed at this response. This reviewer suggests deletion of electrophysiological data in figure 7, as the data is not relevant with other parts of this manuscript and such preliminary, uninterpretable data misleads readers.

Reviewer #3

(Remarks to the Author)
The authors properly addressed my oiginal concerns.

We thank all three reviewers for their constructive criticisms. In the revised manuscript, we have expanded the scope of the study by including (i) a reversible optogenetic actuator to depalmitoylate proteins, and (ii) deployed a PSD95-targeted depalmitoylases to neurons. In addition, we also provide additional control experiments that were requested by the reviewers. Altogether, the revised manuscript includes an extensive amount of new data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have developed a set of new methods to artificially induce the removal of palmitates from proteins, thus altering their localization and/or function. The basic concept is that by splitting the depalmitoylase ABHD17C into two parts (an N-terminal membrane-targeting domain and a C-terminal catalytic domain) a variety of inducible localization systems can be used to accomplish three key goals: (1) depalmitoylation of proteins from specific subcellular locations, (2) targeted depalmitoylation of individual protein complexes, and (3) inducible depalmitoylation in response to cell signaling events.

Using these approaches, the authors were able to induce changes in the localization of a variety of putative substrates. They were able to target specific proteins, and in many cases were able to demonstrate phenotypic effects. I found the overall concept to be very clever, and I appreciated the use of quantitative readouts for many experiments (membrane/cytosol localization ratios, action potentials, whole cell voltage clamp recordings). The ERK biosensor feedback loop system was particularly creative. They also made the intriguing observation that the catalytic domains of ABHD17B and ABHD17C altered neuronal action potential firing rate in opposite ways, even if the underlying mechanism is unclear. The field currently lacks specific approaches to remove palmitate from proteins, and the widely used palmitoylation inhibitor 2BP has many off-target effects and is not suitable for functional studies. Therefore, these methods are likely to be of wide interest, and have the potential to uncover important biological insights.

Despite this, my enthusiasm for the work, as presented, was dampened by a few key issues:
- protein palmitoylation was only examined for one example, and in most cases only changes in localization were studied and assumed to reflect depalmitoylation, which was unsatisfying. It would also be helpful to show the changes were blocked using a catalytically inactive mutant, or reversed using an ABHD17-specific inhibitor.
-the lack of effect of some enzyme chimeras (eg. ER-targeted forms of the enzymes) is not interpretable, since these chimeras may be non-functional. Similarly, no conclusions can be drawn about differences in the activity of different ABHD17A/B/C chimeras without controls.
-there was no indication that the heterologous targeting system preserved the substrate selectivity of endogenous enzymes thus I was not convinced this system could be used to study substrate specificity as suggested.

We thank the reviewer for their positive outlook of the manuscript. We have tried our very best to address these important concerns as outlined below for each specific comment.

Specific comments:

1. The relocalization of the FacePalm constructs to the appropriate cellular locations after addition of rapamycin should be shown. This is particularly important for the targeting to different subcellular organelles in Fig3, and it might explain the patchy substrate distribution in Fig1f. Comparing the time course of FacePalm vs substrate relocalization with the kinetics of palmitate removal (using pulse-chase click methods) would also be desirable.

We completely agree. In the revised manuscript, we provide this information as follows.

1. We have revised Figure 1 to include confocal images that show increased colocalization of the FacePalm NT and CT fragments following rapamycin activation. We quantify this change by measuring Pearson's correlation coefficient. In the same cell, we show an increase in cytosolic localization of $Ca_V\beta_{2A}$ as well as quantify this.
2. To explicitly probe redistribution of FacePalm CT to various organelles, we also considered co-localization with markers for various organelles as provided in Extended Data Figure 3.
    a. When co-expressed with <u>non-subcellularly</u> targeted $FacePalm_{NT}$ ("untargeted"), we found that rapamycin triggered a strong increase in colocalization with plasma membrane (PM) and early endosome markers. We also found mild/modest increase in colocalization with the golgi and late endosomes and minimal change in colocalization with the ER. This is largely consistent with previous studies (26701913) that showed strongest Abdh17a colocalization with PM and early/recycling endosomes. Changes in colocalization was quantified using Pearson's correlation coefficient (ρ).
    b. When co-expressed with <u>subcellularly</u> targeted $FacePalm_{NT}$ ("targeted"), rapamycin triggered a strong increase in colocalization with each of the desired domains. For e.g., with ER-targeted $FacePalm_{NT}$, we found that rapamycin strongly increased colocalization of $FacePalm_{CT}$ with the ER. Similarly, golgi-targeted, PM-targeted, early-endosome targeted, and late-endosome targeted $FacePalm_{NT}$ allowed recruitment of $FacePalm_{CT}$ to the golgi, PM, early-endosome, and late-endosome respectively with similar correlation coefficients.

