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Hyphal growth in Candida albicans requires the phosphorylation of Sec2 by the Cdc28-Ccn1/Hgc1 kinase

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1st Editorial Decision 24 April 2009

Thank you for submitting your research manuscript to The EMBO Journal editorial office. I just received the third report and it becomes obvious that the experts agree on some potentially interesting and significant observations related to the role of Candida albicans Sec2 in hyphal development. However, careful reading of particularly the reports from refs.#2 and #3 reveal that the current dataset seems too preliminary to provide the definitive (rather than circumstantial) evidence needed to support physiological significance of the findings. This seems of particular importance, as substantial evidence for the mechanism of Sec2 localization in S.cerevisiae exists. Therefore, the refs request straightforward, though significant (!) further experimentation that might, depending on the experimental outcome severely change the content of the current manuscript. Given the aim and scope of The EMBO Journal as rapid publication, we are in light of such significant concerns not in the position to invite a single round of limit revisions - and thereby essentially commit to your study.

Please also understand that our journal demands complete papers describing original research of general rather than specialist interest in molecular biology that in addition need urgent publication because they report novel findings of wide biological significance and a sufficient level of molecular understanding. We are therefore only able to pursue manuscripts that receive enthusiastic support from at least the majority of our referees during the initial review. Given the rather too preliminary state of your current study, I am sorry to have to communicate that there was not much choice than to return the paper to you at this point with the message that we are unable to offer further proceedings.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more

positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript the authors report that the cyclin-dependent kinases Cdc28/Ccn1 and Cdc28/Hgc1 regulate the phosphorylation of Sec2 in Candida albicans specifically during hyphal growth. Based on electrophoretic mobility shift in gel after phosphatase treatment, the authors first demonstrated that Sec2 is constitutively phosphorylated during yeast growth and rapidly undergoes further phosphorylation in response to hyphal induction. Importantly, the hypha-specific phosphorylation of Sec2 was found to occur independently of the hypha-specific transcription program. Then, the authors used several approaches to obtain evidence in order to reach the conclusion that Sec2 is directly phosphorylated by Cdc28/Ccn1 and Cdc28/Hgc1 on serine 584. First, deletion mapping revealed that a 8 amino-acid region (583-591) containing 2 serine residues (S584 and S588) is required for the Sec2 phosphorylation even during yeast growth; second, while for unknown reasons mutating S584 to alanine was not successful, mutating it to glutamate (E) appeared to completely abolish Sec2 phosphorylation; third, the hypha-specific phosphorylation of Sec2 is blocked in the cdc28as mutant in the presence of the inhibitor 1NM-PP1; intriguingly, expressing the S584E mutant of Sec2 was sufficient to restore normal hyphal growth in the cdc28as mutant under the inhibition by 1NM-PP1; fourth, Sec2 phosphorylation is fully or partially blocked in ccn1 and hgc1 null mutants; and last, the authors showed coimmunoprecipitation of Cdc28, Ccn1 and Hgc1 with Sec2. By GFP-tagging, Sec2 was found to localize as a tight spot near the hyphal tip which was interpreted as association with the Spitzenkˆrper; and in mutants defective in hyphal growth or under conditions that inhibit hyphal growth, Sec2 became delocalized.

In my opinion, this study has made some exciting discoveries important for understanding the molecular mechanisms underlying C. albicans hyphal growth. Although the actin cytoskeleton and exocytosis are well known for their essential roles in the hyphal growth, it is largely unknown how different parts of the polarity machinery are polarized toward the site of cell growth in a temporally controlled manner. In recent years, studies in both S. cerevisiae and C. albcians have revealed that phosphorylation by CDK of some regulators of the actin cytoskeleton such as the GTPase-activating proteins (GAPs) of the Rho GTPase Cdc42, is critically involved in establishing and maintaining cell polarization. However, it is not known whether any molecules that regulate exocytosis are also controlled by CDK phosphorylation specifically for polarized growth. This study by Bishop et al. demonstrates for the first time that Sec2, the GEF of the Rab GTPase Sec4 which plays a central role in polarized exocytosis, undergoes CDK-dependent phosphorylation in response to hypha-inducing signals. Equally significant, they find that the mechanism of Sec2 phosphorylation resembles the one responsible for the hypha-specific phosphorylation of the septin Cdc11: both processes occur immediately after hyphal induction and require Cdc28/Ccn1 and Cdc28/Hgc1, but do not depend on the MAPK and PKA pathways that activate the expression of hypha-specific genes. The temporally coordinated control of key components of cytoskeleton and regulators of exocytosis is likely of fundamental importance for the cell to rapidly establish polarized growth in response to environmental cues. Furthermore, the new findings of this study contribute significantly to and help establish the newly emerging idea that immediate posttranscriptional activation of the polarity machinery plays an important role in establishing the hyphal development in C. albicans. In general, the discoveries described are very important to the field of C. albicans hyphal morphogenesis. Furthermore, because polarity control is a universal biological issue, the work is expected to have broad impact. Except the overstatements I mentioned above, the manuscript is very well written with great clarity.

For the reasons given above, this reviewer supports publication of this work in the EMBO Journal. However, before it is acceptable for publication the following issues need to be addressed.

