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Nuclear import of the DSCAM-cytoplasmic domain drives signaling capable of inhibiting synapse formation

Sonja M. Sachse, Sam Lievens, Luís F. Ribeiro, Dan Dascenco, Delphine Masschaele, Katrien Horré, Anke Misbaer, Nele Vanderroost, Anne Sophie De Smet, Evgenia Salta, Maria-Luise Erfurth, Yoshiaki Kise, Siegfried Nebel, Wouter Van Delm, Stéphane Plaisance, Jan Tavernier, Bart De Strooper, Joris De Wit, Dietmar Schmucker

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28th May 2018

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find your analysis interesting and support publication here. They raise a number of reasonable concerns that I anticipate you should be able to resolve in a good way. Given the comments I would like to invite you to submit a revised manuscript that addresses the concerns raised by the referees. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to resolve the concerns at this stage.

Let me know if we need to discuss any of the points further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

REFEREE REPORTS:

Referee #1:

In the current study, Sachse et al. identify an interesting potential mechanism through which the Dscams could regulate neurodevelopment. The authors investigated protein interactions of the intracellular domains domains (ICDs) of DSCAM and DSCAML1, using a mammalian two-hybrid technique to identify previously unknown binding partners. One such interactor was Importin 5, which they found to bind to a putative nuclear localization signal (NLS) in the ICD directly adjacent to the cell membrane. The authors provide evidence in cell lines that the ICDs can be cleaved by gamma-secretase and that the liberated ICDs localize to the nucleus and affect gene expression. They also show that overexpression of ICDs with intact NLS can negatively regulate axon outgrowth and synapse formation in cultured neurons. The authors conclude that the Dscams function in part through a membrane-to-nucleus signaling mechanism, and suggest that overexpression of DSCAM ICD could contribute to Down syndrome pathology. This would represent a previously unappreciated mechanism for these important molecules. The paper is generally well written and the figures are clear. The result is quite provocative and will prompt a great deal of additional research in the field, but at the same time, some of the results are not thoroughly validated by multiple approaches and the paper could be improved by addressing the following concerns.

Major concerns:
1. The MAPPIT technique is a powerful tool to identify and map protein interactions, but the candidates should be validated with an independent method such as co-immunoprecipitation from transfected cells.
2. The strongest evidence that gamma-secretase is responsible for liberating the ICDs is the pharmacological data presented in Fig 2F. This conclusion would be strengthened if these inhibitors were used with the cleavage luciferase reporter assay in Fig 2D, and especially with the analysis of nuclear localization in neurons in Fig 3G.
3. Regarding the RNA-seq experiments in HEK cells, the enrichment of nervous system specific pathways is appreciated, but top candidate DEGs should be validated in neurons. This could be done by quantitative in situ hybridization (e.g., RNAScope or Nanostring) on lenti-transduced neurons.
4. The relationship between the differentially expressed genes (>50% overlap between Dscam and Dscaml1 ICD) producing similar effects on axon outgrowth but only Dscam effecting synapse number should be discussed more. Are there genes specific to the Dscam set that may account for this?
5. Figure 3 suggests that there is much more nuclear localization when ICD is expressed than when FL constructs are expressed. This makes it difficult to conclude that nuclear ICD is responsible for the synapse loss seen in FL overexpression. A better comparison would be between FL and FL-delta-NLS.

Minor concerns:
1. The C-terminal YFP tag likely disrupts PDZ interactions. This should be acknowledged and discussed in light of their functional significance (e.g., Yamagata and Sanes, JNeurosci 2010, Garrett et al., eLife 2016)
2. The analyses of neurite length were performed at E14.5 +4DIV. These are still quite immature neurons. These analyses could be extended by culturing the neurons longer and performing Sholl analysis to separate the assessment of dendrite arborization and axon outgrowth.
3. The authors should discuss if any increase in cell death was observed in FL vs. ICD expressing neurons.
4. At the top of page 2 in discussion of Dscam gene dosage and Down Syndrome, the results of Blank et al., J Neurosci 2011 should be mentioned.
5. Page 3, I believe "intellectual disability" is the preferred term over "mental retardation."
6. Page 10, "electroporated at E18..." gives the impression that the electroporations were in vivo and primary cells were then isolated, could this be spelled out to read, "neurons isolated from E18 embryos were electroporated and cultured..."

Referee #2:

Authors identified several novel cytoplasmic signaling effectors of vertebrate DSCAMs using MAPPIT approach, which include IPO5, STAT3,DYRK1A/B, SH2D2A, and USP21. Moreover, they found that the ICD of mammalian DSCAM is liberated by γ-secretase-mediated cleavage and that both the DSCAM and DSCAML1 ICD efficiently translocate to the nucleus, and DSCAMs interact with IPO5 via nuclear localization signal. They further demonstrated that nuclear enrichment of the DSCAM and DSCAML1 ICD in cell lines alters the transcription of genes associated with neuronal differentiation and function. Increased nuclear levels of either the DSCAM or DSCAML1 ICD, impairs neurite outgrowth in primary mouse cortical cultures. Finally, they found that only increased expression of either full-length DSCAM or the DSCAM ICD, but not of the DSCAML1 ICD leads to a strong decrease in synapse numbers in primary mouse hippocampal neurons. The findings of IPO5 mediates membrane-to-nucleus translocatiaon of DSCAM/L1 is novel and important given that increased DSCAM levels have been proposed to contribute to mental retardation in Down syndrome patients. These findings also add new insight of DSCAM family members intracellular signaling mechanisms in general, which we current know very little about. I found the manuscript is well written and data is of high quality.

