molecular systems biology

Cross-species discovery of syncretic drug combinations that potentiate the antifungal fluconazole

Michaela Spitzer, Emma Griffiths, Kim M. Blakely, Jan Wildenhain, Linda Ejim, Laura Rossi, Gianfranco De Pascale, Jasna Curak, Eric Brown, Mike Tyers, Gerard D Wright,

Corresponding authors: Mike Tyers, University of Edinburgh and Gerard Wright, McMaster University

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

1st Editorial Decision 25 February 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees found your study interesting, and they were generally supportive. However, they raised a series of important concerns, which, I am afraid to say, preclude publication of this work in its present form.

Overall, the reviewers, especially the first two, felt that the manuscript lacked important details, and noted several cases where additional clarification is needed. In particular, they had issues with the portion of this work focused on integrating chemical-gene interactions with genetic interaction networks. These issues were significant enough that in some cases they found it hard to fully evaluate the relevance of particular analyses (e.g. point #1 by Reviewer #2). Somewhat related to this issue, the first reviewer also indicated that the chemogenomic profile data should be included as supplemental material.

The first reviewer also had some specific concerns regarding the results shown in Fig. 4, requesting error bars and statistical tests of significance. When revising this work, please indicate clearly in the Figure legend how error bars are calculated, and how many independent experimental replicates (n) were conducted. We also provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this Figure panel. Guidelines are pasted below.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript

will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

Griffiths and colleagues present in this manuscript an evolutionary study of drug synergism. A library of 1120 off patent drugs was tested for the capacity to potentiate the effects of fluconazole in different fungal species. A small subset of those found to potentiate fluconazole was further analyzed, through chemogenomics and microscopy studies, in order to identify the most likely biological mechanism underlying the synergism. The authors argue that drug synergy is poorly conserved across species that integration of chemogenomic with genetic interaction data allows for prediction of drug-synergy and that higher order combinations of drugs can be used to further inhibit fungal growth. Finally, synergistic drug combinations were tested against fluconazole resistant Candida strains and further validated in an in-vivo model of C. neoformans infection. This is a very interesting and exciting study with potential biomedical applications as it suggests venues for computational and experimental exploration of combinatorial therapeutics. I believe it is a very appropriate for publication in MSB and I have only some minor concerns described below.

Minor Concerns:
1 - The authors need to be clearer about how the hits were selected in the first screen (Figure 1).
1.1- The authors state (in supplementary methods) that they selected hits that are 2 median absolute deviations (MADs) away from the diagonal. Was the model used really the diagonal (i.e. y=x) or a linear regression model based on the data for each species?
1.2- The numbers on the supplementary table 1 do not match the explanation of the analysis nor the results described in the main text regarding the number of hits for each species. I believe the labels for the hit types (MAD hit and 80% inhibition) are switched but even so, the total number of hits do not match the main text and figure 1B. For example, from the data in supplementary table 1 it would seem that MAD hits are a actually a combination of the MAD threshold plus some percent inhibition cut-off (~20%?) in the presence of fluconazole. This is not how the MAD hits are described in the methods.
1.3- Is the method used for the results presented in figure 1 not suitable to report antagonistic interactions? It would appear that some of the compounds tested significantly improve the growth fluconazole treated fungi (figure 1A). Even if this is not the focus of this manuscript the authors should analyze these antagonist interactions and report them in the supplementary table. In a related note, are there compounds that potentiate the effect of fluconazole in one species but improve growth in another species?

2- The results presented in the section "Cell biological effects of synergistic combinations" (figure 4) could be significantly improved. At the very least, the images for the drugs that the authors claim were tested but not presented (trifluoperazine, tamoxifen, clomiphene) should be in supplementary

materials. In addition, the growth data shown in figure 4C should have error bars that reflect the reproducibility of the results and significance of the differences.

3- The authors integrated data on chemical-genetics with genetic-interaction data in order to predict the effects of drug-combinations. This integration needs to be better described in the methods section.
3.1- In particular, how were sensitive strains from the chemogenomic screens defined?
3.2- The authors mention that there are "many second order genetic interactions" shared between the sets of sensitive strains. Does this mean the authors also counted the number of indirect genetic interactions between the sensitive strains of different drugs? There is a typo on the p-value of the PPP test (page 12, end of first paragraph: "p-value<0.5")
3.3- The authors claim that they can predict compounds that potentiate the effects of fluconazole by looking at genetic interactions but they use a set of sensitive strains defined with the known synergizers. Could they have made the predictions based on the sensitive strains from the fluconazole screen? When they say that 11 of the 16 represented physcoactive drugs were predicted to synergize, what was the definition used to call this? Was it the p-value cut-off of the overlap?

4 - The chemogenomic data must be available as a supplementary table.

Reviewer #2 (Remarks to the Author):

In this valuable piece of work, the authors strengthen the notion that combining antifungal drugs with synergistic bioactive compounds that do not have antifungal activity per se is a promising antifungal strategy. The authors systematically screen for compounds that synergize with fluconazole to identify many novel combinations. Especially exciting is their examination of species and genus specificity of such combinations, and the discovery of both specific and non-specific combinations. The paper makes little further attempt to examine the factors for specificity or lack of it but provides a foundation for such studies. Instead they measure chemical genetic profiles in yeast to successfully identify plausible common underlying mechanisms for different synergies. They demonstrate the action of combinations in vivo and against resistant strains.

