

**UCSF**

**UC San Francisco Electronic Theses and Dissertations**

**Title**

Development of a High-Resolution Growth Assay for Yeast Functional Genomics and Identification of Orm Family Proteins as Key Mediators of Sphingolipid Homeostasis

**Permalink**

<https://escholarship.org/uc/item/6ds216x8>

**Author**

Breslow, David King

**Publication Date**

2010

**Supplemental Material**

<https://escholarship.org/uc/item/6ds216x8#supplemental>

Peer reviewed|Thesis/dissertation

**Development of a High-Resolution Growth Assay for Yeast Functional Genomics and  
Identification of Orm Family Proteins as Key Mediators of Sphingolipid Homeostasis**

by

David King Breslow

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



Copyright (2010)

By

David King Breslow

**For my family**

## Acknowledgments

Although several years have passed, I can still remember when I first came to UCSF. It was the middle of another cold winter in Boston when I was invited for an interview, and as soon as I got to the campus I could tell it was a special place.

Admittedly it was my first interview, and I was at least partly charmed by the improved weather, but also I felt there was something specific to UCSF that was unique. I couldn't quite put my finger on it at the time, and this feeling did not stop me from agonizing over the decision of whether to go to UCSF or somewhere else (to the surprise of no one). Of course, everyone I asked told me that any of the graduate programs I was considering would have been great choices, but with the benefit of hindsight I'm now convinced that coming to UCSF was an incredibly fortuitous and significant choice. Many people made my time at UCSF so enjoyable, and this short space will have to suffice to acknowledge their immeasurable contributions to my Ph.D. experience.

First, I have to acknowledge the instrumental role of Greg Copeland, the post-doc in Stuart Schreiber's lab who supervised me when I was an undergrad working in the lab. Having now worked with younger students, I am astounded that Greg had the patience and enthusiasm to mentor someone as inexperienced and naïve as I was. It was my time in the Schreiber lab and working with Greg that got me excited about research and convinced me to go to graduate school. And if I remember correctly Greg was also the first person to recommend that I apply to UCSF. For helping me get to UCSF in the first place, I am deeply indebted to Greg.

The vast majority of my time at UCSF was spent in Jonathan Weissman's lab, and two people in particular persuaded me (in their own way) to join the lab. John Newman

was the first person I met in the lab and mentored me during my rotation. It was immediately clear that John was a very sharp scientist, and like Greg he also had a tremendous willingness (most of the time) to teach and mentor me. Many of his habits both practical (his ‘golden mean’ of cloning) and philosophical (‘you have to go slow to go fast’) have stuck with me, although the obsession with white-boards is one notable exception. After joining the lab, John continued to provide me great advice, even if it wasn’t always what I wanted to hear, and I’m glad that we’ve been able to stay in touch even after he left the lab.

Vlad Denic was the second immediate influence on my time in the Weissman lab. I was turned over to Vlad’s supervision for a few days during my rotation while John was away at a meeting, and thus he was briefly subjected to my remedial questions about yeast genetics. As the sixth year student in the lab with no publications and a completely inscrutable lab-meeting presentation style, I wasn’t sure what to make of Vlad at first. It might be fair to say Vlad didn’t know what to make of me either, other than that I was an over-eager kid from Harvard that knew next to nothing about cell biology. Fortunately we built on this questionable foundation a multi-year espresso-fueled friendship that made my time in the lab a lot of fun and that was also very stimulating scientifically. I have to admit that often the direction of information flow was one-way (and not from me to Vlad), but Vlad’s scientific acumen makes it pretty hard to reverse the directionality (and by introducing him to *The Wire* I think my indebtedness is partially mitigated). He even (indirectly) persuaded me to work on what I thought had to be the most obscure and boring topic in all of biology, sphingolipid metabolism, and to even enjoy doing it. Together, Vlad and John were great influences, and given their scientific rigor and

sometimes-jaded outlook, doing science they find exciting will be a worthy goal for me in the years to come.

Sean Collins was another great person to have in the lab, both as a friend and as a data analysis guru with an encyclopedic knowledge of yeast biology. The little I've learned about Matlab and data analysis is largely thanks to him (every time I type "myNaNMean" I will remember that it is really "Sean'sNaNMean"), and his insights often had significant impacts on my project. Despite his emotional volatility, he's also been a lot of fun to hang out with outside of lab, especially when it comes to following our favorite sports teams (or at least he humors my rants about Baltimore sports teams).

Brandon Toyama and Erin Quan were Weissman lab students one year ahead of me. Fortunately for me (and perhaps unfortunately for them), this meant they could advise me on all manner of experiences in the life of a graduate student, as they generally had just gone through the same things themselves. I have to begrudgingly admit that my personality and Erin's have a lot in common. Hopefully our tendencies to (over-) analyze our experiments and keep track of (trivial) details will serve us well scientifically and not cause us too much stress. Brandon helped make sure I didn't get too caught up in the minutiae of experiments and was fun to spend long hours in the lab with, even if his taste in sports teams leaves something to be desired. It should also be noted that my dinners in the Weissman lab were much better off with Brandon and Erin's company.

Mike Bassik, Gloria Brar, and Stirling Churchman were terrific lab members to share a bay with. Coffee and lunch conversation wouldn't have been the same without them. I am also grateful to Mike for patient help with tissue culture and for always being a remarkably friendly and entertaining guy to have at the other end of my bench. Fellow

students Martin Jonikas, Clement Chu, and Cat Foo made sure the Weissman lab was a fun place to come to work every day. Clement in particular could always (literally, *always*) be counted on for helpful advice and for the best non-commercial espresso bar in San Francisco. Nick Ingolia had many thoughtful suggestions for planning and interpreting experiments, Adam Frost shared a rare enthusiasm for the cell biology of membrane proteins, and Jimena Weibezahn introduced me to biochemistry when I knew nothing about the topic. Lastly, Maya Schuldiner, Dale Cameron, and Sigurd Braun in Hiten Madhani's lab were post-docs I had the pleasure of working with more directly. Maya brought EMAPs and ORMs into my life, for which I am deeply grateful. Dale kept everything calm and collected when we were rushing to put together the Nature Methods paper, and I shudder to think about how stressed out I would have been without that influence. Sigurd was also a pleasure to work with – it's too bad our experiments together didn't pan out.

Christine Olson ran the CCB graduate program and was the first person I met at UCSF. Thanks for keeping me on track to graduate. Hiten Madhani and Kevan Shokat served on my thesis committee, and their guidance and helpful suggestions were instrumental in shaping my dissertation project. I imagine being on thesis committees is not always listed as the best part of being a PI, but I am grateful that Hiten and Kevan went out of their way to be involved in my project.

I'd like to thank Justin, Carleton and John for fun times outside of the lab. It was great having old friends in San Francisco to keep me sane.

I am especially grateful that I met Ilana Richman during my time at UCSF. Her friendship and companionship has made the long haul of a Ph.D. a lot of fun. The harder

times in lab were easier, and there were some memorable adventures outside of lab as well. She may not have known when we first met that she would become an expert on epistasis and sphingolipids, but I'm not sure I could have done it without her.

To my parents Michael and Lucille: Thanks for your love and support. You may not be cell biologists, but I couldn't ask for better role models. Also, thanks to my sister Joanna for reminding me not to take myself too seriously.