Major issues

1. One key conclusion the authors made is that Sec2 is phosphorylated on serine 584 by Cdc28 in partnership with Hgc1 or Ccn1. However, the experiments performed in the study are not sufficient for making this conclusion. Several key experiments are usually required according to today's criteria for high quality publications. (1) MS analysis of Sec2 immunopurified from yeast and hyphal cells together with the same samples treated with protein phosphatases. This is required to show that serine 584 is indeed phosphorylated and it may also reveal other phosphorylation sites. (2) in vitro kinase assays using immunopurified Cdc28-as/Ccn1 or Cdc28-as/Hgc1 to phosphorylate immunopurified Sec2 in the presence or absence of 1NM-PP1. P32 ATP is used to visualize protein phosphorylation. This is used to show Sec2 can be directly phosphorylated by Cdc28, although a positive result cannot completely exclude the possibility that Cdc28 activates another kinase in the precipitate that further phosphorylates Sec2. If this is technically difficult, an alternative experiment is to express a region of Sec2 containing serine 584 as GST fusion in E. coli and then use the purified protein as substrate of immunopurified Cdc28/Ccn1 or Cdc28 Hgc1 in in vitro kinase assays. The same fragment containing S584A mutation is included as negative control. Although it is nice to be able to conclude that Sec2 is directly phosphorylated by Cdc28, it is not less significant to make a convincing conclusion that Sec2 is a regulatory target of Cdc28 for hyphal development, direct or indirect. I believe that the current data sufficiently support such a conclusion.
2. In addition to the above point, in several places the authors made conclusions favorable to their hypothesis but left out alternative explanations. One example is that while the deletion mapping results indicated that the 583-591 region is required for Sec2 phosphorylation (loss of band shift without the region), the author concluded that the region 'contains a residue that is phosphorylated and this phosphorylation event is required for normal hyphal growth...' (page 9, end of the first paragraph). An alternative explanation could simply be that the region is required for Sec2 phosphorylation at other sites. This is also one of the reasons why I suggest above using MS to confirm that serine 584 is phosphorylated. A second example is the subtitle on page 9. The presented data are not strong enough for making the statement that Sec2 S584 is phosphorylated by Cdc28-Hgc1 and Cdc28-Ccn1 during hyphal growth. This statement would require the authors to demonstrate that purified Cdc28-Hgc1 and Cdc28-Ccn1 can indeed phosphorylate S584.
3. I would say that the most unexpected result is the ability of the S584E mutant of Sec2 to support normal hyphal growth of the cdc28-as mutant in the presence of 1NM-PP1. This has very important implications. It suggests that as long as Sec2 is phosphorylated on serine 584, it would bypass the requirement of phosphorylation of all other Cdc28 substrates important for hyphal development or even those required for cell-cycle related events. The authors used 5μM 1NM-PP1. In our hand, this concentration is too low to cause significant effects on cell growth although it is high enough to inhibit purified Cdc28-as/cyclin kinases. Have the authors tried higher concentrations like 20 to 25 μM?
4. Another intriguing result is that the S584E mutation, which is suggested to mimic hyphal-specific phosphorylation, not only abolishes the hyphal-induced Sec2 phosphorylation but also its phosphorylation during yeast growth. How is it possible for a yeast event to be dependent on a hypha-specific event?
5. While the authors showed that Sec2 hyperphosphorylation is complete within 20 min of hyphal induction, the coIP of Sec2 with Cdc28, Ccn1 and Hgc1 were performed at much later time points (60 to 120 min). The results would be more robust if time points within the first 20 min of induction are included in the coIP experiments.

Minor points

1. Regarding nomenclature of the Cdc28 as mutant, several versions were used throughout the text and figures including Cdc28 as1, Cdc28 1as, Cdc28-as1, and Cdc28-AS1 and in combinations with the use of upright or italic letters. Please be consistent.
2. In many places in the text the authors misused 5 μm for concentration. It should be 5 μM. Please also check figure legends, Materials and Methods and Supplemental Data.
3. For the description of genotypes, when a mutation or deletion is introduced the gene name should be written in lower cases for example: SEC2 584E or SEC2 1-583 should be sec2 584E or sec2 1-583. Please make changes throughout the text and Table S1.
4. To avoid confusion with fusion proteins, MET3-YFP-SEC2 (and all other strains using the MET3 promoter) should be changed to pMET3-YFP-SEC2 or PMET3-YFP-SEC2.

5. In Table S1, change Wang et al. 2007 to Zheng et al. 2004.
6. Page 10, line 2-3, GFP is used here but YFP is used in the figure.
7. Page 11, line 2 from bottom. 'of the' was typed twice.

Referee #2 (Remarks to the Author):

This paper reports on developmentally regulated phosphorylation of Sec2p, the exchange factor for the rab controlling the final stage of the secretory pathway. They show that Sec2p is hyper-phosphorylated upon induction of hyphal growth and that this phosphorylation is important for the localization of Sec2 to a structure at the tip of the hyphae and for normal hyphal growth.They propose that it is Cdc28 in combination with two different cyclins that is responsible for the modification.