I recommend publication after addressing the following comments:

1) In the abstract it is claimed that: "Synergistic drug interactions were predicted by global genetic interaction networks". The only relevant part of the result section in support of this claim is (p.12): "Of this set, 16 compounds were represented in the Prestwick library, eleven of which were 13 predicted to synergize with fluconazole based on their genetic interactions with the core deletion strains identified above..."
Inexplicably, no explanation is given of the prediction procedure used. The procedure should be explained and justified in details, or the abstract claim should be dropped altogether.

2) On Figs 5A and B authors present evidence for some enrichment in interactions among the sets of deletion strains affected by fluconazole and most of the syncretic drugs. Their strongest claim: "The set of core deletion strains shared by the
membrane active group was highly enriched for shared interactions (p-value < 10-7)", is ambiguously worded. Are these interactions among the "core set" genes, or interactions between the "core set" genes and those potentiating fluconazole inhibition? What exactly is the core set?

3) On the parallel pathway permutation test. Authors say: "By this method, only the core set of deletion strains sensitive to tamoxifen, trifluoperazine, clomiphene, sertraline and suloctidil exhibited significant enrichment (p-value < 0.5; Figure 5D; Table S6)." Is there a typo for the p-value; maybe 0.05?? If figure 5D shows anything, it is lack of significance. So I am confused. If this test is not significant unlike the more basic randomization then what do we learn from that? It should be spelled out.

1)-3) Make the entire section "Integration of chemical-gene interactions with genetic interaction networks" problematic in its current form.

4) The primary high-throughput screen "Systematic antifungal potentiation screens in model and pathogenic fungi" is not a screen for synergy itself but some proxy. A single OD measurement after 48 hours is used. Definitions of outliers are somewhat arbitrary. It will be very helpful to state what are the approximate rates for false positives and false negatives based on the more detailed studies from the next section. Are thresholds too conservative or too weak? This will give more perspective to Fig 1B. This is important for the paper as a whole since the authors advertize systematic screening for synergy and examination of how common these effects are.

5) For Fig 1A, the authors measure 0.5MIC concentration for fluconazole. However, they do not mention whether such concentration has a negligible effect on OD after 48h. If not, is the y-axis on 1A normalized for that. The main text explanation is too vague: "All data were normalized for plate- and row/column-specific effects".

6) The term syncretic, which is used many times in the paper and the title, is introduced with the sentence: "Instead, unbiased screens for small molecules that exhibit unexpected chemical-genetic interactions, sometimes termed syncretic combinations, are needed to fully explore chemical space (Keith et al)." Yet the very paper they site defines it as "it is used to denote a drug that is composed of two or more active ingredients, at least one of which is not used individually to treat the target disease indication". This second definition is in fact a far better fit for the way they use "syncretic" in the rest of the paper.

7) In general the readability of the Results section is quite low. This is partly due to the overwhelming amount of undigested data dumped on the readers, and the very many points the authors try to make. Most of the methods are simply cited without a succinct explanation of exactly what the outputs of the measurements are. Figure captions are not detailed enough, and it is sometimes not clear what we are supposed to see.

8) Fig 3A y-axis is very uninformative "Z-score". What is the biological quantity?

9) 3B homozygous not mentioned in caption. How are the genes shown selected; are these all the genes that have an effect on at least one compound? Where is the analog of fig 3A? How significant is this enrichment for vascular and vesicle mediated transport? How are we supposed to see this enrichment; maybe, cluster together those genes? Which subset of genes is presented in panel 3C?

Reviewer #3 (Remarks to the Author):

The manuscript submitted by Griffiths et al describes a screen for compounds that act synergistically with the antifungal fluconazole (FLC) against Saccharomyces cerevisiae, Candida and Cryptococcus strains. The screen was performed against a library of 1120 compounds in the presence and absence of FLC and identified 115 compounds that had activity in one or more species in combination with FLC. The authors also found that five compounds were active in the screen against all species tested. The authors selected 12 compounds to perform FIC analysis and identified synergistic combinations that render FLC fungicidal and found that these drugs act to disrupt either sphingolipid biosynthesis or membrane permeability. The authors also found that some drug combinations could be used to effectively treat FLC resistant Cryptococcus during an in vivo infection mode in wax moth larvae. The synergy between these two classes of drugs (sphingolipid metabolism and membrane permeability) and FLC is not surprising, as drugs that act on membrane components could be expected to work together with inhibition of ergosterol biosynthesis to inhibit fungal growth. The chemical genetic screen also provides a significant amount of data and further supports the conclusions about synergy between the FLC and alterations in sphingolipid/membrane fluidity.
The manuscript is very well written, the figures are laid out nicely and the conclusions logical and relevant. Studies like this one are useful in identifying compounds that may be used therapeutically to improve the efficacy of FLC against FLC resistant fungi.