Last and certainly not least, I'd like to thank Jonathan Weissman for being my advisor during my dissertation studies. In short, it's hard to imagine a better mentor. From designing and interpreting experiments to thinking about the broader implications of one's work, Jonathan has been an exceptional role model. The lab may have grown since I first joined, but Jonathan always found the time to think and talk about science with an enthusiasm and dedication that is, without exaggeration, inspiring. In fact, on some occasions the time-stamps on his emails suggested an upper bound on his sleep that reached that grey area where inspiring approaches alarming. Granted he never seemed worse for wear, and this ceaseless energy and optimism was often the key catalyst in rebounding from a disappointing result to the next set of experiments. Besides his scientific abilities, I've especially appreciated the fact that he is also a genuinely nice person who cares about the success of his students. As I leave the lab, I am grateful for everything I've learned from Jonathan, and I will have to hope to someday approach his abilities as a mentor and scientist.

Finally, I would like to acknowledge generous research fellowships from the Hertz Foundation and the National Science Foundation. Their financial support was a major contribution to this work.

## Abstract

Advances in DNA sequencing have provided the complete genome sequence for many organisms. However, understanding the functions of each gene product encoded by the genome remains a major challenge. This dissertation describes an improved strategy for identifying functional relationships between genes and the use of unbiased genetic and biochemical approaches to uncover the function of the *ORM* family of genes.

Large-scale measurement of genetic interactions has proven to be a powerful approach for functional genomics in the model organism *Saccharomyces cerevisiae*. The key to such analyses is comparing the effects of mutations to individual genes or pairs of genes on a phenotype of interest, such as growth rate. In the first part of my thesis, I developed a high-throughput, flow cytometry-based strategy for measuring growth rates in *S. cerevisiae* with improved accuracy. This assay made it possible to precisely quantify the contribution of each gene to growth and to measure genetic interactions with increased sensitivity. With high-precision data, I was able to resolve an enigma regarding the apparent prevalence of gene dispensability in yeast, to reveal new relationships between chromatin modifying factors, and to create a functional map of the proteasome.

In the second part of my dissertation, I used genetic interactions as a starting point to identify members of the conserved *ORM* gene family as negative regulators of sphingolipid synthesis. I found that Orm proteins form a complex with serine palmitoyltransferase, the first and rate-limiting enzyme in sphingolipid production, and that a phosphorylation-based regulatory pathway relieves the inhibitory activity of Orm proteins when sphingolipid production is disrupted. This feedback mechanism matches



sphingolipid supply to cellular demand, with changes in *ORM* gene expression or mutations to their phosphorylation sites causing dysregulation of sphingolipid metabolism. Thus, given the essential roles of sphingolipids as structural components of membranes and as signaling molecules, Orm proteins serve a key role in cellular homeostasis. Finally, the functional conservation within this gene family and the recent identification of the human homolog *ORMDL3* as a potential risk factor for childhood asthma raise the possibility that sphingolipid misregulation contributes to asthma development.

## Contributions

Portions of the text and figures presented in this thesis are reproduced with permission from material published previously. Chapter 2, “Development of a High-Resolution Growth Assay for Yeast Functional Genomics,” was published as an article entitled, “A comprehensive strategy enabling high-resolution functional analysis of the yeast genome” in August 2008 in *Nature Methods*, Vol. 5(8), pp. 711-718. In this work, Dr. Dale Cameron created the library of DAmP strains and measured their growth rates. Together, we created strains for the functional map of the proteasome, developed the degran+DAmP hypomorphic allele strategy (initiated by Dr. Cameron), and wrote the paper. Jacob Stewart-Ornstein, Heather Newman, and Dr. Sigurd Braun also assisted in data collection and analysis, and Dr. Hiten Madhani and Dr. Nevan Krogan assisted in the supervision of the work. Chapter 3, “Identification of Orm Family Proteins as Key Mediators of Sphingolipid Homeostasis,” was published as an article entitled, “Orm Family Proteins Mediate Sphingolipid Homeostasis” on February 25, 2010 in *Nature*, Vol. 463(7284), pp. 1048-1053. In this work, Dr. Sean Collins supervised the collection of genetic interaction measurements and processed the genetic interaction data with the assistance of Dr. Shuyi Wang. Dr. Bernd Bodenmiller performed mass spectrometry analysis to identify sites of phosphorylation on Orm1 and Orm2 under the supervision of Dr. Ruedi Aebersold. Dr. Christer Ejsing performed mass spectrometry analysis of lipidomes from the indicated strains with the support of Dr. Andrej Shevchenko and Dr. Kai Simons. Dr. David King and the HHMI Mass Spectrometry Facility at the University of California, Berkeley identified proteins co-purifying with Orm1 and Orm2. The work described in Chapter 4, “Unpublished Data,” was performed in collaboration with Dr.

Jeremy Thorner and Dr. Françoise Roelants at the University of California, Berkeley. Dr. Roelants performed all *in vitro* analyses of Ypk1 activity and also found that mutation of *ORM1* and *ORM2* suppresses growth defects of *YPK1/2* mutants.

With the exceptions of those items listed above, the work presented in this dissertation was performed by its author, David Breslow, under the supervision of Dr. Jonathan Weissman.

# Permissions

## NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Jul 18, 2010

This is a License Agreement between David K Breslow ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

|                              |                                                                                                                                                                                |
|------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| License Number               | 2472080407188                                                                                                                                                                  |
| License date                 | Jul 18, 2010                                                                                                                                                                   |
| Licensed content publisher   | Nature Publishing Group                                                                                                                                                        |
| Licensed content publication | Nature Methods                                                                                                                                                                 |
| Licensed content title       | A comprehensive strategy enabling high-resolution functional analysis of the yeast genome                                                                                      |
| Licensed content author      | David K Breslow, Dale M Cameron, Sean R Collins, Maya Schuldiner, Jacob Stewart-Ornstein, Heather W Newman, Sigurd Braun, Hiten D Madhani, Nevan J Krogan, Jonathan S Weissman |
| Volume number                |                                                                                                                                                                                |
| Issue number                 |                                                                                                                                                                                |
| Pages                        |                                                                                                                                                                                |
| Year of publication          | 2008                                                                                                                                                                           |
| Portion used                 | Full paper                                                                                                                                                                     |
| Requestor type               | Student                                                                                                                                                                        |
| Type of Use                  | Thesis / Dissertation                                                                                                                                                          |
| Billing Type                 | Invoice                                                                                                                                                                        |
| Company                      | David K Breslow                                                                                                                                                                |
| Billing Address              | Byers Hall Room 404 MC2542<br>1700 4th St<br>San Francisco, CA 94158<br>United States                                                                                          |
| Customer reference info      |                                                                                                                                                                                |
| Total                        | 0.00 USD                                                                                                                                                                       |
| Terms and Conditions         |                                                                                                                                                                                |

### Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication)

For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME],  
advance online publication, day month year (doi: 10.1038/sj.[JOURNAL  
ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

**Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.**

**If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10816967.**

**Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.**

**Make Payment To:  
Copyright Clearance Center  
Dept 001  
P.O. Box 843006  
Boston, MA 02284-3006**

**If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2472080407188 and noting the reason for cancellation.**

**Questions? [customercare@copyright.com](mailto:customercare@copyright.com) or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.**