Taken together, these results suggest that the subcellular targeting strategy is actually effective at directing the depalmitoylases to desired intracellular organelles. Importantly, in all cases, the $FacePalm_{CT}$ which encodes the catalytic subunit is identical. Therefore, the minimal change in localization of CaVβ2A with ER targeted FacePalm as well as the difference in the rate of depalmitoylation with FacePalm directed to other domains is unlikely to be due to a non-functional FacePalm or poorly functioning FacePalm. Instead, it likely points to the ineffectiveness of depalmitoylating this protein from the ER.

2. Effects on protein palmitoylation and not just localization should be shown. Furthermore, not all palmitate modifications are dynamic, many are believed to be stable. Does FacePalm remove only those modifications that are normally dynamic or does it remove even stable palmitates from a given protein? Does FacePalm also depalmitoylate and inactivate endogenous APTs such

as APT2?

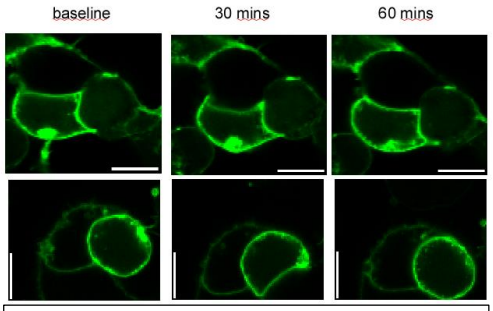
We thank the reviewer for this suggestion. To demonstrate that FacePalm-17a-c alters palmitoylation status, we undertook Acyl-RAC assay to show changes in palmitoylation of CaVβ2A with FacePalm-17a, and FacePalm-17b, as well as of Kchip2, Lyn, and NRas with FacePalm-17c. This information is provided in Figure 2. In all cases, we observed a statistically significant reduction in palmitoylation.

At present, our studies cannot distinguish whether FacePalm can remove stable palmitate modifications. Nevertheless, previous studies have shown that Abdh17s are more selective in targeting dynamically palmitoylated proteins such as NRas. Given the design of FacePalm, we expect it to preserve this specificity. We note this limitation in the discussion section.

We did not test whether FacePalm could inactivate other enzymes by depalmitoylating them. Although this is a very interesting possibility, we feel it is beyond the scope of the present study.

3. It was not demonstrated that the FacePalm system faithfully reproduces enzyme specificity. This could be shown using STREX BK channels as a substrate. Yokoi et al (2016) also showed that ABHD17C is less active than ABHD17A/B on PSD95 when expressed in HEK cells. The same paper also showed that targeting the catalytic domain of ABHD17B to the cell surface with a PM localization signal only partially restores activity, which highlights the concern that not all chimeras described in the paper will be active.

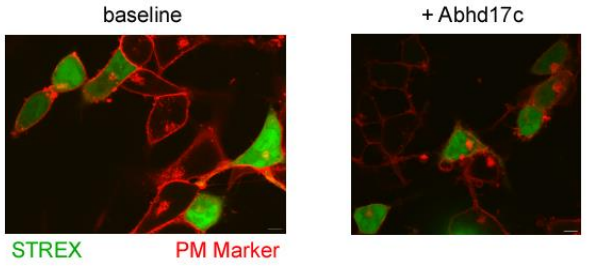
FacePalm system splits the Abhd17 enzyme into NT and CT components by leverage a variable loop, and then uses chemically induced dimerization of FKBP/FRB to reconstitute the holoenzyme. To demonstrate that the change in CaVβ2A (Fig. 1) is due to the activity of the Abdh17 enzyme, we provide additional data showing that recruitment of a catalytically inactive FacePalmCT (S/A mutant) and inhibiting the catalytic activity of FacePalm fails to alter CaVβ2A localization (Fig. 1g). Given the critical modifications of the wild-type enzyme, it is entirely possible that FacePalm does not faithfully recapture the specificity of the parent enzyme. Indeed, this is true for any enzyme that is modified by attachment of additional protein domains.



