STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites

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1st Editorial Decision 08 November 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your work, and referee #3 clearly supports publication. Referee #1 and #2, however, think that more work is required for further consideration here. Better support for your conclusions is needed as well as a more detailed insight into the described cholesterol transport.

Given the interest into the topic and the referees’ input and recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

Importantly,
- the EM analyses require better controls (referee #1, point 3; referee #2, point 6)
- the quantitation issues need to be fixed (referee #1, point 2)
- the ILV data for VAP depleted cells needs to be extended to studying STARD3 mutant cells (referee #1, point 4)
- it would be good to add further insight into which population of endosomes accumulate cholesterol in a STARD3 dependent manner (referee #1, minor point 4; referee #2, point 4)
- it would also be good to better show the physiological significance of your findings (referee #1, first paragraph and point 1; referee #2, point 7 and 10)
REFEREE REPORTS

Referee #1:

Wilhelm et al report that the sterol-binding protein STARD3 mediates cholesterol accumulation in endosomes and that this depends on the lipid transfer activity of STARD3 as well as on its ability to form ER-endosome contact sites. Using an in vitro assay, the authors find that VAP and STARD3 form a complex that promotes cholesterol transfer in membrane contact sites. The authors conclude that STARD3 mediates cholesterol transfer from ER to endosomes. Overall this is a potentially interesting story, but in its present form its biological significance remains unclear, and there are some technical issues.

Major points:

1. This manuscript provides evidence that STARD3 contacts transfer cholesterol from ER to endosomes. But when is this mechanism employed by the cells? Can absence of LDL mediated endosomal cholesterol loading trigger the STARD3 pathway? This should be tested by doing siRNA mediated STARD3 depletion in the presence and absence of LDL and measure endosomal cholesterol levels. Alternatively, changes could be observed in PM cholesterol levels, if the flow based assay was more sensitive. Upon LDL deprivation and STARD3 depletion, the level of endosomal cholesterol should be decreased.

2. The information about quantitative data is inconsistent. In general it is confusing that "n" is sometimes defined as number of cells and sometimes as number of experiments. For each quantification, it should be clearly stated in the figure legend how many independent experiments (from where the statistical testing should be done) and in addition how many cells per condition, to indicate the level of throughput. E.g. in figure 1: Are the values coming from only one experiment, since the total number of cells has been used for statistical testing? The number of independent experiments should be stated in the figure legend and mean values from independent experiments should be used for statistical testing. Likewise, statistical testing should be applied for all quantitations. It would be informative to know which samples were tested against each other. E.g Fig1E. are the mutants tested against ctr or each other? The black line is confusing. Indicate better what is tested against what, either with connecting lines in the graph, or in the figure legend.

3. In Fig. 3, how was the endosomes selected for quantification? The morphology of endosomes in STARD3 stable cells resembles lysosomes, whereas endosomes in the ctr and VAP depleted cells look like late endosomes. It would be more convincing to select endosomes after a pulse and subsequent 30 - 60 min chase of BSA-gold.

4. The authors state (bottom line, first paragraph page 7) that "STARD3-mediated cholesterol transport promotes inner luminal vesicles formation in endosomes". This statement is based on the EM result that VAP depletion reduces ILV formation in the STARD3 stable line. VAP depletion indeed reduces cholesterol accumulation in STARD3 stable cells, but since VAP proteins are required for many types of ER contacts, it is only an indirect argument towards STARD3 function. To be able to conclude directly that STARD3 mediated cholesterol transport promotes ILV formation in endosomes, the STARD3 mutant cell lines (STARD3 dSTART, STARD3MR/ND) should be analyzed by EM.

Minor points:

1. It is not clear from the images that the colocalization between D4 or filipin with LAMP1 or STARD3 is almost 100% as shown in the graphs. The scoring of colocalization was done manually. The authors should use Manders/Pearson scoring from at least 3 independent experiments. The amount of triple colocalication (STARD3/Filipin/LAMP1) or (STARD3/D4/LAMP1) could also be informative.

2. In Fig. S3f, some experiments have only been performed 2 times. Experiments should be repeated at least 3 times, even though this is a control experiment.

3. In Fig S4 there is no co-occurrence of endogenous STARD3 and VAP in HeLa/Ctr. Although the endogenous STARD3 signal is weak, it is visible in the picture as well as in the line plot. Why are there no ER-endosome contacts in this line plot?
4. Eden et al., 2016 have also reported that endosomal cholesterol is important for ILV formation, in an EGF and Rab7 dependent manner (Annexin1-ORP1L dependent). Does the STARD3 dependent cholesterol transfer happen in Rab7 endosomes only? Or is it also active in Rab5 endosomes? Since the cell has at least two mechanisms for transferring cholesterol from the ER to endosomes (AnnexinA1 (Eden et al 2016) and STARD3 (this manuscript), it would be informative to know which part of the endocytic pathway is covered, since ILV formation already start at the Rab5 stage. In Fig. 1, it is evident that STARD3 positive endosomes colocalize with LAMP1. However, there are many STARD3 positive LAMP1 negative endosomes, which also seems to accumulate filipin. Are these Rab5 or Rab7 positive?

5. Some of the floatation assays have only been done 2 times (Fig. S3F), and there is lack of statistical testing. For the DHE transport assay, the MR/ND mutant is tested together with the G7 mutant shown in Figure 5B. In addition, the MR/ND mutant is also tested in Fig. S3G, in combination with the VAP KD/MD mutant, but there is no call out to Suppl Fig 7E in the text.

6. Fig S6, showing that VAP depletion does not decrease PM cholesterol is not quantified, only showing example cells. Fig 7B, using the same assay in STARD3 mutant cell lines is quantified. The data would have been more conclusive if the effect of VAP depletion could also be quantified.