---

---

**NATURE PUBLISHING GROUP LICENSE  
TERMS AND CONDITIONS**

Jul 18, 2010

---

---

This is a License Agreement between David K Breslow ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

|                              |                                                                                                                                                |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| License Number               | 2472070966594                                                                                                                                  |
| License date                 | Jul 18, 2010                                                                                                                                   |
| Licensed content publisher   | Nature Publishing Group                                                                                                                        |
| Licensed content publication | Nature                                                                                                                                         |
| Licensed content title       | Orm family proteins mediate sphingolipid homeostasis                                                                                           |
| Licensed content author      | David K. Breslow, Sean R. Collins, Bernd Bodenmiller, Ruedi Aebersold, Kai Simons, Andrej Shevchenko, Christer S. Ejsing, Jonathan S. Weissman |
| Volume number                |                                                                                                                                                |
| Issue number                 |                                                                                                                                                |
| Pages                        |                                                                                                                                                |
| Year of publication          | 2010                                                                                                                                           |
| Portion used                 | Full paper                                                                                                                                     |
| Requestor type               | Student                                                                                                                                        |
| Type of Use                  | Thesis / Dissertation                                                                                                                          |
| Billing Type                 | Invoice                                                                                                                                        |
| Company                      | David K Breslow                                                                                                                                |
| Billing Address              | Byers Hall Room 404 MC2542<br>1700 4th St<br>San Francisco, CA 94158<br>United States                                                          |
| Customer reference info      |                                                                                                                                                |
| Total                        | 0.00 USD                                                                                                                                       |
| Terms and Conditions         |                                                                                                                                                |

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of

this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication)

For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME],  
advance online publication, day month year (doi: 10.1038/sj.[JOURNAL  
ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME]  
(reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the



use of this material. Thank you.

v1.1

**Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.**

**If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10816963.**

**Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.**

**Make Payment To:  
Copyright Clearance Center  
Dept 001  
P.O. Box 843006  
Boston, MA 02284-3006**

**If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2472070966594 and noting the reason for cancellation.**

**Questions? [customercare@copyright.com](mailto:customercare@copyright.com) or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.**

---

---

## Table of Contents

|                   |                                                                                    |      |
|-------------------|------------------------------------------------------------------------------------|------|
| <b>Preface</b>    | Acknowledgments                                                                    | iv   |
|                   | Abstract                                                                           | ix   |
|                   | Contributions                                                                      | xi   |
|                   | Permissions                                                                        | xiii |
|                   | Table of Contents                                                                  | xix  |
|                   | List of Tables and Figures                                                         | xx   |
|                   | List of Supplementary Materials                                                    | xxi  |
| <b>Chapter 1</b>  | Introduction                                                                       | 1    |
| <b>Chapter 2</b>  | Development of a High-Resolution Growth Assay for Yeast Functional Genomics        | 20   |
| <b>Chapter 3</b>  | Identification of Orm Family Proteins as Key Mediators of Sphingolipid Homeostasis | 77   |
| <b>Chapter 4</b>  | Unpublished data                                                                   | 126  |
| <b>Chapter 5</b>  | Summary                                                                            | 141  |
| <b>Appendix A</b> | References                                                                         | 147  |

## List of Tables and Figures

### Chapter 1

|                   |                                            |    |
|-------------------|--------------------------------------------|----|
| <b>Figure 1.1</b> | Chemical structures of select yeast lipids | 19 |
|-------------------|--------------------------------------------|----|

### Chapter 2

|                   |                                                                                                   |    |
|-------------------|---------------------------------------------------------------------------------------------------|----|
| <b>Figure 2.1</b> | Construction of a library of hypomorphic alleles of essential yeast genes using the DAmP approach | 46 |
|-------------------|---------------------------------------------------------------------------------------------------|----|

|                   |                                                                              |    |
|-------------------|------------------------------------------------------------------------------|----|
| <b>Figure 2.2</b> | A flow cytometry-based technique for high-precision growth rate measurements | 47 |
|-------------------|------------------------------------------------------------------------------|----|

|                   |                                                                                                   |    |
|-------------------|---------------------------------------------------------------------------------------------------|----|
| <b>Figure 2.3</b> | Growth rate measurements of deletion and DAmP yeast strains by the flow cytometry-based technique | 48 |
|-------------------|---------------------------------------------------------------------------------------------------|----|

|                   |                                                            |    |
|-------------------|------------------------------------------------------------|----|
| <b>Figure 2.4</b> | Utility of DAmP strains for identification of drug targets | 49 |
|-------------------|------------------------------------------------------------|----|

|                   |                                                                                                                        |    |
|-------------------|------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 2.5</b> | Highly sensitive growth rate measurements enable resolution of previously inaccessible classes of genetic interactions | 50 |
|-------------------|------------------------------------------------------------------------------------------------------------------------|----|

|                   |                                             |    |
|-------------------|---------------------------------------------|----|
| <b>Figure 2.6</b> | Functional dissection of the 26S proteasome | 51 |
|-------------------|---------------------------------------------|----|

### Chapter 3

|                   |                                                          |     |
|-------------------|----------------------------------------------------------|-----|
| <b>Figure 3.1</b> | Orm1/2 are negative regulators of sphingolipid synthesis | 100 |
|-------------------|----------------------------------------------------------|-----|

|                   |                                                                    |     |
|-------------------|--------------------------------------------------------------------|-----|
| <b>Figure 3.2</b> | Orm/Ormdl proteins form a complex with serine palmitoyltransferase | 101 |
|-------------------|--------------------------------------------------------------------|-----|

|                   |                                                      |     |
|-------------------|------------------------------------------------------|-----|
| <b>Figure 3.3</b> | <i>ORM</i> gene function is conserved in human cells | 102 |
|-------------------|------------------------------------------------------|-----|

|                   |                                                                          |     |
|-------------------|--------------------------------------------------------------------------|-----|
| <b>Figure 3.4</b> | Orm1/2 are regulated in response to disruption of sphingolipid synthesis | 103 |
|-------------------|--------------------------------------------------------------------------|-----|

|                   |                                                                             |     |
|-------------------|-----------------------------------------------------------------------------|-----|
| <b>Figure 3.5</b> | Mutation of phosphorylated Orm1/2 residues impairs sphingolipid homeostasis | 104 |
|-------------------|-----------------------------------------------------------------------------|-----|

### Chapter 4

|                   |                                                            |     |
|-------------------|------------------------------------------------------------|-----|
| <b>Figure 4.1</b> | Deletion of <i>ORM1/2</i> suppresses <i>ypk1/2</i> mutants | 138 |
|-------------------|------------------------------------------------------------|-----|

|                   |                                  |     |
|-------------------|----------------------------------|-----|
| <b>Figure 4.2</b> | Ypk1 phosphorylates Orm proteins | 139 |
|-------------------|----------------------------------|-----|

|                   |                                                                           |     |
|-------------------|---------------------------------------------------------------------------|-----|
| <b>Figure 4.3</b> | Ypk1 is activated upon myriocin treatment in an Fpk1/2-independent manner | 140 |
|-------------------|---------------------------------------------------------------------------|-----|

