Reviewers' comments:  
  
Reviewer #2: The study of Dawson et al. is an interesting study aimed at identifying specific Candida Albicans EVs markers by label-free proteomic analysis. The study is designed properly and the results are interesting and robust enough to guide the future research in Candida Albicans EVs and, more generally in yeast EVs.  
However, few additional experiments could add important information to the manuscript and some additional analysis are probably needed.  
  
Results  
  
Table S1  
Page 15 line 38. The authors state "The EV preparation with the largest amount of visual debris (ATCC90028) also had the highest protein concentration" suggesting in this way that the findings are connected. However, the ug/particles concentration is similar with DAY286Y, suggesting that probably the higher yield of protein it's due to a higher particles concentration. Given this, I think that the sentences is slightly misleading.

This sentence was indeed confusing and has been removed.  
  
Figure 1  
Many of the comparisons between biological replicates are just descriptive without any statistical test. Can the authors please perform statistical analysis and furnish p-value of each comparison?

The comparison of different mode sizes between the replicates has been furnished with p-values which is presented in Supplementary Figure S3A (One-way ANOVA followed by Tukey’s HSD post hoc test). The comparison of different total particle concentrations between the replicates has also been furnished with p-values which is presented in Supplementary Figure S3B (Kruskal-Wallis test as data were non-normal, followed by Dunn’s Test).

Figure 1B  
Since the total yield of particles of ATCC10231 is significantly lower than DAY286Y and ATCC90028, it could be better to show the "percentage" of total particles in the y axis. This trick could help understanding the "size distribution" of all the three sample. This information could be interesting given the differences in average, median and mode size of three biological replicates.

Figure 1B has been changed to a bar chart showing the percentage of total EVs for different size ranges (0-100, 100-200 etc…). This new figure better shows how the size distribution of ATCC EVs is right-shifted (i.e. they are typically larger) compared to DAY286 EVs. The NTA traces remain as Supplementary Figure S2.

Figure 2A  
Page 16 line 45-47. Can the author please give a p-value of this comparison?

This comparison is simply of the number of proteins in the overlap of each Venn diagram in Figure 2A. Specifically, it is a comparison of sets and hence there is no statistics to be done.   
  
Additional experiments and analysis  
\* Can the author please confirm with Western Blot at least a couple of specific EVs markers and WCL specific markers, like in a proof-of-concept experiment  
\* Immunogold TEM labeling of one or more of the EVs exclusive markers in EVs e WCL would undoubtfully help to actually visualize the protein localization (for example in EVs membrane).  
\* Nano-FACS analysis of EVs labelled with Ab against EVs exclusive markers could help understanding the percentage of EVs positive for specific markers.

We agree that it would be helpful to confirm some of these markers via Western blot, immunogold TEM labelling and/or Nano-FACs. However, there are no commercially available antibodies to any of these marker proteins. Making and validating an antibody to anyone of these proteins would be a substantial undertaking. An analogous paper on protein markers for E. coli EVs was published in JEV in 2019 (Hong J, Dauros-Singorenko P, Whitcombe A, et al. Analysis of the Escherichia coli extracellular vesicle proteome identifies markers of purity and culture conditions. J Extracell Vesicles. 2019;8(1):1632099. Published 2019 Jun 24. doi:10.1080/20013078.2019.1632099). This paper uses only proteomics to identify EV markers and does not have any antibody-based validation experiments, probably because of the same issues with antibody availability.

One putative EV exclusive marker, the plasma membrane protein Hgt1 has already been independently confirmed to be *C. albicans* EV cargo using immunogold TEM labelling by Kenno et al. (2019) (see Figure 3D). We refer to this briefly in the discussion, however some additional emphasis has been added.

We have presented our findings at a number of conferences and symposia as well as sharing our findings with other members of the fungal EV community. We were the first to undertake a search for fungal EV marker proteins, but since we have generated and shared our list, we are finding that a subset of our markers have been identified in EVs from other diverse fungal species. For example, the homologs of Sur7 have been found in EVs from *S. cerevisiae* (Sur7, see Zhao et al. 2019) and *Fusarium graminearum* (FGSG\_08692, unpublished data). The Evp1 homolog Pun1 was also detected in *S. cerevisiae* EVs. Looking at some other marker candidates we also find homologs for orf19.2168.3 in *S. cerevisiae* EVs (Yop1, see Zhao et al. 2019) and *F. graminearum* EVs (FGSG\_07419, unpublished data), and the Phr1 homolog FOTG\_02101 in *Fusarium oxysporum f. sp. vasinfectum* EVs (Bleackley et al. 2020 https://doi.org/10.3389/fpls.2019.01610).

It is imperative that this study on *Candida albicans* EV marker proteins is published soon to ensure that we are the first to publish these markers and it is not delayed by the need to generate and validate antibodies to our putative markers and then do the experiments to confirm that the proteins are in fact in the EVs.