Recruiting FacePalm-17C_{CT} to the plasma-membrane with membrane tethered-FRB (i.e. Lyn-FRB) was not sufficient to alter CaVβ2A suggesting that FacePalm-17C_{NT} is required to upregulate activity of the enzyme.

The study by Yokioi et al showed that directing Abhd17A to the membrane by replacing the NT with a myristoylation motif from Src resulted in an inactive enzyme. In line with this, We also found that simply recruiting FacePalm17C CT to the PM with a membrane-tethered FRB construct (Lyn NT-FRB) (Inoue et al 2005 Nat. Methods 2:415-418) was not sufficient to evoke changes in CaVβ2A localization. In fact, this is why we chose to attach FRB to the NT of Abhd17. In this manner, the addition of rapamycin would reconstitute the holo-Abhd17 enzyme instead of simply bringing the catalytic domain to the plasma membrane.

Both studies by Yokoi et al (PMID: 27307232) and Lin and Conibear (PMID: 26701913) showed that all three Abhd17 enzymes are capable of depalmitoylating PSD95. Although Abhd17a appeared to be slightly stronger than Abhd17b and Abhd17c, it is not clear that these differences would actually be statistically significant (Figure 1 in PMID: 27307232 and Figure 4-figure supplement 1 in PMID: 26701913). In our studies, we also found that all three FacePalm variants are capable of depalmitoylating PSD95, although FacePalm-17B and FacePalm-17C were somewhat more effective than FacePalm-17A in reducing PSD95 puncta density.



STREX fails to show appreciable membrane localization at baseline.

We considered using the purported differential selectivity of STREX as an approach to see if the FacePalm system reproduces the preferences of the parent enzyme. However, in our hands, we found that STREX peptide which was shown to be membrane localized had very minimal membrane localization. Therefore, it was difficult to use this protein as a reference for whether FacePalms recapture enzyme selectivity of Abhd17 enzymes. It is possible that these difference stem from difference in the cell lines used or other experimental details such as presence of specific zDHHC enzymes. Given this discrepancy, we would prefer not to include this in the main text.

Given this, we note the following limitation in the discussion section: "First, engineered depalmitoylases critically modify Abhd17 enzymes by heterodimerization motifs. As these motifs are often large, this modification may itself impact the kinetics and selectivity of these proteins. Therefore, differences observed with FacePalm variants may not necessarily reflect those of the parent enzyme."

4. I found the organelle relocalization experiments in Fig3 to be poorly controlled. The ER-targeted FacePalm may not have an effect simply because the enzyme is non-functional. This section would be more convincing if ER-localized FacePalm was shown to act specifically on ER-localized palmitoylated substrates. The organelle targeting determinants were not described in the paper, but it appears from the references that Lyn was used to target the PM. If so this is problematic, because Lyn is palmitoylated and Fig2 shows that it is at least partially mislocalized by FacePalm recruitment. It is not explained how the PM-localized substrate is mislocalized by early or late endosome-targeted FacePalm. Is this because the substrate is recycled through endocytic compartments, or because FacePalm targeting is non-specific?

We apologize for this unintentional oversight. We used the following targeting sequences: Specifically, we modified FacePalmNT which contains the Abhd17NT with FRB and the added various targeting sequences (noted below) to the c-terminus of this sequence.