## List of Supplementary materials

### Chapter 2

|                       |                                                                                             |        |
|-----------------------|---------------------------------------------------------------------------------------------|--------|
| <b>Supp. Fig. 2.1</b> | Strategy used for DAmP library construction                                                 | 52     |
| <b>Supp. Fig. 2.2</b> | Normalization of fitness measurements                                                       | 53     |
| <b>Supp. Fig. 2.3</b> | Simulated versus observed distribution of differences between duplicate growth measurements | 54     |
| <b>Supp. Fig. 2.4</b> | Effect of simulated error on the apparent number of deletion strains with growth defects    | 55     |
| <b>Supp. Fig. 2.5</b> | Effects of gene deletions on growth are correlated with expression levels                   | 56     |
| <b>Supp. Fig. 2.6</b> | Relationship between growth defect and participation in the environmental stress response   | 57     |
| <b>Supp. Fig. 2.7</b> | Genetic interactions between proteasome genes                                               | 58     |
| <b>Supp. Tbl. 2.1</b> | Relative growth rates for TAP, TAP-DAmP and TAP-degron+DAmP alleles                         | 59     |
| <b>Supp. Tbl. 2.2</b> | Estimated true and false positive rates for identification of strains with growth defects   | 60     |
| <b>Supp. Note</b>     | Discussion of previously detected deletion strains with observable growth defects           | 61     |
| <b>Supp. Methods</b>  | Detailed methods                                                                            | 62     |
| <b>Supp. Data 2.1</b> | List of primers used in this study                                                          | online |
| <b>Supp. Data 2.2</b> | Growth rate measurements for flow cytometry assay validation                                | online |
| <b>Supp. Data 2.3</b> | DAmP library growth rates measured by flow cytometry                                        | online |
| <b>Supp. Data 2.4</b> | Deletion library growth rates measured by flow cytometry                                    | online |

## List of Supplementary materials cont.

### Ch. 2 cont.

|                       |                                                            |        |
|-----------------------|------------------------------------------------------------|--------|
| <b>Supp. Data 2.5</b> | Growth rate measurements used for genetic interaction data | online |
|-----------------------|------------------------------------------------------------|--------|

### Chapter 3

|                       |                                                                                                                        |        |
|-----------------------|------------------------------------------------------------------------------------------------------------------------|--------|
| <b>Supp. Fig. 3.1</b> | A functional role for Sac1 in sphingolipid metabolism                                                                  | 105    |
| <b>Supp. Fig. 3.2</b> | <i>ORMDL</i> gene expression after RNAi treatment                                                                      | 106    |
| <b>Supp. Fig. 3.3</b> | Myriocin treatment does not prevent formation of the Orm1/2-Lcb1/2-Sac1 complex                                        | 107    |
| <b>Supp. Fig. 3.4</b> | Orm2 localization changes in response to myriocin treatment                                                            | 108    |
| <b>Supp. Fig. 3.5</b> | Disruptions to sphingolipid synthesis that act downstream of Lcb1/2 but upstream of Aur1 induce Orm1/2 phosphorylation | 109    |
| <b>Supp. Fig. 3.6</b> | Mass spectrometry identifies phosphorylated residues on Orm1 and Orm2                                                  | 110    |
| <b>Supp. Fig. 3.7</b> | Wild-type and phospho-mutant forms of Orm1 and Orm2 show similar expression levels                                     | 111    |
| <b>Supp. Tbl. 3.1</b> | Orm-associated proteins identified by mass spectrometry                                                                | 112    |
| <b>Supp. Methods</b>  | Detailed methods                                                                                                       | 113    |
| <b>Supp. Data 3.1</b> | EMAP and genetic interaction data for selected genes                                                                   | online |
| <b>Supp. Data 3.2</b> | Yeast and HeLa cell lipidomic data                                                                                     | online |

# **Chapter 1**

## Introduction

## Introduction

The publication of the complete genome sequence of the budding yeast *Saccharomyces cerevisiae* in 1997 provided researchers with the first DNA blueprint of a eukaryotic organism and marked the beginning of a new era in biology research<sup>1</sup>. With the full sequence of every yeast gene, it was possible for the first time to address long-standing questions in cellular and evolutionary biology. A great surprise, however, was the finding of 2,000 yeast genes of unknown function and with no homologs of known function<sup>2</sup>. A major challenge since that time in both yeast and more complex organisms is to identify the cellular functions of each gene product encoded by the genome and to understand how these gene products act in concert to carry out complex biological processes. A number of powerful strategies have recently been developed that have greatly improved our ability to meet this challenge. One such approach is based on the large-scale measurement and analysis of genetic interactions<sup>3</sup>.

Genetic interaction describes a phenomenon in which simultaneous mutation of two genes results in a phenotype that would not have been predicted based upon the phenotypes caused by mutation of the genes individually<sup>4</sup>. There are two broad classes of genetic interactions: positive (or ‘alleviating’) genetic interactions, in which the double-mutant phenotype is milder than expected, and negative (or ‘synthetic’) genetic interactions, in which the double-mutant phenotype is more severe than expected. A number of quantitative metrics for the identification and measurement of genetic interactions have been proposed<sup>4,5</sup>. In one such formula, the interaction score,  $\epsilon$ , for genes A and B is equal to the difference between the observed double-mutant phenotype,  $W_{AB}$ , and the expected double-mutant phenotype, which is given by the product of the

single-mutant phenotypes  $W_A$  and  $W_B$  (thus,  $\varepsilon = W_{AB} - W_A \times W_B$ )<sup>5</sup>. Thus, by measuring the effects of single and double gene deletions on a phenotype of interest such as rate of growth, genetic interaction scores can be derived for any gene pair.

Although genetic interactions are rare, theoretical considerations and empirical studies have established the utility of genetic interactions in defining functional relationships between genes<sup>5-9</sup>. Early studies focused on negative interactions, ( $\varepsilon < 0$ ), as these tend to occur between genes participating in closely related cellular processes<sup>5,6,8,9</sup>. More recently, positive interactions ( $\varepsilon > 0$ ) have proven highly valuable, as they often occur between genes that have very closely related functions—for example, between members of the same protein complex or biochemical pathway<sup>5-7</sup>.

In the past decade, systematic measurement of genetic interactions has been used in budding yeast to obtain precise information on gene function on a genomic scale<sup>7-12</sup>. A major advantage of these studies is that large genetic interaction datasets provide an additional metric that can be used to ask if two genes of interest share a functional relationship. This additional metric is complementary to the direct interaction score and is based on the fact that, for each gene, genetic interactions are measured with hundreds of other genes, thus providing a functional signature for each gene. Comparison of two genes' signatures (for example, by calculating the correlation of their genetic interaction scores with all other genes in the dataset) has proven very useful in identifying functional relationships, as genes with highly correlated genetic interaction patterns often have very similar cellular functions<sup>7,13</sup>. This strategy has been used to reveal the functions of many previously uncharacterized genes, including the roles of *GET1/2/3* in tail-anchor protein biogenesis, of *YJL097W/PHS1* in very long chain fatty acid synthesis, and of *RTT109*, in



histone acetylation<sup>7,11,14,15</sup>.