Minor concerns:
1) The authors to extend the conclusions about synergy between azoles and two of the hits

(sertraline, trifluoperazine) by using a second azole, ketoconazole and another ergosterol biosynthesis inhibitor, terbinafine. The authors should mention that FLC and KETO likely have similar mechanisms of drug import and efflux, and should discuss that at this point we don't know if the synergy is due to altered localization of drug efflux or import proteins or general membrane permeability. The authors mention drug efflux and sterol import, but don't mention drug import. The authors need to cite the recent paper showing that azole import is not via passive diffusion (Mansfield et al, PLoS Pathog. 2010 Sep 30;6(9)), and as such the effect isn't necessarily due to general membrane permeability but could be due to altered membrane localization of import or efflux proteins.

2) One minor concern regarding the hits that were identified is that these drugs have other therapeutic uses (antidepressant, antipsychotic, anti-nausea, etc). The authors should provide a line or two about the therapeutic limitations or potential unwanted effects from the use of these combinations.

3) Table 1 A and B should have a top line describing what the measurements are, unless this is against journal standards. The top line should mention that these values are FIC.

4) The results in Fig 6D suggest the Eagling effect for three of these strains - growth or lack if significant difference in growth at higher concentrations. While Eagling is usually for caspofungin, the results here resemble those results. The authors might mention this effect and the resemblance.

5) The Materials and Methods might mention that CLSI is not performed in SC media. Therefore, the use of SC media should be noted as a variation from the protocol.

6) Page 20, line 14 - "corresponding 2" makes no sense.

1st Revision - authors' response 04 April 2011

Please find attached our revised manuscript entitled "Cross-Species Discovery of Syncretic Drug Combinations that Potentiate the Antifungal Fluconazole" by Spitzer et al (previously Griffiths et al) for re-consideration at Molecular Systems Biology. The revised submission consists of a main text document of 58,828 characters (including references but not methods), six figures, one table and a supplementary information file that contains additional method descriptions, eight figures and seven tables. In addition, a separate file contains the extended synopsis, list of bullet points, and a standfirst sentence. A thumbnail figure is also attached. Linked data files are provided for all figures, as appropriate. The license to publish has been signed and faxed to the editorial office.

We would like to thank the Reviewers for their very constructive comments, which we believe that we have been able to address in full, and which have substantially improved the manuscript. A point-by-point response to each of the Reviewers' comments is provided on the following pages.

In brief, the main changes to the manuscript are:

1. As requested by the Reviewers and emphasized in your correspondence, we have clarified the approach for the integration of chemical genetic profiles and genetic interaction networks and the rationalization/prediction of new synergistic interactions. Extensive changes have thus been made to Figure 3 and its legend to precisely define the signature deletion strain set, to the Results sections that describe Figure 3 and Figure 5, and to the Methods sections that explain how the computational analyses were undertaken. As part of this reorganization, we have moved the parallel pathway permutation tests to the supplementary information (Figure S9). As requested, all of the heterozygous diploid and haploid chemogenomic profile data is now also included as supplementary material and data files.

2. We have addressed the statistical concerns on Figure 4C by re-running the experiment in quadruplicate replicates. The histogram now includes appropriate error bars and a statement of replicates in the legend.

3. We have added 4 new Supplementary Figures to address the Reviewers' concerns: (i) Figure S4 to indicate high throughput screen performance; (ii) Figure S7 to show primary data from all haploid deletion strain sensitivity screens; (iii) Figure S8 to show microscopy images for each of the membrane active compounds not shown in Figure 4, and (iv) Figure S9 to remove the PPP test results from the main figures.

4. We have clarified the high throughput screen data analysis methods and corrected Supplementary Table 1 that contains the primary screen data. In addition, we have estimated false negative and false positive rates in the primary screen (Figure S4).

5. As requested, we now provide linked data files for Figure 2A, 3B, 3D, 4C, 5C, 6A, 6C, 6D, S1 and S5A.

6. Finally, we note that the listed order of the two co-first authors has been switched by mutual agreement of all authors.

Thank you for your continued time and efforts in handling our revised manuscript. We look forward to your thoughts.

Detailed Response to Reviewers' Comments
(original comments in italic, response in plain text)

Reviewer #1
Griffiths and colleagues present in this manuscript an evolutionary study of drug synergism. A library of 1120 off patent drugs was tested for the capacity to potentiate the effects of fluconazole in different fungal species. A small subset of those found to potentiate fluconazole was further analyzed, through chemogenomics and microscopy studies, in order to identify the most likely biological mechanism underlying the synergism. The authors argue that drug synergy is poorly conserved across species that integration of chemogenomic with genetic interaction data allows for prediction of drug-synergy and that higher order combinations of drugs can be used to further inhibit fungal growth. Finally, synergistic drug combinations were tested against fluconazole resistant Candida strains and further validated in an in-vivo model of C. neoformans infection. This is a very interesting and exciting study with potential biomedical applications as it suggests venues for computational and experimental exploration of combinatorial therapeutics. I believe it is a very appropriate for publication in MSB and I have only some minor concerns described below.

Minor Concerns:
1 - The authors need to be clearer about how the hits were selected in the first screen (Figure 1).