Construct	
ER- Targeted FacePalm	FacePalm$_N$-Cytochrome b5 (100-134)
Golgi-Targeted FacePalm	FacePalm$_N$-Giantin (3131-3259)

PM -Targeted FacePalm	FacePalm$_N$-CAAX (KRAS-4B, 183-199)
Early-Endosome-targeted FacePalm	FacePalm$_N$-Rab5
Late-Endosome-Targeted FacePalm	FacePalm$_N$-Rab7

For PM localization, we actually used a farnesylated CaaX motif from KRAS-4B which is not palmitoylated in it C-terminus. Therefore, the concern raised does not apply here. In addition, in Extended Data Fig. 3 data, we now show the largely successful recruitment of FacePalm-17C$_{CT}$ (the catalytic domain) to the desired subcellular locations with the targeting strategy. Given that the very same catalytic domain is recruited to the various domains and as the Abhd17NT is present for all of these various, we don't believe the absence of change in CaVβ2A localization is due to inactivity of the enzyme in ER.

Minor comments:

Fig 1G: label the X-axis. Also explain in the legend that "1-3" represents the cells shown in 1F.

This figure has been replaced.

Fig1H: explain what HA stands for in the legend

We have added this to the figure legend.

Fig2: why is the x-axis for all graphs marked with a bar labeled 30mins? I would expect the time point to be indicated by a single point, and not a range

We have replaced the scale bar with an explicit x-axis.

Fig3: explain which sequences were used for targeting

We provide this now in the methods.

Fig4G: why does the control become less cytosolic over time?

This was actually an error in the sheet, we apologize for this. We have verified that the correct data set is included in revised Fig. 4f.

Reviewer #2 (Remarks to the Author):

The manuscript by Jayaraman et al. developed a rapamycin-activated depalmitoylase ABHD17, which is composed of two regions: (1) FKBP rapamycin binding domain (FRP)-fused N-terminal region of ABHD17 and (2) FK506 binding protein (FKBP)-fused C-terminal catalytic region of ABHD17. The authors showed that the addition of rapamycin could reconstitute the ABHD17 depalmitoylase activity (named FacePalm) toward several substrates, including Cavβ2A, Kchip2, Lyn, NRas and PSD95. FacePalm was modified to specifically target the various organelles (ER, golgi, plasma membrane and early/late endosomes) and specific protein complexes. Furthermore, the authors applied FacePalm to make the negative feedback loop that activates the depalmitoylase toward NRas upon NRas -induced Erk activation, leading to the downregulation of the NRas signaling. Finally, the authors expressed Facepalm of ABHD17a, 17b and 17c in cultured neurons and showed their different effects on neuronal activity. Although there are several interesting things in this paper, there are also some significant shortcomings that temper this reviewer's enthusiasm for the paper. Addressing the following points would strengthen this paper.

Major comments

1. The FKBP/FRB/rapamycin system has been widely employed to probe molecular events like protein kinase activity and inositol phosphatase activity. Although FacePalm the authors developed seems useful, the principle of this tool itself is not new. To increase the novelty, further innovations are necessary, such as reversibility of the manipulation.

We thank the reviewer for this suggestion. We agree that FKBP/FRB chemically induced dimerization (CID) system has been used extensively in the past to manipulate protein function. Our intention was not to suggest that the application of this CID is particularly novel, but to highlight the versatility of engineered depalmitoylases. Even still, based on the reviewers suggestion, we felt that addition of reversibility would be useful for future studies. As such, we engineered an optogenetic system where we use enhanced Magnets to dimerize the Abhd17NT and Abhd17CT. With this approach, we are able to reversibly manipulate palmitoylation of CaVβ2A. This data is provided as revised Figure 3.

2. In Fig. 2d-f and 4b, f, the re-localization of target proteins from the plasma membrane to cytosol is not apparent upon rapamycin treatment. The authors should quantify the intensity of target proteins at the plasma membrane in addition to the cytosol. In fact, in Fig. 4b and 4f, the fluorescence intensity at plasma membrane seems unchanged upon rapamycin treatment. In addition, the authors should show the biochemical data using the Acyl-RAC or ABE method as in the Fig. 1h.

We thank the reviewer for this suggestion. We have now included biochemical data using Acyl-RAC assay showing that with rapamycin we see reduced palmitoylation for all the targets we considered (CaVβ2A with FacePalm-17A, FacePalm-17B, FacePalm-17C; Kchip2, Lyn, and NRAS FacePalm-17C) in revised Figure 2.