7. Since cholesterol loaded endosomes cluster perinuclearly, based on the ORP1L-RILP-dynein pathway, the STARD3 dependent loading of cholesterol in endosomes could induce perinuclear localization of endosomes via the ORP1L pathway. Indeed STARD3 overexpression induces perinuclear endosome clustering (suggested to be mediated via actin regulation (Holtau Vuori et al.). This should be discussed.

Referee #2:

This is a well-written manuscript addressing a new and exciting topic. The authors show that overexpression of STARD3, previously shown to induce ER:endosome membrane contacts, causes cholesterol accumulation in endosomes in a manner that depends on both the FFAT motif and sterol binding START domain of STARD3 and is at the expense of plasma membrane cholesterol. This is consistent with the idea that cholesterol is transferred from ER to endosomes at STARD3-dependent contacts under these conditions. Whilst there has been a great deal of discussion in the literature about the potential role of ER:endosome contacts in transport of LDL-derived lipid from endosomes to the ER, much less attention has been paid to their potential role in lipid transport in the reverse direction ie from the ER to endosomes. ORP1L:VAP interactions were implicated in lipid transport from the ER to endosomes containing activated EGF receptor in a recent study (Eden et al., 2016). The contacts described in the current study appear to be a distinct category of contacts that depend on STARD3 overexpression in unstimulated cells. The study is considerably strengthened by the authors' development of a very nice in vitro assay demonstrating directly that STARD3 can transport cholesterol from one membrane to another in a manner that depends on interaction with VAP. The in vitro assay is well validated and represents an important and significant advance over existing studies of ER:endosome contacts.

Specific points:

1. In the Intro '-MENTAL' domain needs to be defined.

2. In the Intro ILVs are mentioned in the final paragraph but have not been defined.

3. The materials and methods description of the D4 and filipin staining of plasma membrane and intracellular compartments is inaccurate- it is clearer from the figure legend of Figure S1 but should be amended in the methods.

4. In Figure 1 the authors use LAMP1 as a marker of endosomes. LAMP1 is a lysosomal protein, though it can also be expressed on late endosomes and this should be made clear in the text. Kant et al., (2013) reported that STARD3 is on endosomes that are ABCA3 positive and an earlier population than those positive for ORP1L/NPC1. The images in figure 1 show only partial costaining with LAMP, consistent with at least some STARD3 being present on an earlier LAMP1-ve population. Very little is made of the
costaining with LAMP1 in the text although quantitative line scans are shown in the figure. Although the accumulation of cholesterol in STARD3 +ve structures is very convincing it would be good to see some costaining with earlier endocytic markers to better identify the population of endosomes in which cholesterol accumulates.

5. The authors state on page 6 that "In situ staining using the GFP-D4 probe or filipin showed the absence of intracellular accumulation of cholesterol in cells expressing the two cholesterol transfer deficient mutants (Fig. 2CD)." However it appears to me that there is more intracellular cholesterol staining in the mutants compared to the control, albeit much less than in the cells overexpressing wild type STARD3. The same applies to the FFAT mutant. The sentence should be adjusted accordingly.

6. What criteria are used to identify the endocytic/lysosomal structures used for quantitation of internal membranes? The endosomes shown in Figure 3D and G appear to be multivesicular endosomes/bodies with discrete intraluminal vesicles and an electron lucent interior whilst those in Figure 3E and F also have irregular membranes and are electron dense. Are all intraluminal membranes quantified? If not, how is an ILV defined? Whilst the differences in internal membranes could be caused by STARD3-dependent sterol transport at membrane contacts and subsequent increases in luminal cholesterol, it could also be that different compartments are being compared i.e. multivesicular bodies in Figures D and G with lysosomes or even autophagosomes in E and F. How do the authors control for this? Also the endocytic vacuoles in STARD3 overexpressing cells appear enlarged compared to controls. Is this a consistent feature? Given that membrane contact sites between endosomes and the ER have been shown to have roles in endosome fission could recycling be affected by STARD3 depletion? This point should at least be discussed.

7. The authors state on page 9 that the finding that STARD3-induced cholesterol accumulation is not dependent on LDL is consistent with the idea that the cholesterol source is predominantly the ER. This should be tested by determining whether the STARD3-induced cholesterol accumulation is prevented by treatment of the cells with statins.

8. The reduced plasma membrane staining upon STARD3 overexpression appears to contrast with the data of Vassilev et al. 2015 Am J Pathol 185:987- who found reduced plasma membrane cholesterol and elevated HMG CoA reductase in STARD3 overexpressing cells. This should be discussed.

9. Page 10 "VAP expression was silenced in cells expressing STARD3 and the PM was labeled with GFP-D4 (Suppl. Fig 5). I think this should be sup figure 6.

10. The authors do not demonstrate effects of STARD3 depletion on cholesterol content of endosomes or indeed on membrane contact sites. Are the contacts reduced upon STARD3 depletion? If they are then the prediction would presumably be that cholesterol content of endosomes would decline under these conditions. Given that cholesterol content of endosomes is very low in control cells this would make a reduction in cholesterol content difficult to measure and so this is probably not possible. However, it begs the question of the physiological relevance of the effects of STARD3 overexpression. Under what conditions might STARD3 overexpressed? This is important and should be discussed as it potentially significantly affects the impact of the paper.

Referee #3:

This is a very clearly written manuscript, describing rigorous experimental work that discovers a cholesterol trafficking pathway from the ER to endosomes and establishes its molecular basis. This is a significant advance because how cholesterol is trafficked within cells is very poorly understood. The role of the STARD3 protein in this process is further elucidated as well as the importance of its FFAT motif, which allows for tethering/ contact site formation between the ER and endosomes. A role for tethering/contact site formation in enhancing lipid transport is established. The work nicely combines in vitro studies with studies in cells, leading to well-supported conclusions.