Referee #3:

Neuronal cell adhesion molecules play a pivotal role in the development and proper function of the neuronal circuitry. Whereas the essential roles of these proteins is well established, the precise cellular mechanisms and in particular the downstream signaling effecting cellular functions of neuronal cells is in many cases unknown. In the manuscript submitted by Sachse et al. a new membrane-to-nucleus signaling of DSCAM/DSCAML1 has been proposed. In an unbiased screen for the identification of proteins interacting with the intracellular domain (ICD) of DSCAM/DSCAML1 several interaction partners were identified including STAT3, USP21, DYRK1 A/B, SH2D2A and importin 5 (IPO5). The authors demonstrate that DSCAM's ICD can be released by γ-secreatase. The interaction of nuclear localization sequence (NLS) of ICDs with IPO5 leads to nuclear translocation of ICD following by profound changes in gene expression. The overexpression of NLS containing but not NLS-lacking DSCAM- or DSCAML1-ICDs leads to significant impairment of neurite outgrowth in primary cortical neurons. Moreover the expression of NLS-containing or full-length DSCAM leads to substantial reduction in synapse number resulting from cell-autonomous as well as cell-non-autonomous effects. Taking in account the variety of new exciting findings described here by the authors as well as the implication of DSCAM in the pathogenesis of the Down syndrome this study is absolutely appropriate to be published in EMBO Journal.
Despite the striking results and high quality data provided by the authors there are some points which should be addressed / clarified / corrected by the authors to support the conclusions of this manuscript:
Major points:
(1) Authors propose that the nuclear translocation of DSCAM/L1-ICDs and consequent changes in gene transcription are responsible for the phenotypes observed in neuronal culture. Indeed profound changes in gene transcription including those involved in neurite outgrowth and synaptic function could be observed in HEK cells overexpressing DSCAM/L1-ICDs by RNAseq experiments. It would strongly support the hypothesized mechanism if authors could demonstrate that similar changes in gene expression (for some selected genes) occur in neuronal cells. This could be done by qPCR using RNA isolated from lentivirus infected primary neurons (as in experiments demonstrated impairment of neurite outgrowth) or any other appropriate methods (ICH, in situ hybridization, Western blot) if preferred by the authors.
(2) In the paragraph 'The ICDs of DSCAM and DSCAML1 Translocate to the nucleus' (page 7) authors highlight the differences of cleavage efficiency and nuclear translocation between HEK cells and primary neurons expressing full-length DSCAM/L1. This conclusion is made based on the observation of ICH data (Fig.3). In the supplementary methods authors state that cortical neurons were treated with proteasome inhibitor MG132 prior to fixing. This is not mentioned in the main text or figure legend and it is not clear if HEK293 cells were treated in the same way. If not, this could of course explain observed differences. This point should be clarified by the authors.
Minor points:
(1) Please correct: Legend to Fig.3 '(B-G) Scale bars, 10μm.', not 10μM.

Non-essential suggestions:
Several SH2-domain containing proteins were demonstrated in this study to interact with DSCAM/L1-ICDs in Y-containing-motif dependent manner. This interaction would require phosphorylation of the Y-residue. Indeed some reports demonstrate Y-phosphorylation of DSCAM upon treatment with netrin-1. This fact might be interesting for the discussion of the results of this study. Additionally, the interaction of DSCAM with SH2D2A which is known to be specifically expressed in T-cells and probably involved in the regulation of T-cell activation is a very interesting finding since individuals with Down syndrome are known to have impairments of the immune system. This might be also an interesting aspect which highlight the importance of the study and would be worth to be mentioned in the discussion.

1st Revision - authors' response 4th Dec 2018

We thank the referees for their generally positive response, as well as their in-depth analysis and highly constructive comments on the previous version of our manuscript.
We have by now addressed all the potential concerns raised by the referees.
We are confident that we can offer satisfying answers to all the questions and points raised.
Together, we feel that the revisions have considerably strengthened the conclusions of our manuscript.
Below we list all changes concerning the figures. Subsequently we address and discuss the referees comments point-by-point. Comments by the referees are in *Italics*.