We chose to quantify changes in normalized cytosolic fluorescence intensity given the simplicity of this measurement. By contrast, demarking an exact region as plasma membrane can be challenging given the resolution limits and cell movement over time. To explicitly show that the

increase in cytosolic localization corresponds to a decrease in membrane localization, we undertook new experiments where we expressed CaVβ2A and used a PM marker (cell mask). This data is provided as Extended Data Figure 1. Upon activation of FacePalm-17C with rapamycin, we see an increase in normalized cytosolic fluorescence. At the same time, computing the Pearson's correlation between CaVβ2A and PM marker showed a decrease in membrane localization of time. The two metrics linearly correlate as evident from an R^2=0.98 and the estimated time-constants based on the two metrics are not statistically different.

3. In Fig. 3, the authors target FacePalm to specific organelles and showed the different effect on Cavβ2A localization. Because most of Cavβ2A is localized at the plasma membrane, their experiments are not suitable to show the subcellular specific depalmitoylase activity of FacePalm. The authors should investigate the effect of organelle specific FacePalm on the ER, Golgi, endosome-localized palmitoylated proteins. In addition, the FacePalm localization after treatment with rapamycin should be presented.

We thank the reviewer for this suggestion. We now provide FacePalm localization after rapamycin addition and quantify changes colocalization with respective subcellular locations using Pearson's correlation (Extended Data Figure 3).

We are not exactly sure how to investigate effect of organelle specific FacePalm on ER or golgi localized palmitoylated protein, as we are not quite aware of specific proteins that are palmitoylated by Abhd17 and are restricted to the ER. The canonical view is that for many proteins, for e.g. NRas, dynamic palmitoylation promotes its plasma membrane localization while depalmitoylation in the case of NRas causes trafficking back to the golgi. This dynamic cycle of palmitoylation is critical for the function of these proteins.

As the reviewer notes, although the vast majority of Cavβ2A appears to be localized to the PM. Even still, activation of FacePalm in golgi or in early or late endosomes is sufficient to result in appreciable cytosolic accumulation. In the case of early endosome localized FacePalm, the effect appears to almost as strong as with PM localized FacePalm. This would suggest that like other palmitoylated protein Cavβ2A also undergoes dynamic trafficking.

4. The authors showed that FacePalm-17A, -17B and -17C commonly reduce the puncta density of PSD-95 in neurons. In contrast, FacePalm-17A, -17B and -17C showed the different effect on neuronal activity. Although several possibilities are discussed, the molecular mechanism remains unclear. The authors should show the molecular mechanism of these different effects, such as identification of specific substrates for ABH17 isoforms.

As the reviewer notes, the differential effect of FacePalm on neuronal activity is quite interesting and unexpected. As such, we felt it was worth reporting in this study. In the revised manuscript, we show that indeed these differential effects are due to the catalytic activity of the enzyme, as expressing FacePalm-17B S/A mutant and FacePalm-17C S/A mutant prevents changes in neuronal activity (and PSD95 localization).

That said, we believe that dissecting the exact targets responsible for these effects and validating them would be beyond the scope of the present manuscript. (Frankly, this is the

subject of our recent R01 grant proposal). This is because ion channels that support neuronal action potentials are complex multi-subunit proteins. Palmitoylation is thought to affect not only the pore-forming subunits, but also auxiliary subunits of many ion channels including Na, Ca, and K channels. Furthermore, the exact effect of (de)palmitoylation on the function of the channel itself could be complex depending on which subunit or isoform is modulated and could involve changes in both trafficking and/or gating of the channel. For instance, in some cases, it is possible that depalmitoylation could upregulate the channel while in other instances it may downregulate channel activity. As such, identification of substrates itself may not suffice to determine the contribution to neuronal activity. Instead, determining the exact mechanism would likely require global proteomic analysis, as well as complementary electrophysiological studies and molecular approaches that disable palmitoylation of specific proteins. These would be a multiyear endeavor that we feel is better suited as a follow up study. As such, we note this limitation in the discussion section. We feel that the differential response of FacePalm-17b versus FacePalm-17c is unexpected, and therefore worth noting this would be valuable even without an explicit mechanism.

As suggested by R3, we also expand the scope of the study in a different direction by demonstrating the possibility of targeted depalmitoylation in these neurons. Specifically, we show that targeting FacePalm-17C to PSD95 using fibronectin intrabodies (FingR) is sufficient to reduce PSD95 puncta density without affecting neuronal activity (Figure 7m-p). This shows the potential utility of the targeted depalmitoylation approach to tune specific aspects of neuronal function.