In my view, this manuscript should be published as is.
Response to Editor:

1- the EM analyses require better controls (referee #1, point 3; referee #2, point 6)

ANSWER 1:
I realize that the EM analysis was incomplete; actually it was meant to illustrate the ultrastructural complexity of the cholesterol loaded STARD3 endosomes. This was not a major finding in this report and it is not mentioned in the abstract. Nevertheless we have significantly modified this part in the manuscript: first we provide a clearer description of the structures that we quantified. Endosomes were chosen based on morphological criteria. In Vacca et al (2016, The late endosome, In Encyclopedia of Cell Biology), the authors reviewed the ultrastructure of the endocytic pathway. Late endosomes are described as multivesicular structures exhibiting multivesicular/multilamellar regions. Lysosomes are spherical electron dense structures while early endosomes are interconnected tubulo-cisternal elements. Multivesicular bodies (MVB), which contain intraluminal vesicles (ILVs) are intermediate compartments originating or reaching late endosomes. For EM quantifications, we selected vesicle sections harboring at least one ILV in order to exclude lysosomes, therefore including late endosomes per se as well as MVBs. In the original manuscript submission, the term ILV was misused: indeed, we did not quantify ILV (defined as spherical 50 nm vesicles) but all internal membranes found in MVB and late-endosomes e.g. ILV, multilamellar and multivesicular regions. This is now explained in the results section, in the methods section and in Figure 3 and Figure EV4 legends.

2- the quantitation issues need to be fixed (referee #1, point 2)

ANSWER 2:
As pointed by the reviewer 1, we did not use a homogenous nomenclature in the different experiments, privileging the quantification a large number of cells. We followed the reviewer recommendations. We modified the way the data are presented: “n” now always indicates the number of independent experiments and mean values are shown on the graphs. Therefore, statistical analyses were performed on mean values from independent experiments. At least three independent experiments were performed in each case. The details are stated in the figures legends. For examples for microscopy experiments, the number of experiments (n) and the total number of cells per conditions are now indicated in figure legends.

For a better clarity of the statistics, as suggested by the reviewer we modified the graphs and added connecting lines to indicate which sample was tested against which.

3 - the ILV data for VAP depleted cells needs to be extended to studying STARD3 mutant cells (referee #1, point 4)

ANSWER 3:
As suggested by referee #1, we analyzed late endosomes internal membranes by TEM in cells expressing the STARD3 DSTART mutant, which is unable to bind sterol, and in cells expressing the STARD3 FA/YA mutant, which is unable to generate ER-endosome contacts. These data are presented Figure EV4 F-J. Compared to HeLa/STARD3 cells which displayed endosomes with more internal membranes, HeLa/STARD3 DSTART and HeLa/STARD3 FA/YA cells possessed endosomes with internal membrane structures similar to control cells, which corroborated the data we obtained with VAP-depleted HeLa/STARD3 cells. Together, these observations suggest that STARD3-mediated cholesterol transport provides building blocks for membrane formation within endocytic vesicles.

3- it would be good to add further insight into which population of endosomes accumulate cholesterol in a STARD3 dependent manner (referee #1, minor point 4; referee #2, point 4)

ANSWER 4:
Regarding the late-endosomal localization of STARD3, the early localization studies on STARD3 were done in collaboration with Jean Gruenberg’s group and published in 2001 (Alpy et al., JBC). Nevertheless, we have added a new figure (Figure EV2) where these endosomes are characterized by co-labelling with a number of late endosomes markers (Rab7, BMP, CD63, Lamp1) and EEA1 as a marker of early endosomes (Figure EV 2A). Colocalization was measured by determining the Pearson correlation coefficient (PCC) (EV 2B); extensive co-localization was found between STARD3 and all late-endosome markers but not with the early endosome marker EEA1. Regarding the cholesterol loading of STARD3-positive endosomes, in Figure 1, we show that STARD3 colocalizes with cholesterol using PCC (Fig. 1 panel E) and in Figure EV2 C that in HeLa:STARD3 cells, Rab7 colocalizes with cholesterol. Figure EV2 (panels D and E) was also meant to better explain the difference between the two cholesterol probes used in the study. Filipin labels all free cholesterol, while GFP-D4 labels membranes with high cholesterol/phospholipid ratio 35 mol% sterol (Ohno-Iwashita et al., 2004). The staining with GFP-D4 is included in the filipin staining but it does not completely overlap, therefore there is a bias of using GFP-D4 to measure cholesterol colocalization. This is why we chose to determine whether all cholesterol loaded vesicle identified by the GFP-D4 probe were also STARD3 and Lamp1 positives (Fig 1B). Altogether, these additional experiments reinforce the message of the study by showing that STARD3 positive vesicles are late-endosomes and cholesterol loaded.

ANSWER 5:

The physiological role of STARD3 in intracellular cholesterol traffic is supported experimentally, by Figure 8 panel C, which shows that STARD3 silencing increases membrane cholesterol. Regarding the physiological significance of the cholesterol transport mediated by STARD3, we have no experimental support yet, but we can raise hypotheses. We believe that sterol transport by STARD3 at MCS is a way to rapidly modulate the sterol composition of subcellular region, while sparing the global cholesterol homeostasis which is under transcriptional control. This idea is better developed in the discussion:

“At the cellular level, cholesterol level needs to be constantly tuned (Steck and Lange, 2010). The regulation of cellular cholesterol occurs at the DNA level through the transcription factors from the SREBP family (Horton et al., 2002). SREBP-2 notably regulates the expression of genes involved in cholesterol synthesis and uptake in the ER and endosomes, respectively. One surprising finding of this study was the lack of SREBP-2 control over the cholesterol accumulation phenotype mediated by STARD3. This suggests that intracellular cholesterol levels can be modulated in discrete organelles without activation of the transcription machinery. Such a local regulation mode offers advantages in term of reactivity and precision, compared to the global regulation mode through transcription. This mode of regulation could also offer the advantage of sparing cellular energy by avoiding cholesterol synthesis and capture. The distribution of cholesterol mediated by STARD3 at MCS might represent an economic and fast way to modulate sterol content in specific subcellular territories such as in late endosomes internal membranes.”