1. We have changed Figure 3D to address the second comment of referee 3 regarding the cleavage efficiency and nuclear translocation between HEK cells and primary neurons expressing full-length DSCAM/L1.
2. We have added a new main figure (Figure 5 in revised manuscript) addressing the third concern of referee 1 and the first concern of referee 3.
3. Main figures 5, 6, 7 of our previous version of the manuscript are now main figures 6, 7, and 8 in the same order in the revised manuscript.
4. We have added an additional expanded view figure (Figure EV1) to address the first concern of referee 1 regarding the validation of MAPPIT candidates by co-immunoprecipitation.
5. To address the second point of referee 1 concerning the cleavage of DSCAM by gamma-secretase, we have added an additional expanded view figure (Figure EV2).
6. We have added an additional figure panel (E) to our former figure S5, which is now expanded view figure EV3.
7. Former Figure S4 is now included in the source data file for main Figure 2F.
8. Former Figures S6, S7, and S8 are now Appendix Figure S4, S5 and S6 in their initial order.
9. We have slightly modified/corrected Figure 4D by adding a differentially expressed extracellular factor (i.e. SFRP4) to the “extracellular” bar graph. We had noted this accidental omission by reviewing expression differences of DSCAM-cyto versus DSCAML1-cyto.

Comments Referee #1
Overall: “*…This would represent a previously unappreciated mechanism for these important molecules. The paper is generally well written and the figures are clear. The result is quite provocative and will prompt a great deal of additional research in the field, but at the same time, some of the results are not thoroughly validated by multiple approaches and the paper could be improved by addressing the following concerns.”*

Response:
We truly appreciate the supportive assessment and comment below on all concerns point by point.

Concerns:
1. *"…The MAPPIT technique is a powerful tool to identify and map protein interactions, but the candidates should be validated with an independent method such as co-immunoprecipitation from transfected cells…"*

Response:
As suggested by the referee, the interactions between DSCAM as well as DSCAML1 and candidates have been successfully validated by co-immunoprecipitation from cells transfected with Ha-tagged DSCAM or DSCAML1 and Flag-tagged binding partners. We were successful in demonstrating co-immunoprecipitation with all six potential downstream signaling partners (IPO5, SH2D2A, STAT3, DYRK1A, DYRK1B, and USP21). We have added an additional figure with the results in our updated version of the manuscript (please see the new Figure EV1).
Interestingly, we noted that successful co-immunoprecipitation of DSCAM/L1 and IPO5 required the strict presence of Tyrosine-, as well as Serine-, and Threonine-Phosphatase inhibitors. This suggests that the interaction between DSCAM receptors and IPO5 is likely regulated by phosphorylation, potentially involving multiple classes of kinases. Note that the interaction between DSCAM and IPO5 was most convincingly confirmed in SH-SY5Y human neuroblastoma cells (also in the presence of phosphatase inhibitors). In HEK293T cells the interactions were more labile and sensitive to phosphatases. Notably, as opposed to HEK293T cells we could observe low levels of nuclear localization of DSCAM-YFP in SH-SY5Y cells even in the absence of proteasomal inhibitor (see Figure EV2A of revised manuscript), suggesting that kinases and/or phosphatases regulating the interaction between DSCAM and IPO5 might be expressed at different levels in those cells.

2. *"…The strongest evidence that gamma-secretase is responsible for liberating the ICDs is the pharmacological data presented in Fig 2F. This conclusion would be strengthened if these inhibitors were used with the cleavage luciferase reporter assay in Fig 2D, and especially with the analysis of nuclear localization in neurons in Fig 3G…"*

Response:
We fully agree with the referee. As proposed, we further confirmed our finding of gamma-secretase mediated cleavage of DSCAM by analyzing DSCAM nuclear localization also in primary neurons (see new Figure EV2 C-D of revised manuscript). We found that the reduction of nuclear YFP intensity in the presence of gamma-secretase inhibitor was distinctive and statistically significant. We would like to note, however, that we did not analyze a large number of neurons, as combined Lactacystin/ gamma-secretase inhibitor treatment caused high levels of cell death in primary neuronal cultures, even at low concentrations, suggesting that neurons are very sensitive to prolonged incubation with those inhibitors. This is not surprising, given the multitude of cellular processes, which are likely affected by gamma-secretase inhibition in neurons. Nevertheless, since cell lines were found to be more robust, we also performed these experiments in human neuroblastoma SH-SY5Y cells in order to strengthen our findings further. We have added these additional findings as a novel figure in our revised manuscript (see new Figure EV2 of revised manuscript).

3. *"…Regarding the RNA-seq experiments in HEK cells, the enrichment of nervous system specific pathways is appreciated, but top candidate DEGs should be validated in neurons. This could be done by quantitative in situ hybridization (e.g., RNAScope or Nanostring) on lenti-transduced neurons…"*

Response:
We agree with the referee and we have validated several nervous system specific DEGs further. We used both quantitative PCR on cells as well as RNAscope on primary neurons. This point was also raised by referee 3 (see below).