\* Even if beyond the strict purpose of this study a comparison of protein content and pathway analysis of proteins from yeast and biofilm of DAY286Y EVs could be helpful and interesting (Results can be added to supplemental files).

A comparison of the DAY286 yeast EV and biofilm EV proteins presented in Figure 2A has been performed. Supplementary Figure S4A has been added which shows the relevant Venn diagram showing this comparison. Supplementary Figure S4B shows a volcano plot comparing the differential abundance of proteins common to EVs from both morphologies. Furthermore, the proteins enriched in DAY286 biofilm EVs vs yeast (and yeast vs biofilm) have been subject to a GO analysis and the top 10 enriched GO terms for both comparisons are presented in Supplementary Figure S5. These new Supplementary Figures are described in the results section.

Reviewer #3: General comments  
The authors propose new protein markers of extracellular vesicles (EVs) secreted from Candida albicans (C. albicans) using label-free quantitative proteomics.   
Despite potent important roles in pathogenic mechanisms, EVs secreted from C. albicans does not have well-established EV markers and this limitation obstruct EV research development. Using several C. albicans strains, different culture conditions, their replicate samples and label-free quantitative proteomics technique, the authors identified proteins specifically expressed in EVs such as Claudin-like Sur7 family proteins Rho GTPases as novel EV protein markers. According to the ISEV2018 guideline, the authors not only proposed EV markers, they also addressed EV negative markers to evaluate cellular compartment contaminations. The number of the proteins detected by proteomics analysis and the number of EV marker candidates finally met their criteria were increased compared to previous proteomic analysis reports for EVs secreted from C. albicans and other fungi.  
Although the proteomic data presented in this manuscript are improved and have interesting implications, this paper still has some problems as indicated below.  
  
Major comments  
1- Page 10, line 7 "an individual biological replicate …… derived from cells in the same culture or biofilm"  
The biological replicates should be cultured independently. Reproducibility of the EV-marker candidate proteins' expressions in EVs should be validated by checking independent trials.

This sentence was confusing and has been replaced. What we mean to say is we did indeed culture our biological replicates separately i.e. we had 3x individual DAY286 yeast cultures, 3x individual ATCC90028 cultures, 3x individual ATCC10231 cultures, and 5x individual DAY286 biofilms (details of these cultures are provided in Supplementary Table S1). When we talk about paired samples we mean to say that, for example, ATCC90028 EV bio rep 1 and WCL bio rep 1 came from ATCC90028 independent culture #1, ATCC90028 EV bio rep 2 and WCL bio rep 2 came from ATCC90028 independent culture #2, and so on.  
  
2- Page 3, line 6 "Unfortunately, mammalian EV markers such as tetraspanins and ESCRT components, either have no fungal homologs or are absent in fungal EVs."  
This sentence sounds to be exaggerated. For example, ESCRT system is conserved among mammalian to fungi such as Saccharomyces cerevisiae (S. cerevisiae)(1). ESCRT complexes in S. cerevisiae are well-studied and C. albicans has homologue genes of S. cerevisiae ESCRT proteins-coding genes. Indeed, Zarnowski et al, identified ESCRT genes homologue to that of S. cerevisiae, which are functionally relevant to EV production in C. albicans(2).

We agree this sentence may have been misleading and it has been replaced with a clearer statement. The ESCRT system does have a role in fungal EV biogenesis as demonstrated by Zarnowski et al. (2018) and Zhao et al. (2019), and we have changed this sentence to acknowledge this. However, in our previous meta-analysis of fungal EV proteomics data (Bleackley et al. 2019) we showed that ESCRT proteins are not proteins commonly detected (or detected at all) in fungal EVs. For example, Zarnowski et al. (2018) only detect Vps27 and Hse1 out of all the ESCRT proteins; and only very low levels in 1 biological replicate of *C. albicans* biofilm EVs and not at all in their planktonic EVs. Tetraspanin-like proteins have been reported for a handful of fungi but not *S. cerevisiae* or *C. albicans*.

3- Page 20, section: "Putative C. albicans EV positive markers include GTPases and Sur7 family proteins"  
Please explain about GTPases and Sur7 which are mentioned in the title and the subheading in this section.

We agree the focus of this paper is not on the GTPases that have been identified as marker candidates, therefore the reference to GTPases in the title of the paper and the title of the section has been removed. The Sur7 family proteins are the focus and the relevant Pfam IDs for this family have been added to the abstract, the specified section of the results, and the discussion. Also, a description of the Sur7 family has been added to the discussion.  
  
4- Page 20, section: "Putative C. albicans EV positive markers include GTPases and Sur7 family proteins"(2)  
Which GO term do marker candidates listed in this section belong to? Which C1-7 categories in the previous sections do marker candidates belong to? Please refer to them as text or in main figures.

The marker candidates have been bolded and Figure 5 to show which clusters they belong to (C1-C4 and C8). Additionally, this information has been included in the results. The GO terms associated with these proteins and other potentially interesting metadata have been included as Supplementary Data S8.  
  