Moreover the physiological relevance of this mode of cholesterol transport makes a lot of sense in the context of the START protein superfamily function (see Discussion paragraph #6 p13). Despite its importance, this aspect is going beyond the scope of our current study and will be explored in the future.

Referee #1:

Wilhelm et al report that the sterol-binding protein STARD3 mediates cholesterol accumulation in endosomes and that this depends on the lipid transfer activity of STARD3 as well as on its ability to form ER-endosome contact sites. Using an in vitro assay, the authors find that VAP and STARD3 form a complex that promotes cholesterol transfer in membrane contact sites. The authors conclude that STARD3 mediates cholesterol transfer from ER to
endosomes. Overall this is a potentially interesting story, but in its present form its biological significance remains unclear, and there are some technical issues.

**Major points:**

1. This manuscript provides evidence that STARD3 contacts transfer cholesterol from ER to endosomes. But when is this mechanism employed by the cells? Can absence of LDL mediated endosomal cholesterol loading trigger the STARD3 pathway? This should be tested by doing siRNA mediated STARD3 depletion in the presence and absence of LDL and measure endosomal cholesterol levels. Alternatively, changes could be observed in PM cholesterol levels, if the flow based assay was more sensitive. Upon LDL deprivation and STARD3 depletion, the level of endosomal cholesterol should be decreased.

Indeed, referee #1 is correct: when STARD3-mediated cholesterol transfer is employed by the cell is elusive. Our data show that STARD3-mediated cholesterol transport occurs in presence and in absence of LDL (Figure 7). Indeed, Figure 7 shows that in the absence of exogenous cholesterol capture by the LDLR pathway, the cholesterol accumulation phenotype remains.

We added new experiments using statins and we showed (Figure 7) that interrupting the biosynthesis pathway in the ER clears the majority of accumulated cholesterol from endosomes.

Referee #1 recommends to silence STARD3 and to observe endosomal cholesterol. This experiment did not give conclusive results because, in control cells and in STARD3-silenced cells, the level of cholesterol in endosomes is below the detection limit of our probes (see below).

Therefore, we labeled plasma membrane cholesterol with the GFP-D4 probe and quantified the staining by flow cytometry in the presence and absence of LDL (Figure 8C). In presence and absence of LDL, STARD3 silencing resulted in increased cholesterol levels in the plasma membrane. These data suggest that LDL absence or presence is not the trigger which activates STARD3-mediated cholesterol transport. Rather, our data (Figure 7) suggest that cholesterol synthesized in the ER is crucial. Indeed, inhibiting sterol synthesis with statin reduced cholesterol transport by STARD3.
2. The information about quantitative data is inconsistent. In general it is confusing that "n" is sometimes defined as number of cells and sometimes as number of experiments. For each quantification, it should be clearly stated in the figure legend how many independent experiments (from where the statistical testing should be done) and in addition how many cells per condition, to indicate the level of throughput. E.g. in figure 1: Are the values coming from only one experiment, since the total number of cells has been used for statistical testing? The number of independent experiments should be stated in the figure legend and mean values from independent experiments should be used for statistical testing. Likewise, statistical testing should be applied for all quantitations. It would be informative to know which samples were tested against each other. E.g Fig1E: are the mutants tested against ctr or each other? The black line is confusing. Indicate better what is tested against what, either with connecting lines in the graph, or in the figure legend.

This point is discussed in ANSWER 2 above. Briefly, we modified the way data are represented and analyzed according to the reviewer’s recommendations:

Almost all the figures have been modified accordingly, including

Figure 1F (formerly labeled Figure 1E): the data presented on this figure were coming from different independent experiments (3 or 6 depending on the sample); we had chosen to display the relative fluorescence intensity of individual cells using a box-and-whisker plot in order to represent data scattering. This graph was modified and now displays the mean values from independent experiments.

Figure 3C: the data presented on this figure were coming from 3 different independent experiments. This graph was modified and now displays the mean values from these independent experiments.

All Statistical analyses were done on mean values from independent experiments and are now represented with connecting line.

3. In Fig. 3, how was the endosomes selected for quantification? The morphology of endosomes in STARD3 stable cells resembles lysosomes, whereas endosomes in the ctr and VAP depleted cells look like late endosomes. It would be more convincing to select endosomes after a pulse and subsequent 30-60 min chase of BSA-gold.

This point was addressed above in the response to the editor section (ANSWER 1). Regarding the BSA-gold experiment we had no experience in this kind of assay. We performed several trials but without success. I just want to stress that we have previously shown that STARD3 expression affects the endosome morphology (Alpy et al, JBC, 2001; Alpy et al, JCS, 2013). The molecular mechanisms involved in this morphological alteration are unclear; from our precedent work, we raised the hypothesis that it is a proper ty of the MENTAL domain (Alpy et al, JBC, 2005) and it does not rely on the formation of contact sites nor on the function of the START domain (Alpy et al., JCS, 2013).