In our hands nucleofection as well as lentiviral-transduction efficiency of DSCAM constructs in neurons was 20% at best. This poses a problem for mRNA quantification by qPCR since at least 80% of neurons are not over-expressing DSCAM and therefore changes in gene expression in transfected neurons will be "masked" by mRNA levels of wt neurons. For this reason we chose to validate some of the changes in gene expression in transfected mouse Neuro 2A cells, which is a neural crest-derived cell line and an established model system to study neuronal differentiation and signaling. In N2A cells we could achieve 60-70% transfection efficiency and were able to confirm differential expression of UNC5A, TUBB3, PCDH17 and NOS1 in cells expressing the DSCAM ICD as compared to control conditions by quantitative RT-PCR.
These data have been added in Figure EV3E in our revised version of the manuscript.
We also agree with the referee that quantitative in situ hybridization methods would be an elegant way to measure mRNA levels in primary neurons. In particular, since these methods allow to analyze mRNA levels in single DSCAM ICD overexpressing cells. Hence low transfection efficiencies and masking of gene expression changes by wt neurons would not pose a problem with those methods. For this reason we adapted the RNAscope technology to primary neurons grown on cover slips and validated expression changes of three potential target genes. We performed single-molecule fluorescent *in situ* hybridization (smFISH) on hippocampal cultures transfected with DSCAM-ICD-YFP or YFP alone using probes against mouse *Unc5a*, *Pcdh17*, and *Ntrk2* (*TrkB*) mRNAs and multiplexed each candidate probe with a probe against YFP mRNA in order to detect single YFP positive neurons. There too, we were able to show clear transcriptional changes for Unc5a, Ntrk2, and PCDH17. These new data were integrated in the revised manuscript as new figure 5A-F and text page 10.

4. *"…The relationship between the differentially expressed genes (>50% overlap between Dscam and Dscaml1 ICD) producing similar effects on axon outgrowth but only Dscam effecting synapse number should be discussed more. Are there genes specific to the Dscam set that may account for this?..."*

Response:
This is a very interesting point. Some DEGs unique to the DSCAM data set could indeed account for the differences observed between DSCAM and DSCAML1 regarding synapse development. For instance, L1CAM (L1 Cell Adhesion Molecule), SFRP4 (Secreted Frizzled Related Protein 4), NTF3 (neurotrophin 3), RGMA (Repulsive Guidance Molecule Family Member A), SEMA3E (Semaphorin 3E), as well as a cluster of Ephrins (i.e. EFNA3, EFNA4, EFNB3) are neuronal genes that are exclusively (i.e. differentially) expressed upon DSCAM nuclear enrichment. In particular the fact that the effect of DSCAM cyto function has a strong cell non-autonomous component suggests that the crucial factor/s might be either secreted or on the cell surface.
We have addded the following note on this to the results:
"Despite the overlap between DEGs in the DSCAM/DSCAML1 datasets (Fig 4C) several gene expression changes were specific for DSCAM including a cluster of Ephrins, namely EFNA3 (Ephrin A3), EFNA4 (Ephrin A4), EFNB3 (Ephrin B3), as well as SFRP4 (Secreted Frizzled Related Protein 4), L1 cell adhesion molecule (L1CAM), SEMA3E (Semaphorin 3E), and RGMA (Repulsive Guidance Molecule Family Member A) (Fig 4D)."
We have further thought that it could be tested most directly by biochemical means. But of course, verification of any potential factor and its role in synapse formation would be a major effort and a new study in itself. We believe, however, that this is beyond the scope of this study.

5. *"...Figure 3 suggests that there is much more nuclear localization when ICD is expressed than when FL constructs are expressed. This makes it difficult to conclude that nuclear ICD is responsible for the synapse loss seen in FL overexpression. A better comparison would be between FL and FL-delta-NLS..."*

Response:
We apologize if our description on this issue was confusing.
In the experiments on synapse numbers (e.g. figure 6) we compare FL-DSCAM with FL-DSCAML1 and control YFP in E18/DIV10 hippocampal neurons but not E14.5/DIV4 cortical neurons (figure 3). Remarkably, only FL-DSCAM but not FL-DSCAML1 impact on synapse formation.

Further, and this might have been the reason for the confusion, the nuclear localization of FL-DSCAM (figure 6) in hippocampal neurons is significantly stronger than nuclear localization of FL-DSCAM in cortical neurons (figure 3).

Importantly, looking at FL-DSCAM in primary hippocampal neurons we did not observe a significant difference regarding the nuclear levels when compared to DSCAM ICD alone tested in multiple cell types.

Overall, we think it is rather remarkable that a very significant amount of DSCAM readily translocates to the nucleus in hippocampal neurons suggesting a high degree of proteolytical processing of DSCAM in this cell type.

Minor concerns:
1. *"...The C-terminal YFP tag likely disrupts PDZ interactions. This should be acknowledged and discussed in light of their functional significance (e.g., Yamagata and Sanes, JNeurosci 2010, Garrett et al., eLife 2016)..."*

Indeed, one would expect that a C-terminal YFP tag might disrupt interactions with PDZ domain containing proteins. Moreover, as the referee points out, the DSCAM/L1 receptors are known to interact via their C-termini with synaptic scaffolding proteins of the MAGI and PSD95 families (Yamagata and Sanes, 2010; Garrett et al., 2016). However, functional studies where *Drosophila* Dscam1 transgenes were tagged C-terminally with GFP, showed - surprisingly - that Dscam1 loss of function can be rescued by these tagged proteins (Wang et al. 2004; Neuron, Vol. 43, 663–672). Furthermore, the Wang et al. study and several other subsequent studies showed convincingly that also subcellular localization is preserved in Dscam1-GFP fusion proteins. Dscam1 localizations is primarily dependent on the transmembrane segment (TM1-17.1 or TM2-17.2). In particular TM1-17.1 is almost exclusively localized to dendrites and a C-terminal fusion with GFP does not interfere with this localization or function (Wang et al. 2004). This suggests either that the PDZ docking site is still accessible despite a fusion to GFP, or that important Dscam1 functions do not depend on interactions with PDZ domain containing proteins.