1.1- The authors state (in supplementary methods) that they selected hits that are 2 median absolute deviations (MADs) away from the diagonal. Was the model used really the diagonal (i.e. y=x) or a linear regression model based on the data for each species?

Response: The reviewer is correct, we did originally use a linear regression model based on the data for each species. However, in reconsidering the matter, we have concluded that it would be more appropriate to use the simple diagonal as our model because the high number of hits in the C. gattii and C. neoformans screens distorts the linear regression model. Use of the diagonal is also justified since the residual growth data are normalized against controls for both conditions (i.e., with and without fluconazole) and the median is 100 for all screens. As described in 1.2 below, we have also simplified the hit definition.
To make the approach clear, the legend for Figure 1A now reads as follows: 'Scatterplots for Prestwick library screens for four fungal species. Growth inhibition caused by compounds in the absence (x-axis) and presence of fluconazole (y-axis) is represented by residual activity after treatment. Yellow and red filled circles indicate compounds that were classified as active (2 median absolute deviations below the diagonal). Compounds that inhibited growth in the presence of fluconazole by at least 80% compared to the effect of that compound alone are highlighted in red.' (page 32)
In addition, the supplementary methods for the screen data analysis now reads as follows: 'The diagonal (y=x) was used to identify hits from the screen data with versus without fluconazole.

Compounds that were more than 2 median absolute deviations (MADs) below the diagonal were defined as hits.' (page 3, 2nd paragraph of supplementary material)
As a consequence of these changes, the number of hits listed for each species has changed slightly: '43 compounds were active against S. cerevisiae, 30 against C. albicans, 70 against C. neoformans and 91 against C. gattii (Figure 1A, B).' (page 6, end of first paragraph)

1.2- The numbers on the supplementary table 1 do not match the explanation of the analysis nor the results described in the main text regarding the number of hits for each species. I believe the labels for the hit types (MAD hit and 80% inhibition) are switched but even so, the total number of hits do not match the main text and figure 1B. For example, from the data in supplementary table 1 it would seem that MAD hits are a actually a combination of the MAD threshold plus some percent inhibition cut-off (~20%?) in the presence of fluconazole. This is not how the MAD hits are described in the methods.

Response: We thank the reviewer for noticing this discrepancy as the column labels for the hit types were indeed switched. Once this labeling error is rectified, the number of hits do match the results described in the main text. To define hits, we had also applied an intensity cut-off to the data from the screen without fluconazole, which might have been the source of the confusion about the number of hits. Also, we recognize that the original hit selection method was convoluted and not particularly well described the table. In the revised version, we have simplified the hit selection procedure such that it is now solely based on median absolute deviation (MAD) statistics of the residuals. Compounds that were 2 MADs below the diagonal were thus classified as hits.
To reflect these changes, we have adjusted the supplementary methods and the description of the screen data file as follows: 'Supplementary file (SupplFile_ScreenData.xls) with normalized screen data. For each of the four fungal strains, all compounds are listed with plate position and name (columns 1-4). Columns 5 & 6 contain the residual activity (averaged over the two replicates) for the screens with Prestwick library alone and in the presence of fluconazole. The 7th and 8th column contain the level of additional inhibition in the presence of fluconazole and the residuals for each compound, respectively. The term 'yes' in column 9 indicates that a compound has been classified as a hit. Compounds with a residual larger than 2*MAD and a residual activity in the Prestwick screen in the presence of fluconazole below the respective cut-off (median residual activity ñ 2*MAD of residual activity values) were defined as hits. The MAD and the residual activity cut-offs were as follows: C. gattii - 71.03 & 31.44, C. neoformans - 69.25 & 36.80, C. albicans - 89.47 & 11.88, S. cerevisiae - 87.86 & 12.71.' (supplementary material, page 6, bottom).

1.3- Is the method used for the results presented in figure 1 not suitable to report antagonistic interactions? It would appear that some of the compounds tested significantly improve the growth fluconazole treated fungi (figure 1A). Even if this is not the focus of this manuscript the authors should analyze these antagonist interactions and report them in the supplementary table. In a related note, are there compounds that potentiate the effect of fluconazole in one species but improve growth in another species?

Response: The reviewer raises an interesting point. However, analysis of the potential antagonistic interactions revealed that there is very little overlap between the 4 fungal strains, much less so than the overlap observed for the fluconazole synergizers. Of the 12, 9, 21 and 21 drugs that might be classified as antagonistic in the C. gattii, C. neoformans, C. albicans and S. cerevisiae screens respectively, only 2 compounds are shared between C. gattii and C. neoformans, and 1 compound is shared between C. neoformans and C. albicans. More importantly, 3 of these putative antagonistic compounds (clofazimine and kawain in C. albicans and suloctidil in C. neoformans) were quantitatively tested against all four species (Figure 2A) but did not exhibit antagonistic interactions with fluconazole in secondary assays and, in fact, suloctidil was synergistic with fluconazole. This lack of reproducibility, although tested incidentally only for a limited number of compounds, suggests that the screen is not sufficiently robust to accurately detect antagonistic drug interactions. This limitation undoubtedly arises because the endpoint assays were close to saturation for control-treated cultures, i.e., the dynamic range of the assay was not tailored to detect increased growth. The reliable detection of antagonistic compounds would thus require the screens to be re-run under different assay conditions.