There are some nice aspects to this paper, however there are also technical shortcomings as well. Moreover, they do not probe the mechanism of Sec2 localization or the role of phosphorylation in controlling this mechanism. Since there is already a substantial literature on the mechanism of Sec2 localization in S. cerevisiae, it should be straightforward to explore this. Unless they can take this story to a mechanistic level I would suggest a more specialized forum.

Specific comments:

1. In Fig 1 it is difficult to tell where the cell boundaries are. It would also be helpful for the general reader to define the difference between a hyphae and a pseudo-hyphae. Also the difference between the apical cap structure and the Spk structure seems subtle to the non-expert.

2. In several figures, such as Fig2 the level of Sec2 seems to vary widely from lane to lane. Some of the difference could represent changes in synthesis or degradation, but why should the level go way up upon CIP treatment in panel B? The apparent fluctuations in level certainly confuse the interpretation of the functionality of the various mutants.

3. If the role of phosphorylation is to regulate the localization of Sec2 to the Spk, does the S584E mutant localize to Spk's even under yeast or pseudo-hyphal growth? If not, why is Sec2 ever de-phosphorylated since this constitutive allele seems to do everything fine? If the authors want to convince us that this regulation is important they need to define roles for both the phosphorylated and non-phosphorylated forms.

4. Why is the S584A mutant lethal since this region is not even needed during yeast growth? It seems that there is more going on here.Have they tried other substitutions? It is also very strange that the S584E mutation can bypass Cdc28 function but not that of the associated cyclins. These results make me somewhat dubious of their model.

5.In Fig 5B they need to run the + and - 1NM PP1 lanes adjacent to each other since it is too difficult to see the mobility difference with an intervening lane.

6. To a naive reader the cells in the two panels of Fig 5C look very similar.

7. In Fig 5D the reader must be able to figure out what fraction of the total pool of Cdc28 is co-precipitating with Sec2. Curiously, the interaction appears more significant in the yeast form when Sec2 is not phosphorylated. The background precipitation is appreciable. Another negative control should be included such as an irrelevant YFP tagged protein.

8. In Fig 6C and 7C the reader needs to be able to figure out the fraction of Sec2 that is co-precipitating.

9. In Fig 7B the level of Sec2 is much lower. Why?

10. In general, an independent marker is needed for the Spk. If Sec2 is not exhibiting Spk localization, we need to know if that reflects a failure to associate with the Spk or a failure to assemble a Spk.

Referee #3 (Remarks to the Author):

Polarized growth involves the targeted secretion of proteins and membranes. The yeast Rab GTPase Sec4 and its GEF Sec2 are critical for vesicle trafficking and for secretion. This study focuses on the role of Candida albicans Sec2 in hyphal development. It is shown that CaSec2 localizes to the Spitzenkorper during hyphal growth and is differentially phosphorylated. Deletion mapping strategies were employed to identify 8 amino acid region necessary for hyphal-specific Sec2 phosphorylation and localization. The identified sequence contains two putative phosphorylatable residues, including a CDK phosphorylation consensus site. Co-IP experiments suggest that Sec2 associates with Cdc28 (and possibly Pho85). The Spitzenkorper localization of Sec2 is dependent on Cdc28 and cyclin. Significantly, expression of phosphomimetic Sec2-S584E appears to rescue the hyphal defects caused by Cdc28 inhibition. The main conclusion of this manuscript is that Cdc28-dependent phosphorylation of Sec2 (on S584) is critical for hyphal development.

The manuscript is clearly written and the experiments and, in general, the interpretations are logical. Nevertheless, there are several critical issues that need to be addressed prior to publication.

1. A central concern is that the evidence for Sec2-S584 phosphorylation by Cdc28 is circumstantial. Throughout the manuscript, the state of Sec2 phosphorylation was inferred by electrophoretic mobility shifts. Attempts to express non-phosphorylatable Sec2-S584A derivatives were unsuccessful. Even if the hyphal-specific band shift is due to S584 phosphorylation, as suggested by immunoblots of Sec2-S584E, S584 phosphorylation may be indirectly influenced by Cdc28. Moreover, the authors report that another kinase Pho85 co-precipitates with Sec2, (might Pho85 be responsible for S584 phosphorylation?). Thus, it remains possible that Cdc28 regulates Sec2 indirectly. For these reasons, experiments should be included to directly demonstrate that 1) Sec2 is phosphorylated on S584 and 2) Sec2-S584 is phosphorylated by Cdc28.

2. It is unfortunate that one of the most important reagents, (the Sec2-S584A mutant), to prove the physiological significance of S584 phosphorylation is lacking from this study. It is unclear which specific constructs were attempted, however it seems that Sec2-S584A derivatives should be able to be expressed in SEC2+/SEC2+ cells. It would be worth trying alternative methods for expressing Sec2-S584A in vivo (different promoters, etc).

3. The authors state that the aberrant Sec2 localization in hgc1 and ccn1 mutants is consistent with a role for Cdc28 regulating Sec2 via phosphorylation S584 during hyhpal development. Experiments should be included to test if the S584E mutation rescues the Sec2 mislocalization defect in cyclin and cdc28 mutants.