Directly related to experiments described in our manuscript: In our proteomics (MAPPIT) screen, we used constructs in which the C-termini of DSCAM as well as DSCAML1 are not tagged and the PDZ- binding motif is freely accessible. Furthermore, in primary neurons and cell lines DSCAM/L1 localization at the membrane and particularly at cell-cell contacts was not disturbed by the C-terminal YFP-tag in our constructs (see figure 3D and G). Based on our analysis of binding motifs, most of the molecular interactions of DSCAM/L1 identified in this study (i.e. IPO5, SH2D2A, USP21 and STAT3) do not involve the C-terminal PDZ-interacting motif, but occur with motifs localized within the ICDs (see Figure S3, Appendix). Placing the YFP tag at other positions within the cytoplasmic domain would therefore seem more risky in terms of interference with DSCAM

function. In particular, the interaction of IPO5 with the NLS of DSCAM/L1 likely requires a freely accessible N-terminal end of the ICD. However, we were careful in placing a flexible 11aa linker between the C-terminus and the YFP. We reasoned that this enhances the chances that the PDZ docking site is still accessible for interacting proteins. Such a scenario has been tested systematically in studies that tested functional preservation of GFP tagged genes in flies (e.g. gene-trapping insertions at endogenous genes: Venken et al. 2011; Nagarkar-Jaiswal et al. 2015).

2. *"…The analyses of neurite length were performed at E14.5 +4DIV. These are still quite immature neurons. These analyses could be extended by culturing the neurons longer and performing Sholl analysis to separate the assessment of dendrite arborization and axon outgrowth…"*

Response:
Indeed the analysis was performed in young neurons although we have also tried to accomplish Scholl analysis in more mature neurons to asses axon outgrowth after longer periods of in vitro culture. However, in order to obtain single neurons after extended times of in vitro culture of more mature neurons, we needed to seed the cultures at extremely low densities to prevent that neurites from neighboring neurons extensively overlap, (which obscures neurite tracing and Scholl analysis). Unfortunately, in our hands neuronal cultures could not tolerate this low density in combination with lentiviral expression of DSCAM constructs.

3. *"…The authors should discuss if any increase in cell death was observed in FL vs. ICD expressing neurons…"*

Response:
Given that overexpression of full length DSCAM in mice has been shown to increase neuronal cell death of developing retinal neurons (Li et al., 2015) it is possible that the cleaved ICD may be involved in the regulation of neuronal cell death. We therefore assessed neuronal cell death levels of cortical neurons (E14.5, DIV4) infected at DIV1 with lentivirus expressing FL DSCAM-YFP, DSCAM ICD-YFP, or YFP by analyzing their nuclear morphology. We quantified the number of nuclei with condensed chromatin based on nuclear dye (DAPI) staining (as described in Vieira et. al., 2016). We did not find a significant difference in the percentage of dead YFP-positive neurons in cultures transduced with DSCAM FL (10%) or DSCAM ICD (7.5%) relative to the total number of YFP-positive neurons (dead YFP-positive cells/ total number YFP-positive cells; see figure 1C below). In general, there were only few YFP-positive cells with condensed nuclei in both conditions. Since YFP-tagged proteins may have been degraded already in dead neurons we also quantified overall cell death levels in DSCAM FL vs. DSCAM ICD infected neuron cultures. There was also no significant difference in the percentage of degenerating nuclei in DSCAM FL (18.84%) and DSCAM ICD (21.3%) infected cultures (dead cells/ total number of cells; see figure 1B below). This suggests, that at least in lentivirus infected DIV4 cortical cultures there is no difference of cell death levels in DSCAM FL vs. ICD expressing neurons.

Nevertheless, we cannot exclude a difference in cell death for other time periods of in vitro culture or different embryonic days at which the cultures are isolated. Especially after culturing neurons for an extended time in vitro (i.e. DIV10) followed by immuno-staining, dead neurons are mostly already lost from the coverslip. Therefore a potential difference in cell death levels between DSCAM FL vs. ICD expressing cultures may be obscured.