The generation of large genetic interaction datasets in yeast has been enabled by a number of recent technological advances. First, a collaborative effort has created a collection of nearly 5000 yeast strains in which each non-essential gene has been deleted<sup>16,17</sup>. Additionally, hypomorphic alleles have been generated for many essential genes (for example, DAmP alleles that use mRNA destabilization to reduce gene expression)<sup>7,18-21</sup>. Second, new methods have emerged for the high-throughput construction and phenotypic analysis of double-mutant strains<sup>9,22</sup>. The predominant phenotypic read-out used has been rate of growth, as this property can be readily measured for each strain, does not require that genes participate in a particular cellular process, and has direct relevance to evolutionary considerations. Two major methods have been developed for the high-throughput measurement of growth rates (and thus genetic interactions) in *S. cerevisiae*. In one approach, strains of interest are robotically transferred in high-density arrays onto agar plates, and colony sizes are determined by automated image analysis after a defined period of growth<sup>7-9</sup>. The second approach takes advantage of the fact that each deletion strain is marked with a unique DNA barcode that can be read out on DNA microarrays. It is then possible to quantify the abundance of each deletion strain in a pooled culture of all deletion strains before and after a defined period of growth or following a perturbation of interest<sup>12,22,23</sup>.

However despite these advances and the success of recent studies, several considerations indicate that more precise measurement of growth phenotypes would improve the usefulness of genetic interactions maps. First, genetic interactions are rare, with only ~1% of randomly chosen gene pairs exhibiting a genetic interaction (even in

biased gene sets, only ~3% of gene pairs interact)<sup>7,8,10</sup>. This rarity demands precise growth measurements so that *bona fide* interactions can be distinguished from measurement error within the much larger number of non-interacting gene pairs.

Second, many genetic interactions correspond to small differences between observed and expected double-mutant growth rate that are difficult to measure accurately and reproducibly. This difficulty stems in large part from the fact that many yeast single-gene deletions result in small growth phenotypes. Indeed, several studies using the yeast non-essential gene deletion library identified statistically significant growth defects for a surprisingly small fraction of gene deletions (~20%)<sup>16,24,25</sup>. From a theoretical standpoint, this result was unexpected; the retention of these genes throughout natural selection implies that their loss is detrimental. A number of hypotheses to account for this apparently high level of gene dispensability have been put forward, including genetic redundancy and condition-specific roles for genes whose deletion had no effect under standard growth conditions<sup>26-28</sup>. Both of these explanations likely account for some of the unexpected gene dispensability but are difficult to reconcile with the fact that nearly 80% of genes are expressed under standard growth conditions, which implies that they are beneficial to the cell<sup>29</sup>. On a more practical level, the fact that many gene deletions have little to no observable effect on growth makes the detection of positive genetic interactions a significant challenge. Indeed if the growth defect caused by a given mutation is small, it is quite difficult to tell if this small effect is diminished by the introduction of a second mutation.

A third reason that more precise growth measurements are needed is that differences in the magnitude of genetic interaction scores can be functionally informative.

For example, different values for  $\varepsilon$  can indicate three different types of positive genetic interaction, in which the effect of a given mutation may be partially reduced, completely masked, or even suppressed by the presence of a second mutation. It is important to note that each of these three scenarios suggests a different functional relationship between the two genes in question. Segrè *et al.* recently addressed this issue by introducing an improved method for calculating genetic interaction scores<sup>5</sup>. This approach normalizes the raw interaction score (as calculate above) by the interaction score expected in the case of a completely masking interaction ( $\varepsilon_{NORM} = \varepsilon / [\min(W_A, W_B) - W_A \times W_B]$ ). Thus, raw interaction scores ( $\varepsilon$ ) are re-scaled such that  $\varepsilon_{NORM} = 1.0$  for complete masking or alleviation,  $\varepsilon_{NORM} = 0$  for no interaction,  $0 < \varepsilon_{NORM} < 1$  for partial alleviation, and  $\varepsilon_{NORM} > 1$  for suppression<sup>5</sup>. However, the differences between these types of positive genetic interactions often correspond to very small differences in growth rate. As a result, initial efforts to use this normalization have had limited success and called into question the value of this metric<sup>30</sup>.

To address these challenges in applying genetic interaction measurements to functional genomics, I developed a high-throughput assay for growth rate measurements in yeast that offers significantly improved precision over existing methods. In this method, wild-type and mutant strains are marked by the expression of different fluorescent proteins. The strains are then grown in competition with each other in a co-culture format, and the fraction of the culture represented by each genotype is monitored over time by flow cytometry. Changes in the composition of the co-culture are used to calculate the relative growth rate of the mutant strains and result in a typical error of <0.5% - a significant improvement over previous methods. Importantly, these growth

measurements can also be carried out in high-throughput.

With this improved assay, I first measured the growth rates >4000 non-essential gene deletion strains and a new collection of ~1000 DAmP alleles of essential genes. Notably, we found a significant growth defect for a considerably larger fraction of deletion strains than previously reported. This result suggests that there is not a high level of gene dispensability in yeast and indicates instead that most genes do contribute to robust growth, even under a single experimental condition. For many gene deletions, the effect on growth can only be detected with precise measurements, but such growth differences would have a strong effect on an evolutionary timescale. The flow cytometry-based assay also made it possible to detect genetic interactions missed in previous studies and to resolve functionally significant differences in genetic interaction magnitude. Using the normalized interaction score metric, different types of alleviating genetic interactions were readily distinguished, leading to novel functional insights for chromatin modifying enzymes and the 26S proteasome. Thus, this novel methodology has the potential to significantly increase the utility of quantitative genetic approaches to functional genomics in yeast.

The second part of my dissertation concerns the functional characterization of members of the *ORM* gene family. *ORM* genes are conserved from yeast to man and include two isoforms in *S. cerevisiae* (*ORM1* and *ORM2*) and three isoforms in humans (*ORMDL1-3*)<sup>31</sup>. Prior to the work described below, little was known about the function of this gene family: they were reported to encode trans-membrane proteins localized to the Endoplasmic Reticulum (ER) but had no characterized protein domains<sup>31</sup>. However,

the strong growth defect upon deletion of both yeast *ORM* genes and their evolutionary conservation together suggested that these genes were likely to have a fundamental cellular role<sup>7,31</sup>.

Interest in the *ORM* gene family was recently stimulated by the identification of a genetic risk factor for childhood asthma that is tightly linked to the human *ORMDL3* gene<sup>32</sup>. Subsequent studies verified the association of single nucleotide polymorphisms (SNPs) at this locus with asthma and indicated that these variants may account for ~20% of cases of this common disease<sup>33-37</sup>. Although these findings have generated substantial enthusiasm in the asthma genetics community, without knowing the function of *ORM* genes it is difficult to determine if and how *ORMDL3* participates in asthma pathogenesis. Indeed, the uncharacterized nature of the *ORM* gene family highlights in specific detail a general need for improved functional genomic strategies. This need is made particularly acute by the rapid recent advances in human genetics. Over the past few years, powerful new genome-wide association studies have been used to scan the human genome for genetic risk factors for virtually all major diseases<sup>38</sup>. These studies are identifying new candidate disease genes at an unprecedented rate, but unfortunately this rate far exceeds our current ability to functionally characterize these genes. Consequently, genome-wide association studies have yielded relatively little insight into how disruptions of specific genes and pathways give rise to disease states<sup>39</sup>.

Thus *ORM* genes emerged as a prototypical case for using functional genomics to understand the cellular role of a novel gene family. I therefore turned to systematic measurements of genetic interactions to gain initial clues. As described below, the genetic interactions exhibited by strains with altered *ORM1/2* activity suggested a role for

these genes in sphingolipid metabolism.