4. Fig 2: it is not clear in the text or the legends if CIP treatment of hyphal form of Sec2 increases electrophoretic mobility of Sec2 to the same degree as the yeast form of Sec2. Figure 2 layout suggests that only the yeast form of Sec2 was treated with CIP. Regarding Fig 2B data, the authors suggest that the hyphal-specific Sec2 phosphorylation occurs in cfh1-/- efg1-/- mutants, but figure 2B doesn't convincingly establish that the Sec2 band shift is the same as in corresponding wild type cells. Appropriate controls should be included on the same gel to unambiguously demonstrate the extent of Sec2 phosphorylation.

5. Control immunoblots for the immunoprecipitation experiments are missing (Fig 5-7). Immunoblots should be shown for the input (total yeast extract) and for both immunoprecipiated proteins.

6. Anti-PSTAIRE immunoblots of Sec2 IPs show two bands, which the authors state are Cdc28 and Pho85. Data should be shown to prove the identity of each band. It might be more fruitful to conduct these experiments with Cdc28-specific reagent (such as Cdc28-FLAG).

7. Some of the microcopy data in this manuscript is not adequately quantified (with the exception of Fig 3C and the hyphal morphology in Fg 5C). The percentage of cells with a given phenotype should be noted throughout.

Resubmission - Authors' Response 28 January 2010

Detailed response to reviewers

Referee 1

Major issues

1. One key conclusion the authors made is that Sec2 is phosphorylated on serine 584 by Cdc28 in partnership with Hgc1 or Ccn1. However, the experiments performed in the study are not sufficient for making this conclusion. Several key experiments are usually required according to today's criteria for high quality publications. (1) MS analysis of Sec2 immunopurified from yeast and hyphal cells together with the same samples treated with protein phosphatases. This is required to show that serine 584 is indeed phosphorylated and it may also reveal other phosphorylation sites. (2) in vitro kinase assays using immunopurified Cdc28-as/Ccn1 or Cdc28-as/Hgc1 to phosphorylate immunopurified Sec2 in the presence or absence of 1NM-PP1. αP32 ATP is used to visualize protein phosphorylation. This is used to show Sec2 can be directly phosphorylated by Cdc28, although a positive result cannot completely exclude the possibility that Cdc28 activates another kinase in the precipitate that further phosphorylates Sec2. If this is technically difficult, an alternative experiment is to express a region of Sec2 containing serine 584 as GST fusion in E. coli and then use the purified protein as substrate of immunopurified Cdc28/Ccn1 or Cdc28 Hgc1 in in vitro kinase assays. The same fragment containing S584A mutation is included as negative control. Although it is nice to be able to conclude that Sec2 is directly phosphorylated by Cdc28, it is not less significant to make a convincing conclusion that Sec2 is a regulatory target of Cdc28 for hyphal development, direct or indirect. I believe that the current data sufficiently support such a conclusion.

We have followed the referees suggestion and expressed the peptide spanning S584 as a GST fusion in E.coli and have clearly demonstrated that it is a substrate of immunopurified Hgc1-Cdc28 while a peptide containing E584 is not. Sec2 is a large and low abundance protein. Despite repeated attempts we were unable to purify it in sufficient quantity and purity for a mass spec analysis of phosphorylation sites. We Instead we used a genetic approach which unambiguously defined an 8-amino acid sequence which contained a site necessary for Sec2 phosphorylation and function during polarized growth. Since Sec2 contains 94 serines and 52 threonines it likely to be phosphorylated at multiple sites. We argue that this is at least as informative as a Mass Spec approach because it identified the phosphorylation site that is physiologically important.

2. In addition to the above point, in several places the authors made conclusions favorable to their hypothesis but left out alternative explanations. One example is that while the deletion mapping results indicated that the (loss of band shift without the region), the author concluded that the region 'contains a residue that is phosphorylated and this phosphorylation event is required for normal hyphal growth...' (page 9, end of the first paragraph). An alternative explanation could simply be that the region is required for Sec2 phosphorylation at other sites. This is also one of the reasons why I suggest above using MS to confirm that serine 584 is phosphorylated. A second example is the subtitle on page 9. The presented data are not strong enough for making the statement that Sec2 S584 is phosphorylated by Cdc28-Hgc1 and Cdc28-Ccn1 during hyphal growth. This statement would require the authors to demonstrate that purified Cdc28-Hgc1 and Cdc28-Ccn1 can indeed phosphorylate S584.

We agree that the minimal conclusion from the deletion analysis is that 583-591 region is required for Sec2 phosphorylation, but does not prove that a residue in this region is phosphorylated. However, we have now shown that Cdc28-Hgc1 can indeed phosphorylate S584 in vitro. Moreover, we go on to show that cells expressing the phosphomimetic E584 allele form hyphae normally but Sec2 is not phosphorylated. Finally, the E584 allele rescues the effect of inhibiting Cdc28. These are all independent observations that each demonstrate that phosphorylation of S584 is necessary for the development of the hyphal pattern of phosphorylation.