4. The authors state (bottom line, first paragraph page 7) that "STARD3 mediated cholesterol transport promotes inner luminal vesicles formation in endosomes". This statement is based on the EM result that VAP depletion reduces ILV formation in the STARD3 stable line. VAP depletion indeed reduces cholesterol accumulation in STARD3 stable cells, but since VAP proteins are required for many types of ER contacts, it is only an indirect argument towards STARD3 function. To be able to conclude directly that STARD3-mediated cholesterol transport promotes ILV formation in endosomes, the STARD3 mutant cell lines (STARD3 dSTART, STARD3MR/ND) should be analyzed by EM.

This point was addressed above in the response to the editor (ANSWER 1). As recommended we performed novel TEM experiments using STARD3 mutants; they are presented Figure EV4 F-J.

Minor points:
1. It is not clear from the images that the colocalization between D4 or filipin with LAMP1 or STARD3 is almost 100% as shown in the graphs. The scoring of colocalization was done manually. The authors should use Manders/Pearson scoring from at least 3 independent experiments. The amount of triple colocalization (STARD3/Filipin/LAMP1) or (STARD3/D4/LAMP1) could also be informative.

Data shown Figure 1B and Figure 1D originated from an object based co-localization scoring. To respond to the reviewer concern about the fact that the colocalization scoring was done manually, we modified our protocol: the new protocol we used is described in the Materials and Methods section. As for every object-based colocalization, this analysis requires a thresholding step; unlike our previous protocol in which thresholding was performed manually, signal thresholding was performed using Yen's thresholding method (Fiji software) in the two channels that were compared. After the thresholding step, which allowed the identification of objects, colocalized objects were identified and quantified. These data showed (Figure 1B) that 76% and 90% of GFP-D4 positive vesicles, are positive for Lamp1 and STARD3, respectively; and (Figure 1D) that 85% and 90% of filipin positive vesicles are positive for Lamp1 and STARD3, respectively.

Moreover, we calculated Pearson correlation coefficient between STARD3 signal and GFP-D4 or Filipin signals in images originating from 3 independent experiments. These data are now shown Figure 1E.

2. In Fig. S3f, some experiments have only been performed 2 times. Experiments should be repeated at least 3 times, even though this is a control experiment.

These experiments (now presented Fig. EV3) have been repeated: the bar graph displays the mean value of 3 (cSTD3 MR/ND and cSTD3 7G) and 4 (cSTD3) independent experiments.

3. In Fig S4 there is no co-occurrence of endogenous STARD3 and VAP in HeLa/Ctr. Although the endogenous STARD3 signal is weak, it is visible in the picture as well as in the line plot. Why are there no ER-endosome contacts in this line plot?

Unfortunately, the antibodies we have can detect the endogenous STARD3 protein only by Western blot. The weak signal that is shown on the immunofluorescence image (Fig S4 Ac) corresponds to background. We checked this by staining WT HeLa cells and HeLa in which STARD3 was silenced using a shRNA strategy: the staining obtained by immunofluorescence using anti-STARD3 antibodies was similar in these different cell lines.

The linescans shown on FigS4 were confusing; indeed they represented a relative fluorescence intensity for each of the two channels (expressed as a percentage of maximum intensity; scale 0-100), therefore artificially increasing the values of low intensity staining, and in particular the background intensity values. Thus, we changed the linescan representation which now displays the absolute pixel intensity values (scale 0-255). Note that pixel intensity values found in the STARD3 staining channel are below 25 consistent with a background signal.

Fig S4 legend was changed accordingly:

“(f) Linescan analyses showing fluorescence intensities of the magenta and green channels along the arrow in d. Black thick lines indicate the positions of LEs.”

4. Eden et al., 2016 have also reported that endosomal cholesterol is important for ILV formation, in an EGF and Rab7 dependent manner (Annexin1-ORP1L dependent). Does the STARD3 dependent cholesterol transfer happen in Rab7 endosomes only? Or is it also active in Rab5 endosomes? Since the cell has at least two mechanisms for transferring cholesterol from the ER to endosomes (AnnexinA1 (Eden et al 2016) and STARD3 (this manuscript), it would be informative to know which part of the endocytic pathway is covered, since ILV formation already start at the Rab5 stage. In Fig. 1, it is evident that STARD3 positive endosomes colocalize with LAMP1. However, there are many STARD3 positive LAMP1 negative endosomes, which also seems to accumulate filipin. Are these Rab5 or Rab7 positive?
This is a joined comment with the editor (see ANSWER 4). To address this we did a better characterization of STARD3 positive cholesterol loaded endosomes and showed that they are Rab7 and Lamp1 positive. Pearson correlation coefficients support the conclusion that STARD3 mediates cholesterol accumulation in late-endosomes (FiG. 1E and EV 2).

5. Some of the floatation assays have only been done 2 times (Fig. S3F), and there is lack of statistical testing. For the DHE transport assay, the MR/ND mutant is tested together with the G7 mutant shown in Figure 5B. In addition, the MR/ND mutant is also tested in Fig. S3G, in combination with the VAP KD/MD mutant, but there is no call out to Suppl Fig 7E in the text.

These experiments (now presented Fig. EV3) have been repeated: the bar graph displays the mean value of 3 (cSTD3 MR/ND and cSTD3 7G) and 4 (cSTD3) independent experiments.

Suppl Fig S3G (now Fig. EV 3G) is now called in the text.

6. Fig S6, showing that VAP depletion does not decrease PM cholesterol is not quantified, only showing example cells. Fig 7B, using the same assay in STARD3 mutant cell lines is quantified. The data would have been more conclusive if the effect of VAP depletion could also be quantified.

As suggested by the reviewer, we quantified PM cholesterol using two different methods and on a large number of cells. A direct method using GFP-D4 labelling of plasma membrane cholesterol quantified by flow cytometry (Figure EV 1) and an indirect method using amphotericin B killing. These data are now shown Fig. EV5 B and Fig. EV5 C, respectively.