Sphingolipids are a diverse, abundant and essential class of lipids with key functions both as unique structural components of membranes and as intra- and extra-cellular signaling molecules. Recent work has elucidated the (apparently) complete enzymatic machinery responsible for sphingolipid synthesis, with especially important contributions coming from genetic screens in yeast that identified the roles of *TSC* (Temperature-sensitive Suppressor or *CSG2*) and *SUR* (Suppressor of *RVS161* and *RVS167*) genes in sphingolipid metabolism<sup>40,41</sup>. Sphingolipid synthesis begins in the ER with the condensation of serine with a fatty acid coenzyme A (CoA) conjugate (for an overview see Figures 1.1 and 3.1d)<sup>42,43</sup>. This reaction is catalyzed by the enzyme serine palmitoyltransferase (encoded by *LCB1* and *LCB2* in yeast), and, after a reduction step mediated by *TSC10*, yields key intermediates known as long chain bases (LCBs). These LCBs can be phosphorylated to yield LCB phosphates (LCB-Ps; such as sphingosine-1-phosphate), or they can be condensed with a fatty acid CoA conjugate (often with a very long alkyl chain) to produce ceramides. Ceramides are then transported by vesicular and non-vesicular means<sup>44</sup> (in mammalian cells, by the ceramide transporters CERT and FAPP2<sup>45,46</sup>) to the Golgi apparatus, where species-specific glycosylation at the lipid head group position occurs. Ultimately, sphingolipids continue to move through the secretory pathway to the plasma membrane, where they are most abundant<sup>47</sup>. It should also be noted that the enzymes that carry out the forward synthetic steps in the pathway are opposed by a complementary set of enzymes that carry out the reverse reactions.

Sphingolipids are distinguished from most other lipids by their use of serine rather than glycerol as the backbone to which acyl chains are attached and by the tendency of

these acyl chains to be long and saturated (Figure 1.1). In model lipid bilayers, these unique features impact the order of the lipid phase, membrane thickness, and membrane curvature<sup>47,48</sup>. Sphingolipids can also act together with sterol lipids to form laterally distinct macroscopic domains in mixed model membranes. Unique physical properties have also been observed for sphingolipids (in concert with sterols) in cellular membranes<sup>47,49</sup>. *In vivo*, these lipid-lipid associations are likely complemented by specific interactions between membrane proteins and lipids<sup>47,48,50</sup>; together these forces can modulate membrane curvature<sup>51,52</sup> and create lipid nanodomains, or rafts, in cellular membranes<sup>47</sup>. These sphingolipid-mediated intra-membrane interactions are proposed to organize intra-cellular transport of lipids and proteins and to modulate the activity of key signaling proteins in the membrane<sup>47,48</sup>.

In addition to their unique effects on membrane properties, sphingolipids have a number of key cellular signaling functions<sup>53</sup>. Recent work has defined an increasingly diverse set of proteins that bind to and are regulated by sphingolipid metabolites such as sphingosine, sphingosine-1-phosphate (S1P), and ceramide<sup>54</sup>. In mammalian cells, extra-cellular S1P that has been released from cells by efflux pumps acts as a high-affinity ligand for a family of G-protein coupled receptors encoded by the genes *S1PR1-5*<sup>53,55,56</sup>. Signaling via S1PR1-5 has been shown to modulate a variety of processes including immune cell trafficking, vascular permeability, inflammation, and cell survival<sup>53,54</sup>. Intra-cellular S1P is also thought to have signaling roles, although intra-cellular S1P targets are only now beginning to emerge. Notably, Sarah Spiegel's group has recently identified S1P as a novel ligand that regulates histone deacetylase activity in the nucleus<sup>57</sup>. Interestingly, *RPD3* and *SIN3*, which encode the yeast homologs of the S1P-

regulated deacetylase, were previously found as *TSC* genes by Beeler *et al.*<sup>40</sup>, raising the possibility that this signaling function is conserved. Sarah Spiegel's group has also recently reported that S1P activates TRAF2, a ubiquitin ligase that acts in the TNF/NF- $\kappa$ B pathway<sup>58</sup>.

Ceramide is another key signaling sphingolipid with roles in apoptosis<sup>59</sup>, cell cycle arrest<sup>60</sup>, and response to stress<sup>61-64</sup>. The role of ceramide in apoptosis has been studied in detail, although the precise mechanism by which it induces programmed cell death remains unknown<sup>59,61,65</sup>. Interestingly, ceramide's pro-apoptotic function is opposed by the pro-survival effects of S1P signaling<sup>66</sup>. As ceramide and S1P are also metabolically connected, this finding has led to the proposal of a ceramide/S1P rheostat that underlies sensitivity to cell survival and cell death signals<sup>67</sup>. Finally, it is important to note that even specific sub-classes of sphingolipids can comprise many distinct molecular entities that may have different or even opposing functions. For example, mutation of a long-chain ceramide synthase conferred resistance to anoxic stress in *C. elegans*, whereas disruption of a short-chain isozyme had the opposite effect<sup>68</sup>.

Despite these insights into the signaling functions of sphingolipids and despite great progress in identifying the enzymatic machinery required for their synthesis and degradation, much less is known how cells sense and regulate sphingolipid levels. Sphingolipid metabolism is likely to be tightly controlled however, given the critical roles of sphingolipids in determining the physical properties of membranes and in the aforementioned signaling pathways. Broadly speaking, one would expect that pathways for sphingolipid homeostasis are likely to include components that sense physiologically relevant variations in the levels of sphingolipid metabolites and use this information to



control the activity of enzymes that participate in sphingolipid synthesis and degradation. This basic framework though leaves a number of important questions unanswered. How would a sphingolipid sensor work at a mechanistic level? What would be the most useful sphingolipid metabolite(s) for cells to sense and respond to? What steps in the metabolic pathway require tight regulation and how might cells efficiently translate information about sphingolipid levels into changes in metabolic output? Consideration of a few pathways that regulate other lipid classes, such as sterols and glycerolipids, suggest the possible form of the answers to these questions.

In the case of sterol homeostasis, a number of classic studies have elucidated the numerous transcriptional and post-translational mechanisms that cells use to match sterol production to demand. This pathway is comprised of multiple sterol-binding sensors that regulate proteolytic degradation of HMG-CoA-reductase, a key enzyme in sterol biosynthesis, and that regulate trafficking-dependent proteolytic release of the transcription factor SREBP from the ER membrane so that it can activate gene expression in the nucleus<sup>69,70</sup>. SREBP target genes include a number of sterol pathway components, including the LDL receptor that mediates cholesterol uptake and HMG-CoA-reductase<sup>71</sup>. Furthermore, HMG-CoA-reductase activity is further regulated by sterol-dependent phosphorylation<sup>72</sup>, and other sterol biosynthetic intermediates also regulate HMG-CoA-reductase degradation<sup>70</sup>. Notably, in sterol metabolism, key regulators in the pathway were shown to directly bind and sense the lipids in question<sup>73</sup>.