Minor comments

1.For time-lapse experiments, a protein synthesis inhibitor (e.g., cycloheximde) should be added to neglect the effect of newly synthesized proteins.

We provide this data as Extended Data Figure 2l-m. Indeed, the addition of cycloheximide does not prevent FacePalm-17C dependent changes in membrane localization of Cavβ2A suggesting that the changes in localization are due to depalmitoylation of existing proteins and not the failure to palmitoylate newly synthesized proteins.

2. line 141; Extended Data Fig.1e should be 1d.
line 147; Extended Data Fig.1f should be 1e.

This has been revised to refer the revised figures.

Reviewer #3 (Remarks to the Author):

By adapting a depalmitoylase in a chemically inducible dimerization tool, Jayaraman et al. develop a chemical genetics technique to induce depalmitoylation of various proteins simultaneously or individually. Implementation of such a technique in cultured neurons led to alteration of their electrical properties. While this is claimed to be the first achievement of depalmitoylation that is both inducible and substrate-specific, the technological advance does not seemingly stand out among a large number of similar strategies employed for different enzymes (Ref #65, as well as PMID 20353181, 29590011, 22584056, 23299847). The manuscript is in general written well with justification of significance and approaches, along with helpful visual aids in the main figures. However, some of the key control experiments are missing, while there are statements that do not appear to be well supported by the provided dataset.

We thank the reviewer for this comment. We have addressed concerns raised as noted below.

The primary advance of this manuscript is to develop engineered depalmitoylases with desired properties such as being inducible, subcellularly-regulated, or having substrate specificity. To achieve this, we drew inspiration from the citations mentioned here and utilized chemically induced dimerizers (CID) as a means to inducibly trigger protein depalmitoylation. Therefore, we did not focus on advancing or improving the CID system, but rather on adapting it to an interesting class of protein. Nevertheless, we believe the outcome here, strategies to fine tune palmitoylation of proteins would be a useful tool for the community to dissect the functional consequences of palmitoylation. This is very nicely pointed out by R1: *"The field currently lacks specific approaches to remove palmitate from proteins, and the widely used palmitoylation inhibitor 2BP has many off-target effects and is not suitable for functional studies. Therefore, these methods are likely to be of wide interest, and have the potential to uncover important biological insights."*

In addition, we expand the scope of the study in two directions: First, we engineer an optogenetically activated depalmitoylases by inducing dimerization of the Abhd17NT and CT using photoactivated (and reversible) dimerization of enhanced magnets. Second, as suggested by the reviewer we employ a targeted depalmitoylation approach to reduce clustering of PSD95 in neurons without affecting action potential firing rates. We believe altogether, the manuscript presents a bevy of actuators that could help us better understand and manipulate protein palmitoylation.

Major criticism:
Experimental design and physiological relevance: Despite the clear goal set up in the Introduction (lines 91-96), the corresponding functional assays shown in Fig. 6 do not associate with critical experiments. First, the depalmitoylation conducted is non-specific (why not use the target specific nanobody design here?). Second, molecular characterization is not adequate, thus leaving key questions unaddressed: what molecules are depalmitoylated and which ones are responsible for the phenotypes? Are the phenotypes really caused by depalmitoylation? Some of these questions can be readily assessed by rescuing palmitoylation on proteins of interest, and/or repeating the experiments with a control construct where the catalytic activity

of a given depalmitoylase is defective. The same argument holds true for the data shown in Fig. 3. Alteration in the output responses could be due to indirect effects. In addition, the study is conducted only for overexpressed protein substrates. If so, it is imperative to demonstrate that the tool can be applied to "endogenous" proteins.

We agree and we have addressed these concerns as follows:

(1) In the revised manuscript, we now deployed a PSD95 targeted depalmitoylases by attaching a PSD95 binding fibronectin intrabody generated by mRNA display (FinGr) to demonstrate that we can manipulate endogenous PSD95 in hippocampal neurons without affecting neuronal activity (Figure 7m-p).