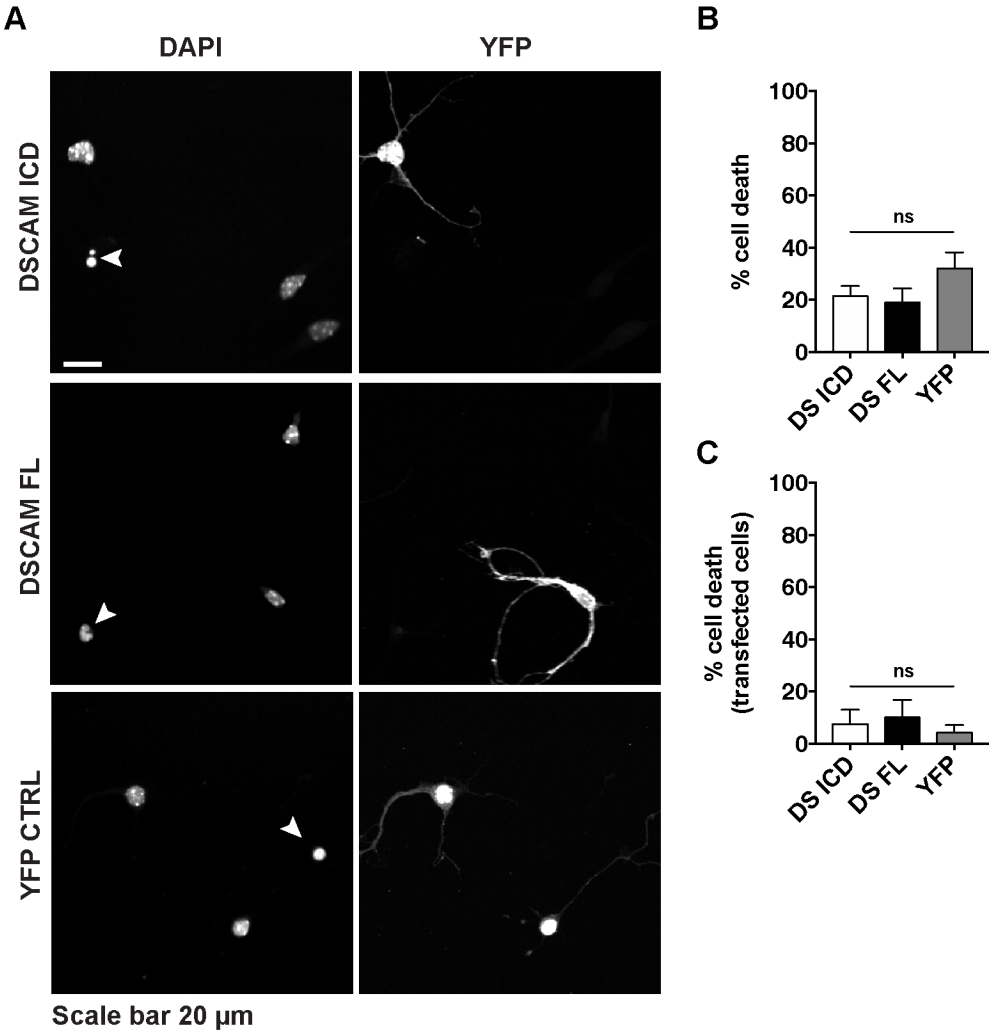

Figure 1. Quantification of cell death levels in primary cortical neurons expressing DSCAM FL vs. DSCAM ICD. (A) Primary cortical cultures from E14.5 mouse embryos were transduced with lentiviral particles expressing DSCAM FL YFP, DSCAM ICD -YFP, or YFP (control) and immuno-stained at DIV4 for YFP, and nuclei were visualized with DAPI. Neuronal cell death was determined by quantifying the number of neurons exhibiting condensed nuclei (arrow heads) based on the DAPI staining. Single confocal projections are shown. (B-C) Quantification of cell death. Bar graphs show the mean ±SEM. P-values ≤ 0.5 , non-significant (ns), calculated by Kruskal-Wallis test with Dunn's multiple comparisons test. (B) Percentage of dead cells relative to the total number of cells. (C) Percentage of dead transfected cells relative to the total number of transfected cells.

4. *"...At the top of page 2 in discussion of Dscam gene dosage and Down Syndrome, the results of Blank et al., J Neurosci 2011 should be mentioned..."*

Response:
We have added a small paragraph summarizing the results of Blank et al. 2011 in the introduction of our revised manuscript (line 64-69).

5. *"...Page 3, I believe "intellectual disability" is the preferred term over "mental retardation."*

Response:
This is absolutely correct. The use of the term "mental retardation" has been replaced throughout the manuscript with "intellectual disability".

6. "*…Page 10, "electroporated at E18..." gives the impression that the electroporations were in vivo and primary cells were then isolated, could this be spelled out to read, "neurons isolated from E18 embryos were electroporated and cultured..."*

Response:
The sentence has been rewritten and now reads: "Primary mouse hippocampal neurons isolated from E18 embryos were electroporated with wt and NLS-deficient YFP-fusions of the DSCAM/L1 ICDs, YFP-tagged full-length DSCAM/L1, as well as a nuclear YPF control. At DIV10 we determined the density of excitatory synapses… ."

Referee #2:

"Authors identified several novel cytoplasmic signaling effectors of vertebrate DSCAMs using MAPPIT approach, which include IPO5, STAT3,DYRK1A/B, SH2D2A, and USP21. Moreover, they found that the ICD of mammalian DSCAM is liberated by γ-secretase-mediated cleavage and that both the DSCAM and DSCAML1 ICD efficiently translocate to the nucleus, and DSCAMs interact with IPO5 via nuclear localization signal. They further demonstrated that nuclear enrichment of the DSCAM and DSCAML1 ICD in cell lines alters the transcription of genes associated with neuronal differentiation and function. Increased nuclear levels of either the DSCAM or DSCAML1 ICD, impairs neurite outgrowth in primary mouse cortical cultures. Finally, they found that only increased expression of either full-length DSCAM or the DSCAM ICD, but not of the DSCAML1 ICD leads to a strong decrease in synapse numbers in primary mouse hippocampal neurons. The findings of IPO5 mediates membrane-to-nucleus translocatiaon of DSCAM/L1 is novel and important given that increased DSCAM levels have been proposed to contribute to mental retardation in Down syndrome patients. These findings also add new insight of DSCAM family members intracellular signaling mechanisms in general, which we current know very little about. I found the manuscript is well written and data is of high quality."