Similar cases of direct lipid sensors are also seen in glycerolipid homeostasis. Specifically, Loewen *et al.* identified a feedback pathway in which the localization of Opi1, a key transcriptional repressor of the yeast enzymes for glycerolipid and inositol

production, is dynamically regulated in response to glycerolipid levels<sup>74</sup>. When lipid synthesis is disrupted (for example, by inositol starvation), Opi1 is inactive because it is retained on the ER membrane via binding to accumulated phosphatidic acid; when lipid synthesis is normal, phosphatidic acid levels are low (owing to its rapid conversion to downstream glycerolipids such as phosphatidylinositol) and Opi1 can leave the ER and act in the nucleus to repress its target genes<sup>74</sup>. In bacteria, an even more direct feedback mechanism has been elucidated for transcriptional control of the ratio of saturated to unsaturated fatty acids. In this case, a transcriptional repressor that regulates expression of enzymes required for production of unsaturated fatty acids directly binds to both saturated and unsaturated fatty acids, but with opposite transcriptional outcomes<sup>75</sup>. Saturated fatty acid binding promotes release of the repressor from DNA, whereas unsaturated fatty acids have the opposite effect. Crystallographic studies have recently elucidated the conformational changes upon saturated and unsaturated fatty acid binding and thus provide a detailed view of how bacteria can sense the ratio of saturated to unsaturated fatty acids<sup>76</sup>.

In contrast to the direct lipid sensors seen in the aforementioned examples, Kurat *et al.* have recently identified an indirect mechanism that controls yeast glycerolipid synthesis, in which cell cycle progression is linked to cyclin-dependent-kinase mediated activation of Tgl4<sup>77</sup>. Tgl4 is a lipase that mobilizes fatty acids stores in lipid droplets for glycerolipid synthesis, and thus its activation provides the raw material needed for *de novo* membrane synthesis during bud formation and growth. Another twist on lipid sensing is seen in the mechanism of SREBP regulation in *Drosophila melanogaster*, in which the pathway has been re-purposed to sense phosphatidylethanolamine (PE) and

control phospholipid synthesis<sup>78</sup>. Although the mechanism of PE sensing by *Drosophila* Scap remains unclear, one hypothesis is that changes in PE are detected via effects on bulk membrane order rather than by a sensor with a discrete lipid ligand<sup>78</sup>. Collectively, the above examples reveal direct and indirect strategies to sense the need for lipid synthesis and a variety of mechanisms to modulate lipid production at both transcriptional and post-transcriptional levels.

What then is known about mechanisms for sensing and regulating sphingolipid levels? First, there is evidence that sphingolipid synthesis is dependent on the levels of the key substrates serine and palmitoyl-CoA. The affinity of yeast serine palmitoyltransferase for these species is very similar to their physiologic concentrations in yeast cells, such that changes in intracellular levels of these substrates are likely to directly affect sphingolipid output (for example, in response to heat shock)<sup>79</sup>. Two additional regulators of sphingolipid synthesis were discovered through efforts to identify enzymes that produce sphingolipids. For example, Aronova *et al.* found that the TORC2 kinase complex is required for maximal ceramide synthase activity and is opposed by the phosphatase calcineurin<sup>80</sup> by pursuing the initial finding of Beeler *et al.* that two genes encoding TORC2 components are *TSC* genes<sup>40</sup>. However, what factors control the balance of TORC2 and calcineurin activity and by what mechanism these enzymes impact ceramide synthase activity are still unclear. Similarly, Vacaru *et al.* were prompted to identify SMSr as a regulator of ceramide production because SMSr is homologous to the known sphingomyelin (SM) synthases SMS1 and SMS2<sup>81</sup>. However, unlike other SM synthases, SMSr lacks the ability to produce SM and instead produces ceramide phospho-ethanolamine (CPE), but only in trace amounts<sup>81</sup>. Surprisingly,

knockdown of SMSr by RNA interference (RNAi) causes a substantial accumulation of ceramide that cannot be accounted for by SMSr's rather modest ability to convert ceramide to CPE<sup>81</sup>. Instead, it is proposed that SMSr may sense ceramide levels to control their production, although further work will be required to clarify if SMSr acts as a sensor and how it regulates sphingolipid synthesis.

There is also preliminary evidence that the sphingolipid synthesis rate is adjusted in response to sphingolipid supply. In cultured cerebellar neurons, growth in the presence of exogenous LCBs was found to suppress incorporation of radio-labeled serine into sphingolipids<sup>82</sup>. This effect was correlated with reduced *in vitro* activity of serine palmitoyltransferase, the first and rate-limiting enzyme in *de novo* sphingolipid synthesis<sup>83</sup>. However, the mechanistic basis for and significance of this regulation remains unknown. A more convincing example of apparent feedback regulation is seen in CERT-mediated transport of ceramide from the ER to the Golgi in mammalian cells. In this case, CERT activity is inhibited when it is phosphorylated by casein kinase<sup>84,85</sup>. Interestingly, CERT is normally partially phosphorylated but was found to become dephosphorylated when sphingolipid levels were reduced (either by external application of recombinant sphingomyelinase to degrade plasma membrane sphingolipids or when sphingolipid synthesis was blocked by treatment with myriocin, a potent small molecule inhibitor of serine palmitoyltransferase<sup>86</sup>)<sup>84</sup>. These results imply that cells can sense sphingolipid levels and that de-phosphorylation of CERT may provide a useful mechanism to respond to disruptions in sphingolipid production. However, the physiologic significance of feedback regulation of CERT and whether sphingolipid synthesis is regulated in a similar fashion to CERT-mediated transport require further

investigation.

While mechanisms that ensure sphingolipid homeostasis remain unclear, there has been notable recent progress in identifying sphingolipid-dependent signaling pathways. In yeast, the best understood of these pathways center around the AGC family kinases Ypk1/2 and Pkh1/2<sup>43,87</sup>. Ypk1/2 are phosphorylated by Pkh1/2 and TORC2<sup>88-91</sup>, and Ypk1 and Pkh1 are further implicated in sphingolipid metabolism as they confer resistance to the serine palmitoyltransferase inhibitor myriocin<sup>91,92</sup>. It has been reported that these Ypk and Pkh kinases are directly activated by LCBs<sup>93,94</sup>. Additionally, a recent study from Fröhlich *et al.* indicates that Pkh1/2 activity is also sensitive to plasma membrane sphingolipid levels<sup>95</sup>. Fröhlich *et al.* found that the yeast transmembrane protein Nce102 dynamically localizes to membrane domains known as eisosomes in a sphingolipid-dependent manner<sup>95</sup>. Disruption of sphingolipid synthesis causes Nce102 to exit eisosomes, leading to de-repression of the Pkh1/2 kinases<sup>95</sup>. Although how Nce102 controls Pkh1/2 activity remains unclear, this study provides an example of an alternative mechanism that can be used to sense sphingolipid levels – namely reversible protein localization to sphingolipid-dependent membrane domains such as eisosomes.

Finally, when considering sphingolipid metabolism, it is important to note that sphingolipids are metabolically and functionally connected to other lipid classes. For example, sphingolipid degradation products can be used to synthesize PE, and thus sphingosine and ceramide levels can regulate the activity of *Drosophila* SREBP<sup>78</sup>. A link between glycerolipids and sphingolipids is also indicated by the recent finding that alterations to sphingolipid synthesis affect phosphorylation of the Fpk1/2 kinases<sup>96</sup>. Fpk1/2 in turn regulate glycerolipid flippases, indicating that sphingolipid levels and

phospholipid asymmetry in the plasma membrane may be coordinately regulated<sup>96</sup>. An additional link between glycerolipids and sphingolipids is seen in the case of the *Drosophila melanogaster* ceramide synthase homolog *schlank*, which was recently found to regulate lipogenesis and lipolysis of triacylglycerides at an organismal level<sup>97</sup>.