2- The results presented in the section "Cell biological effects of synergistic combinations" (figure 4) could be significantly improved. At the very least, the images for the drugs that the authors claim

were tested but not presented (trifluoperazine, tamoxifen, clomiphene) should be in supplementary materials. In addition, the growth data shown in figure 4C should have error bars that reflect the reproducibility of the results and significance of the differences.

Response: We thank the reviewer for both suggestions. We have now included microscopy images for clomiphene, tamoxifen and trifluoperazine as Supplementary Figure 8A, 8B and 8C. The figure for the sorbitol rescue experiment in Figure 4C has been amended to include error bars and the figure legend adjusted accordingly: 'Mean of four independent measurements is shown; error bars represent standard error.' (page 32)

3- The authors integrated data on chemical-genetics with genetic-interaction data in order to predict the effects of drug-combinations. This integration needs to be better described in the methods section.

Response: We have clarified the approach for network integration with the following text in the methods section: 'The 50 most sensitive deletion strains from duplicate chemical-genetic profiles for clomiphene, L-cycloserine, sertraline, suloctidil, tamoxifen and trifluoperazine were tested against the top 50 fluconazole-sensitive deletion strains (from replicate arrays at 8 M). Shared genetic interactions between the sets of deletion strains were determined based on genetic interaction data obtained from BioGRID (Breitkreutz et al, 2008; BIOGRID release 2.62, www.thebiogrid.org).' (page 23, first paragraph)
We have also reworded the corresponding section in the Results: 'Deletion strains that were sensitive to treatment with single drugs were used to assess the number of genetic interactions linked to the chemical-genetic space of fluconazole and each of the synergizers. A core set of haploid deletion strains affected by the membrane active group of compounds, referred to as the signature strain set (Figure 3D), exhibited many genetic interactions with the top 50 fluconazole-sensitive strains (Figure 5A). The top 50 most sensitive deletion strains for each individual drug (Z-scores above ~2.0) also showed many genetic interactions with the fluconazole profile.' (page 12, middle section)
Finally, the signature set is now also explicitly defined in Figure 3D and in the accompanying legend: "Asterisks indicate deletion strains that comprise the core signature set for membrane active compounds". (page 32)

3.1- In particular, how were sensitive strains from the chemogenomic screens defined?

Response: Figure 3C shows every deletion strain that has a Z-score of ±3 or more significant for at least one of the 6 synergizers, as described in the legend for Figure 3C: '(C) Main cluster of haploid deletion strain sensitivities to the six syncretic drugs in the absence of fluconazole, as assessed by barcode microarray hybridization. Strains that have a Z-score more significant than ±3 for at least one of the drugs in duplicate profiles are shown. ' As described above, for the integration of chemical-genetic profiles with genetic interaction data, we used the 30 and the 50 most sensitive deletion strains for each of the synergistic drugs.

3.2- The authors mention that there are "many second order genetic interactions" shared between the sets of sensitive strains. Does this mean the authors also counted the number of indirect genetic interactions between the sensitive strains of different drugs? There is a typo on the p-value of the PPP test (page 12, end of first paragraph: "p-value<0.5")

Response: Upon consideration, we realized that the term 'second order genetic interactions' is ambiguous in this context (we did not count indirect interactions) and so we have removed it (page 12, middle section). We thank the reviewer for noticing the typo, the p-value is indeed < 0.05 and is now corrected. (page 13, end of first paragraph).

3.3- The authors claim that they can predict compounds that potentiate the effects of fluconazole by looking at genetic interactions but they use a set of sensitive strains defined with the known synergizers. Could they have made the predictions based on the sensitive strains from the fluconazole screen?When they say that 11 of the 16 represented psychoactive drugs were predicted to synergize, what was the definition used to call this? Was it the p-value cut-off of the overlap?

Response: Because most of the predictions involve the interpretation of pre-existing datasets, we

have changed the word ëpredictí to ërationalizeí in the abstract in order to be more conservative. While in principle predictions could be made based on fluconazole sensitive strains, such predictions would either only identify other Erg11 inhibitors or would require the use of second order interactions that would by definition be far less specific. We are currently exploring the latter approach but at this point the analysis is too noisy and the datasets too sparse to make compelling predictions.
We have also rewritten the relevant paragraph in the discussion: "The documented synthetic lethal genetic interactions that occur between strains in the fluconazole and membrane active chemical-genetic profiles retrospectively predicted the synergistic effects of other hits in our primary screens. Moreover, when combined another source of chemical-genetic interaction data (Ericson et al, 2008), the membrane active signature strain set correctly identified additional synergistic hits in our primary screen data. In addition, the genetic interaction profile of L-cycloserine correctly predicted a novel synergistic interaction between the sphingolipid biosynthesis inhibitor myriocin and fluconazole. The potentiation of fluconazole activity by CADs and/or inhibition of sphingolipid biosynthesis may allow new general approaches to antifungal therapy in the clinic. As genetic and chemical-genetic space is elaborated, mechanism-based predictive approaches should become a powerful means of identifying new synergistic combinations." (page 18, 1st paragraph)
We have clarified the definition of synergism for these predictions in the methods and included the p-value: ‘To predict potential synergistic candidates based on overlap with published chemical-genetic profiles (Ericson et al, 2008), we used a binary data matrix based on a Z-score cutoff of ± 3. The significance of enrichment was calculated based on the number of genes that overlapped with the signature strain set; a subset of 4 out of 10 genes was significant with a p-value < 0.05.’ (page 23, bottom)

4 - The chemogenomic data must be available as a supplementary table.