7. Since cholesterol loaded endosomes cluster perinuclearly, based on the ORP1L-RILP-dynein pathway, the STARD3 dependent loading of cholesterol in endosomes could induce perinuclear localization of endosomes via the ORP1L pathway. Indeed STARD3 overexpression induces perinuclear endosome clustering (suggested to be mediated via actin regulation (Holtta-Vuori et al.). This should be discussed.

We did not specifically investigate the perinuclear clustering of STARD3 cholesterol-loaded endosomes, therefore we cannot provide a definite answer to the reviewer. Actually, as reported before in Holtta-Vuori et al and in Alpy et al., JBC 2005, STARD3 expression is associated with a perinuclear clustering of endosomes. We previously shown that it is independent of the presence of the START domain and that it is also observed in the absence of ER-endosomes contacts (Alpy et al JBC, 2005, Alpy et al., 2013). Altogether these results support the notion that the MENTAL domain functions in endosome morphology and positioning. Whether cholesterol loading accentuates this function is possible but we have no experimental support, for the sake of clarity we did not discuss this point further.

Referee #2:

This is a well-written manuscript addressing a new and exciting topic. The authors show that overexpression of STARD3, previously shown to induce ER:endosome membrane contacts, causes cholesterol accumulation in endosomes in a manner that depends on both the FFAT motif and sterol binding START domain of STARD3 and is at the expense of plasma membrane cholesterol. This is consistent with the idea that cholesterol is transferred from ER to endosomes at STARD3-dependent contacts under these conditions. Whilst there has been a great deal of discussion in the literature about the potential role of ER:endosome contacts in transport of LDL-derived lipid from endosomes to the ER, much less attention has been paid to their potential role in lipid transport in the reverse direction ie from the ER to endosomes. ORP1L:VAP interactions were implicated in lipid transport from the ER to endosomes containing activated EGF receptor in a recent study (Eden et al., 2016). The contacts described in the current study appear to be a distinct category of contacts that depend on STARD3 overexpression in unstimulated cells. The study is considerably strengthened by the
authors' development of a very nice in vitro assay demonstrating directly that STARD3 can transport cholesterol from one membrane to another in a manner that depends on interaction with VAP. The in vitro assay is well validated and represents an important and significant advance over existing studies of ER:endosome contacts.

Specific points:

1. In the Intro ‘MENTAL’ domain needs to be defined.

The introduction has been modified as follows:

“Interestingly, the functional characterization of STARD3 revealed that it distinguishes itself from the other START domain proteins by the presence of a conserved amino-terminal domain named MENTAL (MLN64 NH2-terminal) and shared with its paralog STARD3NL (alias MENTHO) (Alpy et al., 2002).”

2. In the Intro ILVs are mentioned in the final paragraph but have not been defined.

In the Introduction, the ILV is not used anymore; it has been replaced by the term “membrane”:

“Finally, we show that STARD3 acts as a lipid transfer protein that redirects sterol to the endosome at the expense of the PM and favors membrane formation in endosomes.”

3. The materials and methods description of the D4 and filipin staining of plasma membrane and intracellular compartments is inaccurate – it is clearer from the figure legend of Figure S1 but should be amended in the methods.

The materials and methods section has been modified. Moreover, we now refer to Figure EV1 in this section.

“In order to allow a better visualization of intracellular cholesterol pools, cells were treated with 10mM Methyl-b-Cyclodextrin (MbCD, Sigma) for 30min at 37°C to remove cholesterol from the plasma membrane (Figure EV1).”

4. In Figure 1 the authors use LAMP1 as a marker of endosomes. LAMP1 is a lysosomal protein, though it can also be expressed on late endosomes and this should be made clear in the text. Kant et al., (2013) reported that STARD3 is on endosomes that are ABCA3 positive and an earlier population than those positive for ORP1L/NPC1. The images in figure 1 show only partial costaining with LAMP, consistent with at least some STARD3 being present on an earlier LAMP1-ve population. Very little is made of the costaining with LAMP1 in the text although quantitative line scans are shown in the figure. Although the accumulation of cholesterol in STARD3 +ve structures is very convincing it would be good to see some costaining with earlier endocytic markers to better identify the population of endosomes in which cholesterol accumulates.

This comment joins the editor and referee #1 comments (see ANSWER 4). We would like to recall that STARD3 was originally described as a late endosome protein by co-localization experiments with markers such as Cathepsin D, CD63 and LBPA (aka BMP) (Alpy et al, JBC, 2001).

To better illustrate this subcellular localization, new experiments were done and a new figure was added to the manuscript (Figure EV2) on which are shown co-staining of STARD3 and an early endosome marker (EEA1) and different late endosome/lysosome markers (Lamp1, CD63, Rab7 and BMP). Moreover, we quantified the co-localization between STARD3 and these markers using Pearson correlation coefficient (Fig EV2 B). These experiments show that STARD3 co-localizes strongly with markers of the late endocytic compartment but scarcely with the early endosome marker EEA1. Consistently with a late endocytic compartment localization, Pearson correlation coefficient were high between STARD3 and Lamp1, CD63, Rab7 and LBPA and low between STARD3 and EEA1.
5. The authors state on page 6 that “In situ staining using the GFP D4 probe or filipin showed the absence of intracellular accumulation of cholesterol in cells expressing the two cholesterol transfer deficient mutants (Fig. 2CD).” However it appears to me that there is more intracellular cholesterol staining in the mutants compared to the control, albeit much less than in the cells overexpressing wild type STARD3. The same applies to the FFAT mutants. The sentence should be adjusted accordingly.