(2) To show that the FacePalm induced change in PSD95 puncta density as well as neuronal action potentials are the result of depalmitoylation, we expressed a control construct where catalytic activity of the given depalmitoylases is disabled. We find that changes in PSD95 puncta density and neuronal activity are absent with these control constructs. This data is provided as Extended Data Figure 6.

Nevertheless, identifying the exact culprits responsible for differential effects of activating FacePalm-17B and FacePalm-17C on excitability is beyond the scope of the present study (and the subject of a recent grant proposal). First, ion channels are multisubunit and often heterogenous molecular complexes. Palmitoylation could impact one or more of ion channel subunits, many of which are large proteins. Moreover, many channel subunits such as CaVb2a are alternatively spliced, and only specific variants are regulated by palmitoylation. While pharmacological tools are available for many pore forming subunits, there are few available to manipulate these auxiliary subunits . Beyond this, the exact consequence of palmitoylation on channel function could vary, for e.g. it could alter trafficking/localization or impact gating as with cavb2a. Therefore, establishing a causal link between changes in a set of ion channels and excitability would be requite a full in depth study in and of itself.

(3) We also agree that some of the effects reported with the subcellularly targeted depalmitoylases could be due to indirect effects perhaps resulting from mislocalization or poor recruitment of the FacePalm$_{CT}$ to desired subcellular domains. To address this, Extended Data Figure 3 shows that rapamycin activation of the subcellularly targeted FacePalm results in recruitment of FacePalm-17C$_{CT}$ to the intended subcellular domain as compared to the non-targeted FacePalm.

(4) The reviewer mentions that the study is conducted only for overexpressed protein substrates and that it is essential to demonstrate the tool for endogenous proteins. We note that in the hippocampal neurons, PSD95 is endogenously present and our manipulations in Fig. 7 are of the endogenous protein.

Miscellaneous comments:

1. Introduction is too thorough for a research paper. A more focused Introduction (only describing information centrally related to the study) would be helpful for readers to engage with the study. Similarly, Discussion can also be more concise than now by removing similar statements used elsewhere.

We agree. We have revised the introduction and the discussion to be more streamlined.

2. Too many “thus”; line 173 is representative.

We have removed these.

3. “high precision” (line 110); it is not clear in which parameter (space, time, target specificity?)
We agree and we have removed this.

4. Refs #57, 58 do not appear to match the statement (line 125).

We have replaced these.

5. Line 284 claims that this tool “provides a potential therapeutic strategy” for cancers, which sounds like an overstatement, as if one were to introduce a gene into cancer cells, it would be more straightforward to introduce a non-mutated form of a gene that is mutated (such as N-Ras WT vs. N-Ras Q61K).
We agree and we have removed this.

6. Similarly to #5, the use of the term “exquisite” in line 315 is not well justified, because there seems to be no dataset that helps judge how “exquisite” the present tool is over existing ones.

We agree and we have removed this and apologize for the unnecessary florid language.

7. Figure legends may need to be more instructive. For example, what is “HA” in Fig. 1h, and “control” in Fig. 1l?
We have added this.

Response to Reviewers

In this resubmitted manuscript, the authors have added several new experiments and made significant revisions. I was satisfied by the new controls showing that palmitoylation of several different substrates was reduced in a FacePalm activity-dependent manner, and that the FacePalm constructs were recruited to the targeted organelles. The new reversible optogenetic system and the specific targeting of PSD95 in neurons extend the scope of the study. Overall, these changes strengthen the manuscript and address my concerns.

We thank the reviewer for their careful consideration and positive assessment of our manuscript. We are very excited that the reviewer found our revision to have expanded the scope of our study and addressed concerns.

I have only a few minor comments that can be addressed primarily by edits to the figure legends:
-molecular weight markers are rarely labeled on the figures and instead are described in the legends or not at all. The molecular weight markers should be consistently labeled on the figure itself.
-scale bars are shown on the figures but the size of the bar is not mentioned in the legends.
-the value of “n” is not always supplied in all legends, especially in supplementary data
-legends should be checked for typos (eg. “trials” not “trails”)

We apologize for this oversight. We have corrected this for all the figures.