Response: We now provide two supplementary tables, one for the haploid deletion pool profiles and one for the essential heterozygous deletion pool:
Supplementary data file ‘HaploidTransformedData.csv’: Z-scores for each of the chemical-genetic screens against the haploid deletion pool.
Supplementary data file ‘HetEssTransformedData.csv’: Z-scores for the haplo-insufficiency screens against the ~1000 strains heterozygous for essential genes.

Reviewer #2:
In this valuable piece of work, the authors strengthen the notion that combining antifungal drugs with synergistic bioactive compounds that do not have antifungal activity per se is a promising antifungal strategy. The authors systematically screen for compounds that synergize with fluconazole to identify many novel combinations. Especially exciting is their examination of species and genus specificity of such combinations, and the discovery of both specific and non-specific combinations. The paper makes little further attempt to examine the factors for specificity or lack of it but provides a foundation for such studies. Instead they measure chemical genetic profiles in yeast to successfully identify plausible common underlying mechanisms for different synergies. They demonstrate the action of combinations in vivo and against resistant strains.

I recommend publication after addressing the following comments:

1) In the abstract it is claimed that: "Synergistic drug interactions were predicted by global genetic interaction networks". The only relevant part of the result section in support of this claim is (p.12): "Of this set, 16 compounds were represented in the Prestwick library, eleven of which were 13 predicted to synergize with fluconazole based on their genetic interactions with the core deletion strains identified above..." Inexplicably, no explanation is given of the prediction procedure used. The procedure should be explained and justified in details, or the abstract claim should be dropped altogether.

Response: The prediction procedure is now explained in detail, as described in the response to point 3.3 of Reviewer #1. As noted above, we have changed the word "predict" to "rationalize" in the abstract.

2) On Figs 5A and B authors present evidence for some enrichment in interactions among the sets of deletion strains affected by fluconazole and most of the syncretic drugs. Their strongest claim: "The

set of core deletion strains shared by the membrane active group was highly enriched for shared interactions (p-value < 10-7)", is ambiguously worded. Are these interactions among the "core set" genes, or interactions between the "core set" genes and those potentiating fluconazole inhibition? What exactly is the core set?

Response: We thank the reviewer for flagging this ambiguity. As described above in response to point 3 of Reviewer #1, we have now explicitly defined the signature set in Figure 3 and have changed the sentence to clarify: "The signature deletion set shared by the membrane active group was significantly enriched for genetic interactions with fluconazole-sensitive deletion strains (p-value < 10-7)." (page 12, bottom)

3) On the parallel pathway permutation test. Authors say: "By this method, only the core set of deletion strains sensitive to tamoxifen, trifluoperazine, clomiphene, sertraline and suloctidil exhibited significant enrichment (p-value < 0.5; Figure 5D; Table S6)." Is there a typo for the p-value; maybe 0.05?? If figure 5D shows anything, it is lack of significance. So I am confused. If this test is not significant unlike the more basic randomization then what do we learn from that? It should be spelled out.

Response: We thank the reviewer for noticing the typo; as noted above, the p-value is indeed < 0.05. The PPP test is a much more stringent measure of randomization because it only involves the selected test set of genes, rather than all genes. By this measure, the signature set of sensitive strains is still significantly enriched, and so we chose to include the analysis. However, since all but one of the synergistic drug sets were not significant, we have moved the corresponding figure panels to the supplementary information (Figure S9).

1)-3) Make the entire section "Integration of chemical-gene interactions with genetic interaction networks" problematic in its current form.

Response: We believe that our revisions have addressed points 1-3 raised by the Reviewer, and hope that the section on integration of chemical-gene and genetic interaction networks is now a convincing aspect of the manuscript.

4) The primary high-throughput screen "Systematic antifungal potentiation screens in model and pathogenic fungi" is not a screen for synergy itself but some proxy. A single OD measurement after 48 hours is used. Definitions of outliers are somewhat arbitrary. It will be very helpful to state what are the approximate rates for false positives and false negatives based on the more detailed studies from the next section. Are thresholds too conservative or too weak? This will give more perspective to Fig 1B. This is important for the paper as a whole since the authors advertize systematic screening for synergy and examination of how common these effects are.

Response: The reviewer raises a very important issue that we did not adequately address in our original manuscript. This issue was also noted indirectly by Reviewer #1. We have added a short statement about the false positive and false negative rates of the screen: ‘Most hits from the screens were confirmed as synergistic with fluconazole, except for azoperone and kawain in S. cerevisiae, and azaperone, L-cycloserine and suloctidil in C. albicans (Figure S4). Quantification of interactions at different drug concentrations revealed some additional synergies with fluconazole: trifluoperazine exhibited synergy against C. albicans, tamoxifen against C. gattii and C. neoformans, and suloctidil against C. neoformans (Figure S4). Based on detailed analysis of these 12 compounds, the high throughput screens proved a reliable means to identify synergistic drug interactions, with an estimated false positive rate of 0.20 and a false negative rate of 0.28.’ (page 7, bottom & page 8, top)
We have also added a supplementary figure (Figure S4) to visualize the concordance between the high throughput screens and the results from the detailed synergy assessments.