Response:
We thank the reviewer for this supportive and positive evaluation of our manuscript.

Referee #3

"...Taking in account the variety of new exciting findings described here by the authors as well as the implication of DSCAM in the pathogenesis of the Down syndrome this study is absolutely appropriate to be published in EMBO Journal.
Despite the striking results and high quality data provided by the authors there are some points which should be addressed / clarified / corrected by the authors to support the conclusions of this manuscript":
Response:
We truly appreciate the supportive assessment and comment below on all concerns point by point.

Concerns:
1. *"...Authors propose that the nuclear translocation of DSCAM/L1-ICDs and consequent changes in gene transcription are responsible for the phenotypes observed in neuronal culture. Indeed*

profound changes in gene transcription including those involved in neurite outgrowth and synaptic function could be observed in HEK cells overexpressing DSCAM/L1-ICDs by RNAseq experiments. It would strongly support the hypothesized mechanism if authors could demonstrate that similar changes in gene expression (for some selected genes) occur in neuronal cells. This could be done by qPCR using RNA isolated from lentivirus infected primary neurons (as in experiments demonstrated impairment of neurite outgrowth) or any other appropriate methods (ICH, in situ hybridization, Western blot) if preferred by the authors…"

Note: this concern was raised by both, referee 1 and 3. We fully agree with the referees and we have validated several nervous system specific DEGs further. We used both quantitative PCR on cells as well as RNAscope on primary neurons. In our hands nucleofection as well as lentiviral-transduction efficiency of DSCAM constructs in neurons was 20% at best. This poses a problem for mRNA quantification by qPCR since at least 80% of neurons are not over-expressing DSCAM and therefore changes in gene expression in transfected neurons will be "masked" by mRNA levels of wt neurons. For this reason we chose to validate some of the changes in gene expression in transfected mouse Neuro 2A cells, which is a neural crest-derived cell line and an established model system to study neuronal differentiation and signaling. In N2A cells we could achieve 60-70% transfection efficiency and were able to confirm differential expression of UNC5A, TUBB3, PCDH17 and NOS1 in cells expressing the DSCAM ICD as compared to control conditions by quantitative RT-PCR.

These data have been added in Figure EV3E in our revised version of the manuscript.

We believe that quantitative in situ hybridization methods is the most direct way of measuring mRNA levels in primary neurons. In particular, since these methods allow to analyze mRNA levels in single DSCAM ICD overexpressing cells. Hence low transfection efficiencies and masking of gene expression changes by wt neurons would not pose a problem with those methods. For this reason we adapted the RNAscope technology to primary neurons grown on cover slips and validated expression changes of three potential target genes. We performed single-molecule fluorescent *in situ* hybridization (smFISH) on hippocampal cultures transfected with DSCAM-ICD-YFP or YFP alone using probes against mouse *Unc5a*, *Pcdh17*, and *Ntrk2* (*TrkB*) mRNAs and multiplexed each candidate probe with a probe against YFP mRNA in order to detect single YFP positive neurons. There too, we were able to show clear transcriptional changes for Unc5a, Ntrk2, and PCDH17. These new data were integrated in the revised manuscript as new figure 5A-F and text page 10.

2. *"…In the paragraph 'The ICDs of DSCAM and DSCAML1 Translocate to the nucleus' (page 7) authors highlight the differences of cleavage efficiency and nuclear translocation between HEK cells and primary neurons expressing full-length DSCAM/L1. This conclusion is made based on the observation of ICH data (Fig.3). In the supplementary methods authors state that cortical neurons were treated with proteasome inhibitor MG132 prior to fixing. This is not mentioned in the main text or figure legend and it is not clear if HEK293 cells were treated in the same way. If not, this could of course explain observed differences. This point should be clarified by the authors…"*

Response:

We agree that this part was not clear in the original manuscript and we thank the reviewer for noticing this discrepancy. Indeed, the images now shown in Figure 3D corresponded to non-treated cells in the previous version of the manuscript. The images have been updated to precisely match the culture conditions (i.e. in the presence of proteasome inhibitor). We apologize for this error. In fact, cortical neurons as well as HEK293 cells expressing full-length DSCAM/L1 in Figure 3 were not treated with MG132 but Lactacystin and this was not mentioned in the first submission. Importantly, for HEK293 cells we do not observe nuclear localization in the presence or absence of proteasome inhibitors.

MG132 but not Lactacystin was used in experiments in primary neurons shown in Figure 5 (now now figure 6 in revised manuscript) and 6 (now figure 7 in revised manuscript)M to stabilize intracellular fragments generated by γ-secretase as well as DSCAM/L1 ICDs as these are rapidly degraded by the proteasome (Cupers et al., 2001). We have revised the contents of the material and methods section, as well as the figure legends of figure 3, 5 (now figure 6 in revised manuscript), 6 (now figure 7 in revised manuscript) to clarify this point.