5- Page 20, section: "Putative C. albicans EV positive markers include GTPases and Sur7 family proteins"  
This conclusion is over-interpreted because only a few strains are tested and reproducibility of this conclusion is not sufficiently validated. Authors does not simply descript proteomic analysis result, but this section and the manuscript's title claims that GTPases and Sur7 family protein as reliable EV markers for C. albicans. I recommend to confirm marker candidates' expressions in EVs using other C. albicans strains including clinical strains and drug-resistant strains even if good commercial antibodies are not available for western blotting.

We tested three strains, two clinical isolates from ATCC (90028 and 10231) as well as the lab strain DAY286 as both biofilm and yeast. This is more than the two strains that were assessed in the Hong et al, JEV 2019 paper on E. coli EV biomarkers. As mentioned above some of our markers are being identified in non-Candida fungal species which gives us confidence that these proteins will be useful as markers for fungal EVs. We also hope that other labs will independently validate our list of candidate marker proteins in different strains in the future. The Kenno et al. Frontiers in Microbiology 2019 paper reported immunogold staining of Hgt1, on one of the putative markers in this paper, on EVs from a different strain than we assessed in our paper (SN152)  
  
6- Page 25, line 3 "Among other similarities, these proteins have predicted topologies reminiscent of mammalian tetraspanins, which are key markers for mammalian EVs [15] (Figure 8)."  
The authors need to describe more for topological similarities.  
1) Sur7 and Evp1 have similarity to Tetraspanins but not to other transmembrane proteins. What are the reasons for this suggestion?  
2) Not all Tetraspanin family proteins are also expressed is related to EVs. Describing topological similarity of Sur7 and Evp1 with general Tetraspanin family proteins does not support these proteins' possibility as EV markers. The figure does not have enough information.

1) By focusing their similarities to tetraspanins we are not trying to imply they are not similar to other transmembrane proteins. The sentence indicated has been moved to later in the text and a more detailed description of their topologies/similarities has been provided.

2) Their topological similarity to tetraspanins is not the reason they were chosen as markers (rather they were chosen due to their consistent enrichment in EVs vs WCL in the proteomics data sets). However, their similarity to tetraspanins gives some motivation to pursue Sur7 and Evp1 for validation above the remaining 20 candidates. It is the topology of tetraspanins that make them useful as antibodies which bind to CD9, CD81 etc. bind to their extracellular loops allowing for EV immunocapture amongst other techniques. If Sur7 and Evp1 are validated to be in the membrane of *C. albicans* EVs, then antibodies could be raised against their extracellular loops and used for a variety of EV related experiments.

The legend for Figure 8 has been edited for clarity.  
  
7- Page 25, line 11 "mammalian claudins [97] which have been detected in exosomes isolated from a variety of cancer cell lines [98-100]"  
The purpose of this study is to find general EV markers. The mammalian Claudins are featured as cancer cell-derived EV markers, which imply that Claudin proteins are not broadly expressed in EVs from non-cancer cells. The line of discussion does not match the aim of this manuscript.

This sentence was included just to highlight that claudins are known to be EV cargo in mammalian EVs and not that they function as cancer EV biomarkers. We agree it was a bit out of place and it has been removed.  
  
Minor comments  
1- Page 8, line 18, 19 and 22  
In addition to the rotor number, please indicate k factors for the centrifugation method section(3).

Adjusted k factors have been provided for each centrifugation step.  
  
2- Page 10, line 5 "four different C. albicans strains were used ……"  
The authors distinguished floating and biofilm forming DAY286 as different "strains". It sounds weird to call physiologically different but genetically identical bacteria as distinct strains.

We agree that calling DAY286 yeast and biofilm samples as different fungal strains is confusing (although is less wordy than saying 3 strains and 2 morphologies every time). References in the main text to four strains has been removed.  
  
3- Figure 3  
What is criterion for GO term selection? The GO terms shown in Fig.3 does not match GO terms listed in Table S3.

An arbitrary selection of the most ‘interesting’ GO terms present in Supplementary Data S3 (see the first 4 sheets) were chosen to display in Figure 3. This has been changed to the top 8 (or less if there is not 8 terms that meet the p-value cut-off) most significantly enriched GO term. Specifically, the top 8 terms with the lowest p-value for BP, CC, and MF respectively for DAY286 yeast, ATCC90028, ATCC10231, and DAY286 biofilm.  
  
1. Leung KF, Dacks JB, Field MC. Evolution of the multivesicular body ESCRT machinery; retention across the eukaryotic lineage. Traffic. 2008;9(10):1698-716.  
2. Zarnowski R, Sanchez H, Covelli AS, Dominguez E, Jaromin A, Bernhardt J, et al. Candida albicans biofilm-induced vesicles confer drug resistance through matrix biogenesis. PLoS Biol. 2018;16(10):e2006872.  
3. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Journal of extracellular vesicles. 2018;7(1):1535750.