5) For Fig 1A, the authors measure 0.5MIC concentration for fluconazole. However, they do not mention whether such concentration has a negligible effect on OD after 48h. If not, is the y-axis on 1A normalized for that. The main text explanation is too vague: "All data were normalized for plate- and row/column-specific effects".

Response: The reviewer is correct, in the main text we did not explicitly mention that we analyzed

residual activities. We have changed the sentence to mention this fact: ‘Residual activity was calculated for each compound and the data was normalized for plate- and row/column-specific effects’. (page 5, first paragraph)

6) The term syncretic, which is used many times in the paper and the title, is introduced with the sentence: "Instead, unbiased screens for small molecules that exhibit unexpected chemical-genetic interactions, sometimes termed syncretic combinations, are needed to fully explore chemical space (Keith et al)." Yet the very paper they site defines it as "it is used to denote a drug that is composed of two or more active ingredients, at least one of which is not used individually to treat the target disease indication". This second definition is in fact a far better fit for the way they use "syncretic" in the rest of the paper.

Response: We thank the reviewer for this suggestion to improve the precision of the definition. We have changed the above sentence to: ‘Instead, unbiased screens for synergistic enhancers of a specific bioactivity that are not themselves active, sometimes termed syncretic combinations, are needed to fully explore chemical space (Keith et al, 2005).’ (page 4, bottom)

7) In general the readability of the Results section is quite low. This is partly due to the overwhelming amount of undigested data dumped on the readers, and the very many points the authors try to make. Most of the methods are simply cited without a succinct explanation of exactly what the outputs of the measurements are. Figure captions are not detailed enough, and it is sometimes not clear what we are supposed to see.

Response: We hope that the changes we have made to the manuscript in response to all three Reviewers' constructive comments now make the Results section more intelligible. While we agree that quite a number of points are made, we feel that all bear on the central theme of the manuscript, i.e., species-specific syncretic drug combinations.

8) Fig 3A y-axis is very uninformative "Z-score". What is the biological quantity?

Response: We have changed the y-axis label to ‘log2 sensitivity score (Z-score)’, i.e., the extent of deletion strain depletion in response to drug treatment.

9) 3B homozygous not mentioned in caption. How are the genes shown selected; are these all the genes that have an effect on at least one compound? Where is the analog of fig 3A? How significant is this enrichment for vascular and vesicle mediated transport? How are we supposed to see this enrichment; maybe, cluster together those genes? Which subset of genes is presented in panel 3C?

Response: We have changed the legend for Figure 3 as follows: "Chemical-genetic interactions of six syncretic synergizers. (A) Sensitivity of heterozygous essential deletion strains to six different syncretic drugs, as assessed by barcode microarray hybridization. Genes implicated in membrane organization and vesicle mediated transport are indicated. (B) Core set of haploid deletion strains that are sensitive to fluconazole, as assessed by barcode microarray hybridization. Several concentrations of fluconazole were tested to correlate the signature with MIC. The effect of the six syncretic drugs on the core fluconazole profile was examined in the presence or absence of a threshold concentration of fluconazole (6 µg/mL). Values in parentheses indicate drug concentration in µg/mL. (C) Main cluster of haploid deletion strain sensitivities to the six syncretic drugs in the absence of fluconazole, as assessed by barcode microarray hybridization. Strains that have a Z-score more significant than ±3 for at least one of the drugs in duplicate profiles are shown. Gene names in red indicate deletion strains that were chosen for verification by quantitative growth curve assays. (D) Log-ratio scores calculated from individual growth curve assays to confirm chemical-genetic interactions of the six syncretic drugs. Gene names in bold indicate heterozygous deletion strains for essential genes. Negative Z-scores and log-ratios indicate sensitivity of a strain to a given drug, whereas positive scores represent resistance. Asterisks indicate 14 deletion strains that comprise the core signature set for membrane active compounds.’ (page 31)
We hope that this more detailed legend clarifies the data shown in Figure 3, especially for panel C. In addition, we have rearranged the deletion strains in Figure 3B such that deletion strains deleted for genes involved in vesicle mediated transport and membrane organization cluster together. The non-adjusted p-values for these two categories are 1.9x10-8 and 0.00036, respectively. We have also added a supplementary figure (Figure S6) for the haploid deletion strains that is analogous to 3A.