-the sequences used to direct constructs to different organelles are mentioned only in the methods. I would prefer that the identity of the targeting sequences also be mentioned in the main text or in the figure legend to make this information easier to find.

We have revised Figure 4 to include the identity of the targeting sequences for each location.

Reviewer #2 (Remarks to the Author):

The authors tried to address my comments and incorporated some new data into the revised manuscript. Especially, the addition of “a reversible optogenetic system” to ABHD17 manipulation increases the novelty of this manuscript (figure 3). To validate this new opto-probe, the authors should add the biochemical data (e.g., acyl-Rac) in figure 3 (at time: 0, 60 min, 180). Also, the local, reversible effect of opto-ABHD17 stimulation on PSD-95 clusters should be examined (figure 7). These data strengthen the paper.
We thank the reviewer for this suggestion. As requested, we validated that altered $Ca_V\beta$ localization observed with the new opto-depalm probe is indeed due to changes in palmitoylation by two strategies: (1) we disabled the catalytic activity (opto-depalom S/A mutant) of the probe and showed that there is no change in localization. (2) We have performed acyl-RAC to show time dependent changes in palmitoylation. These data are provided in Figure 3.

With regards to demonstrating “local, reversible effect of opto-ABHD17 stimulation on PSD-95 clusters,” there are some challenges with our current experimental setup. Currently with FacePalm, we use immunostaining to monitor changes in PSD95 clustering properties. This is

convenient as we can activate FacePalm in a whole dish and establish changes in PSD95 clustering statistically. While opto-depalm will allow for local depalmitoylation potentially within a small region of interest including a single cell, this would require live cell imaging to dynamically monitor changes in PSD95 localization. To do so, we would need to either overexpress a fluorescently tagged PSD95 or alternate approaches such as FingR/intrabodies to monitor endogenous PSD95. Over-expression of fluorescent-PSD95 by itself can lead to alterations in spine density and morphology (PMID: 11082065), which clouds interpretation. PSD95-FingR (PMID: 23791193) circumvents this limitation by labeling endogenous PSD95 and using a transcriptional regulatory system to ensure that the sensor is not overexpressed. Unfortunately, these overlap spectrally with opto-depalm. Therefore, establishing a new live-cell framework to demonstrate local changes in PSD95 clustering is beyond our capabilities.

Regarding differential effects of FacePalm-17A, -17B, and -17C on neuronal excitability in figure 7, the authors did not add any data to show/support the molecular mechanism, and just mentioned that this would be beyond the scope of this manuscript. This reviewer was disappointed at this response. This reviewer suggests deletion of electrophysiological data in figure 7, as the data is not relevant with other parts of this manuscript and such preliminary, uninterpretable data misleads readers.

We thank the reviewer for this suggestion. We agree that the electrophysiological data does not provide an exact molecular mechanism per se, i.e. the identification of a particular target or a palmitoylation site within a target. Nevertheless, we disagree that our data is “uninterpretable.” We show that changes in excitability upon activation of FacePalm-17B and FacePalm-17C are due to altered palmitoylation, since catalytically inactivate variants fail to perturb AP firing. This data was requested by other reviewers during the previous submission and was essential to satisfy concerns raised by other reviewers. To ensure that we do not mislead the reader about whether our study identifies a molecular mechanism, we state the following in our discussion:

Although our study does not identify an exact molecular mechanism underlying this change, altered AP properties likely reflect depalmitoylation of one or more targets as the catalytically inactivate FacePalm-17B and FacePalm-17C fail to appreciably perturb AP firing properties.

To our knowledge, there are no reports on changes in neuronal action potentials due to any of the Abhd17 enzymes. Therefore, we believe the overall finding is biologically interesting and valuable to the field as it opens up new avenues of study, even if the biophysical details are currently unknown. To draw a parallel, the effect of adrenergic agonists on increasing AP firing rate of nodal cells in the heart has been known for decades. The exact molecular mechanism, i.e the contribution of increased cAMP production and/or phosphorylation of different targets, still remains to be fully established. Nevertheless, the finding that there are changes in the AP firing has been fundamentally important to understand heart function as whole.

Reviewer #3 (Remarks to the Author):

The authors properly addressed my oiginal concerns.

We thank the reviewer for their careful consideration and positive assessment of our manuscript.