Minor points:
1. *"...Please correct: Legend to Fig.3 '(B-G) Scale bars, 10µm.', not 10µM..."*

Response:
This typing mistake has been corrected in the revised manuscript.

2. *"Non-essential suggestions: Several SH2-domain containing proteins were demonstrated in this study to interact with DSCAM/L1-ICDs in Y-containing-motif dependent manner. This interaction would require phosphorylation of the Y-residue. Indeed some reports demonstrate Y-phosphorylation of DSCAM upon treatment with netrin-1. This fact might be interesting for the discussion of the results of this study. Additionally, the interaction of DSCAM with SH2D2A which is known to be specifically expressed in T-cells and probably involved in the regulation of T-cell activation is a very interesting finding since individuals with Down syndrome are known to have impairments of the immune system. This might be also an interesting aspect which highlight the importance of the study and would be worth to be mentioned in the discussion."*

Response:

We thank the reviewer for this highly constructive comment and we commented on this at the end of the discussion.

2nd Editorial Decision 21st Dec 2018

Thanks for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referees #1 and 3 and their comments are provided below.

As you can see both referees appreciate the introduced changes and support publication here. Referee #3 suggests to add the data on the nuclear translocation of the DSCAM ICD provided in the point-by-point response into the paper. I think it is a good suggestion, but will leave it up to you if you would like to do so.

There are just a few editorial things to sort out before I can see you the formal acceptance letter.

- Appendix Fig S 4C and Tables S3-4 are not called out.

- The reference format should be 20 authors before et al at the moment it is 10 author names before et al.

- Figure legends: For Fig EV2, a panel E is mentioned in the legend but there is no panel E for this figure.

- Our publisher has done a publication pre-check on the manuscript and made some comments regarding the figure legends. Will you please incorporate their suggestions. When you log into the system you will see the file - it is called Wiley pre-acceptance check. Please take a look at the word document as this is the file that has the marked changes.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

You can use the link below to upload the files

That should be all - congratulations on a nice study!

REFEREE REPORTS:

Referee #1:

The revised paper by Sachse et al. addresses all previous concerns. Specifically, results of the MAPPIT screen are validated by independent IP methods, and results obtained in HEK cells are confirmed in neurons. This paper provides important insights into DSCAM signaling and direct regulation of transcription by the intracellular domain following gamma secretase cleavage. This is an important step forward in understanding the mechanisms through which DSCAMs exert their effects in neurodevelopment and the results will stimulate a large number of future studies.

My only comment on the revised manuscript it to please insert a paragraph break at line 250 after the call out for figure 2F.

Referee #3:

The revised version of the manuscript is substantially improved. The concerns raised from my side and by other reviewers were adequately addressed in the manuscript and in the point by point responses.
In particular authors clarified the experimental setup applied to detect the reported differences in the nuclear translocation of the DSCAM ICD upon the cleavage by gamma-secretase in HEK293 cells and primary hippocampal neurons. Interestingly, in the response to the point 1 raised by the reviewer- 1 authors mention the differences in nuclear translocation in HEK293 and SHSY5Y cells and speculate about involvement of phosphorylation in the interaction between DSCAM ICD and IPO5. This is an interesting point in the context of the nuclear translocation of DSCAM-ICD providing possible explanation to the observed differences of DSCAM ICD in different cell types. Why don't add this information to the manuscript?
Another important point which was raised by me and the reviewer 1 was the validation of transcriptional changes, observed in HEK cells overexpressing DSCAM/DSCAML1-ICDs, in neuronal cells. This was addressed by the authors by additional experiments in N2A neuroblastoma cells using qPCR and in primary neurons utilizing single molecule RNA FISH. In both experimental setups substantial changes in the transcription of the selected genes could be observed. Remarkably, for some of the tested genes these changes diverge (up- or down-regulation) in different cell types, thus, emphasizing, as also discussed by the authors, the importance of the cellular context for the effect of DSCAM-ICD on gene transcription.
In my opinion, the revised version of the manuscript is now acceptable for publication in EMBO Journal. There are no further concerns from my side. Congratulations to the authors!

2nd Revision - authors' response 4th Jan 2019

All requested editorial changes were made.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dietmar Schmucker
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99669No

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	An approximate number of samples to analyze was pre-specified for each experiment based on published literature describing similar experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Image aquisistion of DSCAM ICD nuclear localization (Fig EV2) upon gamma-secretase inhibition in neurons and SH-SY5Y cells was performed single blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, to the best of our knowledge.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Normal distribution was tested using the D' Agostino and Pearson normality test. If any group of data did not pass the test, non-parametric tests were used.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	A list of antibodies with catalog numbers, citations, or clone number has been included in the appendix supplementary methods section (see appendix table S4).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines is descibed in the appendix supplementary methods section (see "cell culture").

* for all hyperlinks, please see the table at the top right of the document

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNAseq data were deposited at GEO. The link is given in the "Data Availability" section.
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