Reviewer #3:
The manuscript submitted by Griffiths et al describes a screen for compounds that act synergistically with the antifungal fluconazole (FLC) against Saccharomyces cerevisiae, Candida and Cryptococcus strains. The screen was performed against a library of 1120 compounds in the presence and absence of FLC and identified 115 compounds that had activity in one or more species in combination with FLC. The authors also found that five compounds were active in the screen against all species tested. The authors selected 12 compounds to perform FIC analysis and identified synergistic combinations that render FLC fungicidal and found that these drugs act to disrupt either sphingolipid biosynthesis or membrane permeability. The authors also found that some drug combinations could be used to effectively treat FLC resistant Cryptococcus during an in vivo infection mode in wax moth larvae. The synergy between these two classes of drugs (sphingolipid metabolism and membrane permeability) and FLC is not surprising, as drugs that act on membrane components could be expected to work together with inhibition of ergosterol biosynthesis to inhibit fungal growth. The chemical genetic screen also provides a significant amount of data and further supports the conclusions about synergy between the FLC and alterations in sphingolipid/membrane fluidity.
The manuscript is very well written, the figures are laid out nicely and the conclusions logical and relevant. Studies like this one are useful in identifying compounds that may be used therapeutically to improve the efficacy of FLC against FLC resistant fungi.

Minor concerns:
1) The authors to extend the conclusions about synergy between azoles and two of the hits (sertraline, trifluoperazine) by using a second azole, ketoconazole and another ergosterol biosynthesis inhibitor, terbinafine. The authors should mention that FLC and KETO likely have similar mechanisms of drug import and efflux, and should discuss that at this point we don't know if the synergy is due to altered localization of drug efflux or import proteins or general membrane permeability. The authors mention drug efflux and sterol import, but don't mention drug import. The authors need to cite the recent paper showing that azole import is not via passive diffusion (Mansfield et al, PLoS Pathog. 2010 Sep 30;6(9)), and as such the effect isn't necessarily due to general membrane permeability but could be due to altered membrane localization of import or efflux proteins.

Response: We thank the Reviewer for pointing out this important additional potential mechanism for synergism and for the supporting citation. We have added the following sentence to the discussion on mode of action: "It is also possible that the synergizers affect active import of azoles through altered localization of drug transporters or general membrane perturbation (Mansfield et al., 2010)." (page 17, bottom)

2) One minor concern regarding the hits that were identified is that these drugs have other therapeutic uses (antidepressant, antipsychotic, anti-nausea, etc). The authors should provide a line or two about the therapeutic limitations or potential unwanted effects from the use of these combinations.

Response: We have addressed this caveat in the following sentence in the discussion about therapeutic implications: ‘Importantly, while it is a potential concern that undesirable side effects may arise from drug combinations, as occurs for known contraindicated drugs, it has recently shown that synergistic combinations usually yield enhanced selectivity without adverse side-effects (Lehar et al, 2009).’ (page 20, last paragraph)

3) Table 1 A and B should have a top line describing what the measurements are, unless this is against journal standards. The top line should mention that these values are FIC.

Response: We thank the Reviewer for the suggestion and have added the following lines to the table on page 34: "Table 1A: FICI values for drug combinations in different fungal strains." and "Table 1B: FICI values for double and triple drug combinations in S. cerevisiae."

4) The results in Fig 6D suggest the Eagling effect for three of these strains - growth or lack of significant difference in growth at higher concentrations. While Eagling is usually for caspofungin, the results here resemble those results. The authors might mention this effect and the resemblance.

Response: The heatmaps in Figure 6D represent Bliss scores calculated from the checkerboards in Figure 6C, and do not represent growth effects per se. The apparent lack of synergy between the drugs at the higher concentrations is due to the fact that the highest amounts of fluconazole in C. albicans isolates 2007 and 2008 as well as C. parapsilosis inhibit growth completely, and therefore by definition cannot yield synergism. This effect is thus unlikely to be related to the paradoxical effect that has been described elsewhere for caspofungin (e.g., Stevens et al. 2005). We have noted this effect in the Figure 6 legend: "(D) Bliss synergy analysis for combination assays shown in panel C. The apparent absence of synergy at the highest fluconazole concentrations for C. albicans and C parapsilosis is due to growth inhibition caused by fluconazole alone." (page 34)

5) The Materials and Methods might mention that CLSI is not performed in SC media. Therefore, the use of SC media should be noted as a variation from the protocol.

Response: We now explicitly refer to the use of SC media in the methods section: 'MIC determinations were based on Clinical and Laboratory Standards Institute (CLSI) protocols (Eliopoulos, 1991; Odds, 2003), with the exception that yeast SC medium was used instead of mammalian cell RPMI 1640 medium.' (page 21, first paragraph)

6) Page 20, line 14 - "corresponding 2" makes no sense.

Response: We thank the reviewer for noticing the error and have corrected the wording to "corresponding to 2".

Acceptance letter 26 April 2011

Thank you again for sending us your revised manuscript. The referees have indicated that they are now satisfied with the modifications made and supportive of publication. I am therefore pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor
Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

The authors addressed in this revised manuscript all the concerns I had previously raised. They provide a simplified and clearer explanation of how the primary synergistic drug hits were obtained. They have also improved the description of how the genetic and chemical-genetic data were combined and provide the chemical-genetic data in appropriate supplementary tables. I have no further concerns and feel this article is very appropriate for the audience of MSB.

Reviewer #2 (Remarks to the Author):

The authors adequately addressed the points of concern, which resulted in a much improved manuscript.
I therefore recommend publication.