3. I would say that the most unexpected result is the ability of the S584E mutant of Sec2 to

support normal hyphal growth of the cdc28-as mutant in the presence of 1NM-PP1. This has very important implications. It suggests that as long as Sec2 is phosphorylated on serine 584, it would bypass the requirement of phosphorylation of all other Cdc28 substrates important for hyphal development or even those required for cell-cycle related events. The authors used 5μM 1NM-PP1. In our hand, this concentration is too low to cause significant effects on cell growth although it is high enough to inhibit purified Cdc28-as/cyclin kinases. Have the authors tried higher concentrations like 20 to 25 μM?

Because this result was so surprising we re-sequenced the key region of CDC28 and verified that it still carried the mutation that makes it analogue sensitive. We show the sequencing trace as supplemental data. We agree that one implication of the result is that bypassing phosphorylation of Sec2 bypasses the phosphorylation of all other Cdc28 substrates, which does seems unlikely. A possible explanation is that the Cdc28 1as allele is a hypomorph; that is, addition of the inhibitor may reduce its activity to the point where Sec2 phosphorylation is reduced sufficiently to affect its function; but residual Cdc28 activity may allow it to phosphorylate other sites. This may explain why the S584E allele does not rescue the hgc1 mutation, which is a null allele. Two arguments support this suggestion. First our in vitro assays with the analogue sensitive kinase showed that the inhibitor reduced, but did not abolish Cdc28 activity, even at the higher 25 M concentration. Second, the context of S584 is not a perfect match to the Cdc28 target site so the affinity of Cdc28 for this target may be less than other targets, and its action at this site may be more easily inhibited.

Another intriguing result is that the S584E mutation, which is suggested to mimic hyphal-specific phosphorylation, not only abolishes the hyphal-induced Sec2 phosphorylation but also its phosphorylation during yeast growth. How is it possible for a yeast event to be dependent on a hypha-specific event?

This is a good point and it made us re-examine our data. Careful inspection of figure 4 reveals that the GFP-Sec2 S584E protein does show a small band shift in stationary phase yeast cells which the identical to small band shift evident in the wild type GFP-Sec2 protein. Upon hyphal induction there is no change in the migration of the Sec4-584E protein but a pronounced additional band shift in the wild type GFP-SEC2 protein. The small bandshifts in the yeast proteins are also evident in other autoradiographs not included in the paper and we are confidant that it is reproducible. Thus in yeast cells the Sec2 S584E protein is phosphorylated in the yeast pattern, but does not show the additional hyphal pattern of phosphorylation, suggesting that S584 is phosphorylated specifically during hyphal growth. Note that the pattern of band shifts in the N-terminal fusions is different from the C-terminal fusions presented elsewhere in the paper and so cannot be directly compared. Our deletion analysis showed that the Sec2-1-583 protein no longer shows even the yeast pattern of phosphorylation so this region also plays an important role in regulating the ground state of Sec2 phosphorylation

4. While the authors showed that Sec2 hyperphosphorylation is complete within 20 min of hyphal induction, the coIP of Sec2 with Cdc28, Ccn1 and Hgc1 were performed at much later time points (60 to 120 min). The results would be more robust if time points within the first 20 min of induction are included in the coIP experiments.

We accept it would have been better to use earlier time points, but the main principle that the Cdc28 kinase and its cyclins interacts with Sec2 would not be altered by showing that also occurs after 20 minutes instead of 60 minutes.

Minor issues

1. Regarding nomenclature of the Cdc28 as mutant, several versions were used throughout the text and figures including Cdc28 as1, Cdc28 1as, Cdc28-as1, and Cdc28-AS1 and in combinations with the use of upright or italic letters. Please be consistent.

The instances of inconsistent nomenclature have been corrected. However, it should be noted the use of italics/non-italics and whether the initial letter is upper case follow the standard rules of genetic nomenclature in S. cerevisiae, which have been used in this paper. An allele or gene is written in italics ñ the dominant allele in upper case, the recessive allele in lower case. The protein product of gene is written in non-italics with first letter in upper case. Thus, cdc28-as1 refers to the

analogue sensitive allele of the CDC28 gene; Cdc28-as1 refers to the analogue sensitive protein product of the cdc28-as1 allele. Thus, the format will vary according to the context.

2. In many places in the text the authors misused 5 µm for concentration. It should be 5 µM. Please also check figure legends, Materials and Methods and Supplemental Data.

I presume the reference was to 5 µm instead of 5 µM. This has been checked and corrected.

3. For the description of genotypes, when a mutation or deletion is introduced the gene name should be written in lower cases for example: SEC2 584E or SEC2 1-583 should be sec2 584E or sec2 1-583. Please make changes throughout the text and Table S1

This has been corrected, but note there are some instances where the context refers to a protein product rather than the allele and in these cases the correct format is Sec2 1-583 etc. Also note that SEC2 S584E shows a gain rather than loss of function and, although not formally tested, is probably a dominant allele and is therefore written in upper case.

4. To avoid confusion with fusion proteins, MET3-YFP-SEC2 (and all other strains using the MET3 promoter) should be changed to pMET3-YFP-SEC2 or PMET3-YFP-SEC2.