As the reviewer rightly states, intracellular cholesterol accumulation in cells expressing the two STARD3 cholesterol transfer deficient mutant and the FFAT mutants is much lower than in the cells overexpressing wild type STARD3; however, intracellular cholesterol accumulation is not restored to the basal level observed in control cells. Therefore, we modified the sentence as follows:

“In situ staining using the GFP-D4 probe or filipin showed that intracellular accumulation of cholesterol in cells expressing the two cholesterol transfer deficient mutants was highly decreased compared to that observed with WT STARD3 (Fig. 2C-D). Moreover, quantitative image analysis of filipin staining of over 100 cells showed that cholesterol accumulation was lowered in mutant STARD3-positive endosomes (Fig. 1F).”

6. What criteria are used to identify the endocytic/lysosomal structures used for quantitation of internal membranes? The endosomes shown in Figure 3D and G appear to be multivesicular endosomes/bodies with discrete intraluminal vesicles and an electron lucent interior whilst those in Figure 3E and F also have irregular membranes and are electron dense. Are all intraluminal membranes quantified? If not, how is an ILV defined? Whilst the differences in internal membranes could be caused by STARD3-dependent sterol transport at membrane contacts and subsequent increases in luminal cholesterol, it could also be that different compartments are being compared ie multivesicular bodies in Figures D and G with lysosomes or even autophagosomes in E and F. How do the authors control for this? Also the endocytic vacuoles in STARD3 overexpressing cells appear enlarged compared to controls. Is this a consistent feature? Given that membrane contact sites between endosomes and the ER have been shown to have roles in endosome fission could recycling be affected by STARD3 depletion? This point should at least be discussed.

This point was raised by the editor and the reviewer 1 (see ANSWER 1). Briefly, the use of term “ILV” was misleading; therefore we rewrote this part to better define the structures that were quantified at the ultrastructural level.

I want to stress that we already described in previous papers that STARD3 expression affects late endosome morphology and dynamics (Alpy et al, JBC, 2001; Alpy et al, JCS, 2013). Concerning recycling, we do not have any evidence of a recycling defect in STARD3 expressing cells. Moreover, the ultrastructure analysis of HeLa/STARD3 DSTART which display extensive ER-endosome contacts, showed that endosomes in these cells do not accumulate intraluminal membranes (Figure EV4).

7. The authors state on page 9 that the finding that STARD3-induced cholesterol accumulation is not dependent on LDL is consistent with the idea that the cholesterol source is predominantly the ER. This should be tested by determining whether the STARD3-induced cholesterol accumulation is prevented by treatment of the cells with statins.

As mentioned by the reviewer 2 and to reinforce the idea that the cholesterol source is predominantly the ER, we performed new experiments. We treated HeLa/STARD3 and HeLa/Ctrl cells with statin. These data are now presented Figure 7. While LDL-removal did not diminish cholesterol accumulation in endosomes in HeLa/STARD3 cells (Fig. 7 B), statin treatment reduced cholesterol accumulation (Fig. 7C). We quantified cholesterol accumulation in these different conditions using filipin and the data are presented Fig. 7D and Fig. 7E.
8. The reduced plasma membrane staining upon STARD3 overexpression appears to contrast with the data of Vassilev et al. 2015 Am J Pathol 185:987- who found reduced plasma membrane cholesterol and elevated HMG CoA reductase in STARD3 overexpressing cells. This should be discussed.

We have no definitive answer for the results reported by Vassilev and colleague. We can argue that they used a GFP-tagged construct, therefore one can speculate that the GFP-tag compromises some of the functions of STARD3; indeed we observed increased plasma membrane staining in loss of function experiments. Another possibility comes from the different cellular model used. Vassilev and colleagues used the breast cancer cell line MCF-7. These cells are polarized epithelial cells dependent on estrogen from their growth. One can hypothesizes that these cells which have distinct plasma membrane territories, use a different cholesterol homeostasis mode of regulation compared to HeLa cells. Of interest the Kobayashi group, reported that cholesterol homeostasis is not balanced between both pathways in all cells. They showed that cellular cholesterol in CHO cells was largely dependent on exogenous lipoproteins (Ishitsuka et al., 2011, JLR vol 52:2084-2094). It might be a similar situation in MCF7 cells; therefore since we showed here that cholesterol accumulation by STARD3 depends from ER-Cholesterol, it might not happen in MCF-7 cells under normal culture conditions. This point is now mentioned in the discussion page 12-13.

9. Page 10 "VAP expression was silenced in cells expressing STARD3 and the PM was labeled with GFP-D4 (Suppl. Fig 5). I think this should be sup figure 6.

Indeed, this was an error that has been corrected in the text.

10. The authors do not demonstrate effects of STARD3 depletion on cholesterol content of endosomes or indeed on membrane contact sites. Are the contacts reduced upon STARD3 depletion? If they are then the prediction would presumably be that cholesterol content of endosomes would decline under these conditions. Given that cholesterol content of endosomes is very low in control cells this would make a reduction in cholesterol content difficult to measure and so this is probably not possible. However, it begs the question of the physiological relevance of the effects of STARD3 overexpression. Under what conditions might STARD3 overexpressed? This is important and should be discussed as it potentially significantly affects the impact of the paper.

In control cells, ER-endosomes contacts are focal and limited in size (Alpy et al., JCS 2013). Under STARD3 silencing conditions, using TEM and stereology, we did not observe a significant decrease in the surface of membrane involved in contacts compared with control cells. One can argue that the methodology is not sensitive enough and/or that redundancy with other ER-endosome tethers can compensate the loss of STARD3 and maintain the number of ER-endosome contacts. This latter observation was reported for a number of contact sites tethers as explained in Eisenberg-Bord et al, Developmental Cell, 2016.