All instances have been changed to pMET3 as requested

5 . Page 10, line 2-3, GFP is used here but YFP is used in the figure

This has been corrected

Referee 2

1. In Fig 1 it is difficult to tell where the cell boundaries are.

I suspect this is caused by two problems. First the output from the Delta Vision software is in RGB format, which has to be converted to CMYK for submission to EMBO J. During the conversion the bright blue lines outlining the cells become much duller. Secondly, while the outline of the cells is still very clear on my computer monitor, I have found that the blue is not very bright in the output from a HP Deskjet laser printer. In several place we also had DIC images which we have included for greater clarity. In addition, I have increased the brightness in those images still relying on the blue outline from Calcofluor White staining

It would also be helpful for the general reader to define the difference between a hyphae and a pseudo-hyphae. Also the difference between the apical cap structure and the Spk structure seems subtle to the non-expert.

The pattern of localization of proteins to a spot representing a Spitzenkörper in hyphae or to a crescent/cap at the tip of pseudohyphae is well established in the literature following our pioneering paper on this topic (Crampin et al (2005) J Cell Sci 18:2935-2947) and it has found wide-spread acceptance (see for example Steinberg (2007) Euk Cell 6:351-360). These different patterns of localization were reviewed in the second paragraph of the Introduction. However, following the referee's suggestion this section has been expanded to review general differences between hyphae and pseudohyphae.

2 In several figures, such as Fig2 the level of Sec2 seems to vary widely from lane to lane. Some of the difference could represent changes in synthesis or degradation, but why should the level go way up upon CIP treatment in panel B? The apparent fluctuations in level certainly confuse the interpretation of the functionality of the various mutants

Each of the lanes in these autoradiograms is derived from a separate culture. Every effort was made to equalize loading, but the purpose of these autoradiograms is to analyze qualitative patterns of phosphorylation not quantify protein levels. We do not accept that the interpretation is made more difficult to interpret by variations in loading and to repeat these very difficult experiments does not

seem justified.

3 If the role of phosphorylation is to regulate the localization of Sec2 to the Spk, does the S584E mutant localize to Spk's even under yeast or pseudo-hyphal growth? If not, why is Sec2 ever de-phosphorylated since this constitutive allele seems to do everything fine? If the authors want to convince us that this regulation is important they need to define roles for both the phosphorylated and non-phosphorylated forms.

Sec2 584E does not form hyphae constitutively. As we argued in point 3 referee 1, phosphorylation of S584 is necessary but not sufficient for hyphal formation. It is not surprising that S584E is not sufficient for hyphal formation because hyphal growth requires a coordinated shift in the pattern of vesicle traffic that will probably involves other processes apart from Sec2 phosphorylation.

4. Why is the S584A mutant lethal since this region is not even needed during yeast growth? It seems that there is more going on here. Have they tried other substitutions? It is also very strange that the S584E mutation can bypass Cdc28 function but not that of the associated cyclins. These results make me somewhat dubious of their model.

One explanation of the apparent lethality of the S584A mutation is that the region surrounding S584 acts as to inhibit Sec2 function unless phosphorylated. We suggest that the reason why S584E does not rescue the lack of Hgc1 is that the activity Cdc28-as1 kinase is reduced but not abolished by the inhibitor. The residual activity of Cdc28 allows it to perform other functions required for hyphal growth, so that bypass of Sec2 phosphorylation by S584E is all that is required to allow hyphal growth. In contrast, cyclin mutations are null alleles with no activity, so bypass of Sec2 phosphorylation is not sufficient for hyphal growth as other Cdc28-dependent functions are still affected. We argue this point at greater length and provide supporting arguments in the response to referee 1 point 3 above.

5. In Fig 5B they need to run the + and - 1NM PP1 lanes adjacent to each other since it is too difficult to see the mobility difference with an intervening lane.

We include an addition to Fig 5b showing the + and - 1NM PP1 running side by side taken from a separate experiment.

6. To a naive reader the cells in the two panels of Fig 5C look very similar.

We have substituted different images which we hope make the point more clearly. Note that if the differences are not clear by visual inspection they are quantified in the text.

7. In Fig 5D the reader must be able to figure out what fraction of the total pool of Cdc28 is co-precipitating with Sec2. Curiously, the interaction appears more significant in the yeast form when Sec2 is not phosphorylated. The background precipitation is appreciable. Another negative control should be included such as an irrelevant YFP tagged protein.

We have included the inputs of Sec2 and Cdc28

8. In Fig 6C and 7C the reader needs to be able to figure out the fraction of Sec2 that is co-precipitating.

We have removed these Co-IPs as the inputs are not available

9. In Fig 7B the level of Sec2 is much lower. Why?

We've dealt with this in the text: Sec2 is destabilized in the absence of Ccn1

10 In general, an independent marker is needed for the Spk. If Sec2 is not exhibiting Spk localization, we need to know if that reflects a failure to associate with the Spk or a failure to assemble a Spk.

The referee raises an interesting point which we may wish to follow up, but the focus of this paper is

on the requirements of Sec2 to localize to the Spitzenkˆrper, not whether formation of the Spitzenkörper is dependent on Sec2.

Referee 3

1. A central concern is that the evidence for Sec2-S584 phosphorylation by Cdc28 is circumstantial. Throughout the manuscript, the state of Sec2 phosphorylation was inferred by electrophoretic mobility shifts. Attempts to express non-phosphorylatable Sec2-S584A derivatives were unsuccessful. Even if the hyphal-specific band shift is due to S584 phosphorylation, as suggested by immunoblots of Sec2-S584E, S584 phosphorylation may be indirectly influenced by Cdc28. Moreover, the authors report that another kinase Pho85 co-precipitates with Sec2, (might Pho85 be responsible for S584 phosphorylation?). Thus, it remains possible that Cdc28 regulates Sec2 indirectly. For these reasons, experiments should be included to directly demonstrate that 1) Sec2 is phosphorylated on S584 and 2) Sec2-S584 is phosphorylated by Cdc28.

We've now shown that Sec2-S584 is phosphorylated by Cdc28. As argued in referee 1 point there are there are multiple independent lines of evidence to show that S584 is phosphorylated.

2. It is unfortunate that one of the most important reagents, (the Sec2-S584A mutant), to prove the physiological significance of S584 phosphorylation is lacking from this study. It is unclear which specific constructs were attempted, however it seems that Sec2-S584A derivatives should be able to be expressed in SEC2+/SEC2+ cells. It would be worth trying alternative methods for expressing Sec2-S584A in vivo (different promoters, etc).

We have tried many strategies to generate the Sec2 S584 allele including those suggested by the referee, for some reason this allele just can't be constructed. It is important to remember that C. albicans genetic manipulation is not so facile as S. cerevisiae. The trade off for this limitation is dramatic polarized growth allowing more sophisticated cell biology and its relevance to the real world as a pathogen.

3. The authors state that the aberrant Sec2 localization in hgc1 and ccn1 mutants is consistent with a role for Cdc28 regulating Sec2 via phosphorylation S584 during hyhpal development. Experiments should be included to test if the S584E mutation rescues the Sec2 mislocalization defect in cyclin and cdc28 mutants.

We have constructed these strains and reported the results in the text

4. Fig 2: it is not clear in the text or the legends if CIP treatment of hyphal form of Sec2 increases electrophoretic mobility of Sec2 to the same degree as the yeast form of Sec2. Figure 2 layout suggests that only the yeast form of Sec2 was treated with CIP.

CIP treatment completely dephosphorylates Sec2 and there is no difference in the migration of CIP-treated whether it is isolated from yeast or hyphae. We routinely use the yeast product as a dephosphorylated control because it is technically easier to prepare. We have added a statement to this effect in the legend of figure 2.

5. Control immunoblots for the immunoprecipitation experiments are missing (Fig 5-7). Immunoblots should be shown for the input (total yeast extract) and for both immunoprecipiated proteins.

As we argued before this won't change the basic point of the experiment

6. Anti-PSTAIRE immunoblots of Sec2 IPs show two bands, which the authors state are Cdc28 and Pho85. Data should be shown to prove the identity of each band. It might be more fruitful to conduct these experiments with Cdc28-specific reagent (such as Cdc28-FLAG).

We know the upper band is Pho85 and the lower band Cdc28 from previous work in lab on S. cerevisiae where we have shown the upper band disappears in a Pho85 deleted strain. Claudia Martinez-Anaya ,PhD thesis, Sheffield University 2002). The relevant data has been included in

supplemental figure 2.

7. Some of the microcopy data in this manuscript is not adequately quantified (with the exception of Fig 3C and the hyphal morphology in Fg 5C). The percentage of cells with a given phenotype should be noted throughout.

We have presented quantitation of the percentage hyphae in figure 1. In figure 4 the response was uniformly as reported for the examples presented. We have provided a detailed quantitation for the data reported in figure 5. In figure 6 the example video shown was one of ten videos taken in parallel which all showed identical results.

2nd Editorial Decision 19 February 2010

Your revised manuscript has now been re-assessed by two of the original referees whose comments you will find enclosed.
As you will see both are in support of publication but demand further amendments that should improve the quality of the data. The comments of ref#1 are very specific what needs to be done. Importantly, both referees encourage you to solve the issue whether blocking phosphorylation prevents formation of or Sec2's recruitment to the Spk. I therefore urge you to take these additional points seriously into consideration and address them in full before submitting an ultimate version of your paper for final assessment.

Yours sincerely,

Editor
EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is well-written revised manuscript regarding a key regulator of polarized secretion and fungal hyphal development. The revisions greatly improve the manuscript and provide further evidence that Sec2 is directly regulated by Cdc28 kinase. I am satisfied with the revisions and the authors' responses to the previous critiques. I recommend publication upon addressing the minor comments below:

Although I believe the authors' conclusions that Sec2 is a direct substrate of Cdc28, in my opinion the kinase assay figures 7C and D are not publication quality. The phosphorylated bands of GST-S584 in fig 7C and D are not clearly delineated, perhaps as a consequence of image over-cropping or poor image contrast. The data would be more convincing if the relevant lanes were presented as were the immunoblots of fig 7E, i.e. side-by-side and not vertically cropped between every lane.

I also suggest that the relative phosphorylation levels of GST-S585 and histone H1 be quantified and presented graphically to back up the claim that S584 phosphorylation was reduced by 1NM-PP1 addition to the same degree as the histone H1 control.