Regarding the endosome cholesterol content under STARD3 silencing condition, this issue was also raised by Referee #1, as explained and showed in the answer to Referee #1 Major point 1, we cannot detect and quantify cholesterol in the endosomes of control cells, as stated by this referee a reduction in cholesterol content cannot be seen. This is the reason why we focused our effort on the plasma membrane cholesterol (Figure 8C).

The question of the physiological significance joins comments from the editor and referee #1 (see ANSWER 5). We have further discussed the potential significance of our findings. Our current hypothesis favors a role of STARD3 as a modulator of cholesterol transport in discrete subcellular regions over a role as a global modulator of cellular cholesterol, which is insured transcriptionally by the SREBP pathway.

Referee #3:

This is a very clearly written manuscript, describing rigorous experimental work that discovers a cholesterol trafficking pathway from the ER to endosomes and establishes its molecular basis. This is a significant advance because how cholesterol is trafficked within
cells is very poorly understood. The role of the STARD3 protein in this process is further elucidated as well as the importance of its FFAT motif, which allows for tethering/contact site formation between the ER and endosomes. A role for tethering/contact site formation in enhancing lipid transport is established. The work nicely combines in vitro studies with studies in cells, leading to well-supported conclusions.

In my view, this manuscript should be published as is.

2nd Editorial Decision 28 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees again whose comments are enclosed. As you will see, the referees appreciate the amendments and support publication in The EMBO Journal. I am thus happy to accept your manuscript in principle for publication here.

REFEREE REPORTS

Referee #1:

The authors have successfully addressed my concerns, and I am happy to recommend publication of this revised manuscript.

Referee #2:

I am satisfied with the response to my previous comments and the new data provided. I recommend publication of this manuscript that sheds new light on the role of membrane contacts in the STARD3-dependent transfer of cholesterol from the ER to endosomes and the consequent accumulation of intraluminal endosomal membranes.
**EMBO PRESS**

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**PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER**

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<td>Journal Submitted to: The EMBO Journal</td>
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**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

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 authority guidelines in preparing your manuscript.

**A- Figures**

**1. Data**

The data shown in figures should satisfy the following conditions:

1. 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.
2. Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
3. Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
4. If S, 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 authorship guidelines on Data Presentation.

**2. Captions**

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

- A description of the experimental system investigated (e.g. cell line, species, age) 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/name/detected in a controlled manner.
- The exact sample size(s) 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 was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - Common tests, such as t test (please specify whether paired or unpaired), simple ANOVA, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only; more complex techniques should be described in the methods section.
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Mean statistical test results, e.g., if P value = x but not P value < x; definition of “center values” as median or average;
  - Definition of error bars as SD or SEM.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. 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**

<table>
<thead>
<tr>
<th>Question</th>
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<tr>
<td>1. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?</td>
<td>Sample size for the studies was chosen according to previous studies in the same area of research. In general, 3 to 6 independent experiments were performed.</td>
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<tr>
<td>2.a. For animal studies, include a statement about sample size estimate even if no statistical methods were used</td>
<td>NA</td>
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<td>2.b. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria prespecified?</td>
<td>No sample was excluded</td>
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<td>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.</td>
<td>NA</td>
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<tr>
<td>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.</td>
<td>NA</td>
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<td>4.b. For animal studies, include a statement about blinding even if no blinding was done</td>
<td>NA</td>
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<td>5. For each figure, are statistical tests justified as appropriate?</td>
<td>The test used for each data set is specified in the figure legend.</td>
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<tr>
<td>6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.</td>
<td>Data were tested for normal distribution with Shapiro-Wilk normality test. If data points were not normally distributed, a non-parametric test was used.</td>
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<td>7. If there is an estimate of variation within each group of data</td>
<td>Yes, see figures and their legend with error bars.</td>
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<td>8. If the variance varied between the groups that are being statistically compared</td>
<td>ANOVA’s ANOVA test was used to test variance equality. If the variance was not similar between the groups, a non-parametric test was used.</td>
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**C- Reagents**

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D- Animal Models

4. Report species, strain, gender, age of animals and general condition where applicable. Please detail housing and husbandry conditions and the source of animals.

5. For experiments involving non-human primates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

6. We recommend consulting the ARRIVE guidelines (see link list at top right) [1], [2] to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under "Reporting Guidelines", for more information on ARRIVE recommendations. Please confirm compliance.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. Please include a statement confirming that informed consent was obtained from all subjects and that the experiments were conducted according to the ethical guidelines of the responsible authorities.

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E- Human Subjects

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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments were conducted according to the ethical guidelines of the responsible authorities.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For data involving human genomic datasets, please consult the MIRIAM guidelines (see link list at top right) for guidelines on data availability and whether you have followed these guidelines.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under "Data Accessibility".

Data deposition in a public repository is mandatory for:

a. Proteins, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Functional genomics data

20. Depositors strongly recommend for any datasets that are central and integral to the study, please consider the journal’s data policy. If a structured public repository exists for a given data type, we encourage the provision of data in the manuscript in a Supplementary Document (see author guidelines under "Expanded View" or in machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (SRA, GCF) should be used instead of scripts (e.g., NAF/AR). Authors are strongly encouraged to follow the MIABH guidelines (see link list at top right) and deposit their model in a public database such as GenBank or a database (see link list at top right) (e.g., EGA). If a computer-readable format is provided with the paper, it should be deposited in a public repository or included in supplementary information.

22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (SMA, GMR) should be used instead of scripts (e.g., NAF/AR). Authors are strongly encouraged to follow the MIABH guidelines (see link list at top right) and deposit their model in a public database such as GenBank or a database (see link list at top right) (e.g., EGA). If a computer-readable format is provided with the paper, it should be deposited in a public repository or included in supplementary information.

G- Dual use research of concern

1. To ensure that data sets are dual-use, please check the following requirements: (see link list at top right) and list of select agents and toxins (APHS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it is applicable.