Fig 3D convincingly establishes that the electrophoretic mobility of Sec2-1-583 is the same regardless of CIP-treatment and does not change upon hyphal induction, thereby supporting the conclusion that Sec2 1-583 is un- or hypophosphorylated. However, Fig 3D does not convincingly show a hyphal-specific pattern of phosphorylation for Sec2-1-591 -the patterns of phosphorylation for the yeast and hyphal forms are not distinct. The band shifts do demonstrate, as the authors note, that Sec2 1-583 is significantly less phosphorylated than Sec2-1-591.

Although, Sec2 localizes similarly as the Spiztenkorper, I still feel that co-localization experiments are needed to unambiguously establish whether Sec2 localizes to the Spiztenkorper or to another tip

associated complex, such as the exocyst.

4th page of results, paragraph 1: Figure 3C should read Figure 3D.

Referee #2 (Remarks to the Author):

The paper has been improved and has addressed most of my prior concerns. I would have liked to have seen an independent marker for the Spk, but they chose not to include this. As it stands they can't determine if blocking phosphorylation blocks formation of the Spk or recruitment of Sec2 to the Spk. There are a fair number of grammatical errors and the legends to the figures are unduly wordy.

1st Revision - Authors' Response

Referee 1

Although I believe the authors' conclusions that Sec2 is a direct substrate of Cdc28, in my opinion the kinase assay figures 7C and D are not publication quality. The phosphorylated bands of GST-S584 in fig 7C and D are not clearly delineated, perhaps as a consequence of image over-cropping or poor image contrast. The data would be more convincing if the relevant lanes were presented as were the immunoblots of fig 7E, i.e. side-by-side and not vertically cropped between every lane.

I also suggest that the relative phosphorylation levels of GST-S585 and histone H1 be quantified and presented graphically to back up the claim that S584 phosphorylation was reduced by 1NM-PP1 addition to the same degree as the histone H1 control.

We have repeated the kinase assays and have generated a completely new figure 7. The figure now shows the crucial signals on the same autoradiograph without vertical cropping of the lanes. The inhibition by 1NM-PP1 is more clearly evident than the originals. We have also quantitated the signals combining the results of three independent experiments. The quantitation shows that the S584E substitution consistently reduces phosphorylation by 50%, and that this difference is significant. Furthermore, the inhibition by 1NM PP1 is now much clearer and the quantitation shows it inhibits the reaction to same degree in the Sec2 peptide as in the histone H1 control. The Histone H1 lanes required a separate exposure as the signal is much stronger due to the multiple Cdk target sites in histone H1.

Fig 3D convincingly establishes that the electrophoretic mobility of Sec2-1-583 is the same regardless of CIP-treatment and does not change upon hyphal induction, thereby supporting the conclusion that Sec2 1-583 is un- or hypophosphorylated. However, Fig 3D does not convincingly show a hyphal-specific pattern of phosphorylation for Sec2-1-591 -the patterns of phosphorylation for the yeast and hyphal forms are not distinct. The band shifts do demonstrate, as the authors note, that Sec2 1-583 is significantly less phosphorylated than Sec2-1-591.

We agree that the Sec2 1-591 does not show the hyphal pattern of phosphorylation. We therefore carried out further experiments to identify the residue specifically phosphorylated in hyphae. A new series of truncation alleles showed that the residue was located between 597 and 607, which contained 3 potential phosphorylation sites. We mutated each of these and found that the critical residue was S598. An alanine substitution at this site was still able to support hyphal growth. Thus, while phosphorylation of S584 is essential for hyphal growth, the additional phosphorylation evident in hyphae is not essential. We have shown the new data in figure 3D and modified the interpretation in the manuscript appropriately to reflect this conclusion. We thank the referee for their critical input that has resulted in a change in emphasis in our paper. However, it has not altered the main conclusion that phosphorylation of S584 is the critical event.

Although, Sec2 localizes similarly as the Spiztenkorper, I still feel that co-localization experiments are needed to unambiguously establish whether Sec2 localizes to the Spiztenkorper or to another tip associated complex, such as the exocyst.

We have carried these co-localization experiments. Sec2-YFP co-localizes with FM4-64, a Spitzenkörper marker, and localizes separately from Exo70 GFP, an exocyst component. The additional images are shown in figure 1 D,E.

4th page of results, paragraph 1: Figure 3C should read Figure 3D

This has been amended.

Referee 2

The paper has been improved and has addressed most of my prior concerns. I would have liked to have seen an independent marker for the Spk, but they chose not to include this. As it stands they can't determine if blocking phosphorylation blocks formation of the Spk or recruitment of Sec2 to the Spk

We used FM4-64 as an independent marker of the Spitzenkörper and showed that the Spitzenkörper does not form when Sec2 phosphorylation was inhibited in the Cdc28 1as strain in the presence of the 1NM-PP1 inhibitor. The additional data is shown in figure 5E.

There are a fair number of grammatical errors and the legends to the figures are unduly wordy.

We have revised the paper thoroughly and we hope the amendments are satisfactory. In particular we have shortened the figure legends as much as possible while still retaining the essential technical information.