Given the functional connections between sterols and sphingolipids, there is also strong evidence of inter-connected regulatory mechanisms for these two lipid types. For example, sphingomyelinase treatment has been shown to inhibit SREBP activity and sterol synthesis in mammalian cells<sup>98</sup>. Additionally, the same study that characterized regulation of CERT in response to myriocin treatment found that sterol depletion induces the same response<sup>84</sup>. Recent reports also provide preliminary evidence that CERT, OSBP (a sterol-sensing protein), and VAP (an ER protein bound by both CERT and OSBP) act together to increase SM synthesis upon exposure of cells to oxysterols that are sensed by OSBP<sup>99</sup>. Lastly, a recent report from Howard Riezman's group identified functional connections between sphingolipids and sterols in yeast<sup>100</sup>. Using genetic and lipidomic approaches, many genetic interactions between sphingolipid and sterol genes were found. Furthermore, mutations to either the sterol or sphingolipid pathways often affected levels of the other lipid class, thus suggesting that these pathways are co-regulated<sup>100</sup>. Consistent with this idea, yeast *LCB1* and *LCB2* are both targets of Upc2 and Ecm22, the major sterol-regulated transcription factors that activate a number of yeast sterol biosynthetic enzymes<sup>101</sup>. More work will be required however to elucidate the detailed mechanisms by which sphingolipid and sterol metabolism are linked.

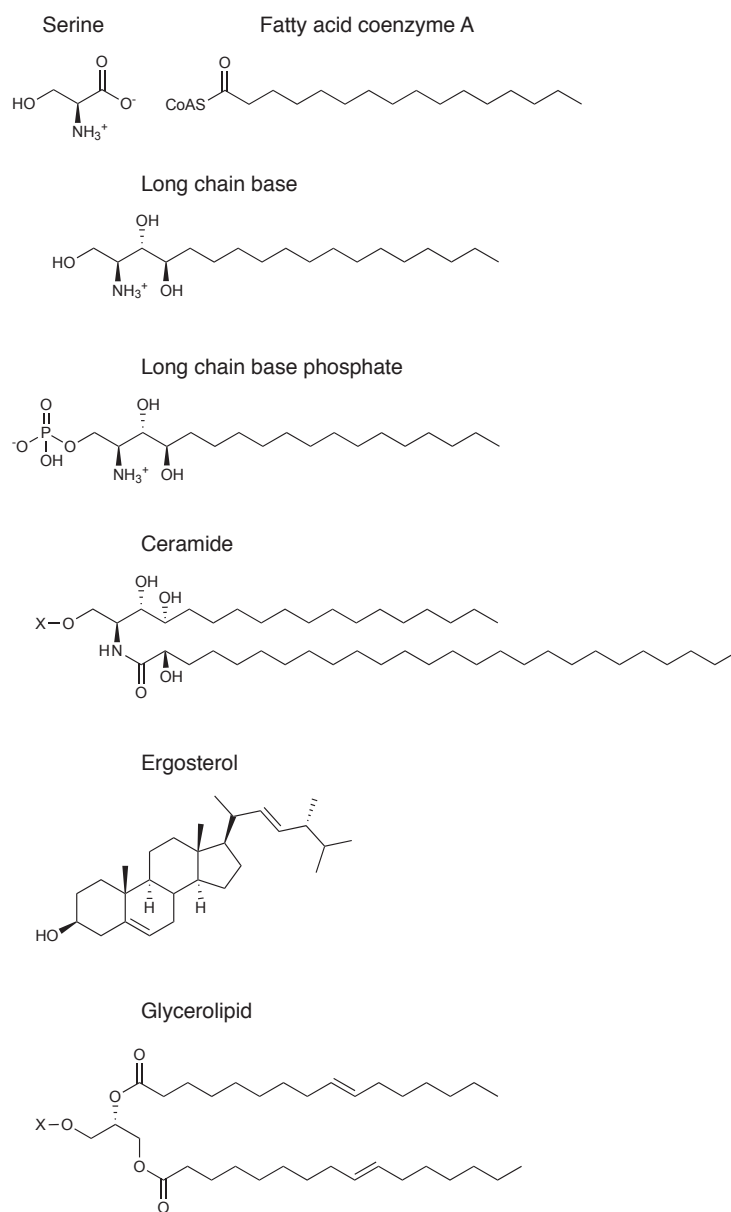
In summary, our understanding of sphingolipid metabolism has grown greatly in recent years. The enzymatic machinery that produces sphingolipids has been identified,

and signaling pathways that are regulated by sphingolipids or that regulate sphingolipid metabolism are now emerging. However, despite these advances, little is known about how cells ensure sphingolipid homeostasis. As described below, I have found that *ORM* genes provide a critical mechanism to match sphingolipid production to cellular demand.

Beginning from yeast genetic interaction data, I found that *ORM* genes encode negative regulators of sphingolipid synthesis. Over-expression of *ORM* genes produces a genetic interaction signature that is highly correlated with that seen for mutations that reduce serine palmitoyltransferase activity, whereas loss of *ORM* genes has the opposite relationship. I also found that Orm proteins form a conserved complex with serine palmitoyltransferase and the phosphoinositide phosphatase Sac1, which we termed the SPOTS complex. As expected for negative regulators of Lcb1/2, loss of Orm proteins causes toxic over-production of sphingolipids, while *ORM* gene over-expression reduces sphingolipid levels.

The activity of Orm proteins is in turn regulated by phosphorylation, which occurs in response to changing sphingolipid levels. Specifically, phosphorylation by the kinase Ypk1 relieves the inhibitory activity of Orm proteins when sphingolipid production is disrupted (for example, by myriocin treatment). This compensatory feedback mechanism matches sphingolipid supply to cellular demand, with changes in *ORM* gene expression or mutations to their phosphorylation sites leading to dysregulation of sphingolipid metabolism. Thus, Orm proteins serve a key role in sphingolipid homeostasis. In light of the conservation within this gene family and the identification of *ORMDL3* as a potential risk factor for childhood asthma, these findings raise the testable hypothesis that sphingolipid misregulation contributes to asthma development.

Figure 1.1



**Figure 1.1. Chemical structures of select yeast lipids.**

Structures of representative yeast lipids are shown for select sphingolipid metabolites and for major sterol and glycerolipid species. 'X' indicates variable lipid head group structures.



## **Chapter 2**

Development of a High-Resolution Growth Assay for Yeast Functional Genomics

# **A Comprehensive Strategy Enabling High Resolution Functional Analysis of the Yeast Genome**

David K. Breslow<sup>1,2,3,4\*</sup>, Dale M. Cameron<sup>1,2,3\*</sup>, Sean R. Collins<sup>1,2,3</sup>, Maya Schuldiner<sup>1,2,3</sup>, Jacob Stewart-Ornstein<sup>1</sup>, Heather W. Newman<sup>5</sup>, Sigurd Braun<sup>2,5</sup>, Hiten D. Madhani<sup>2,5</sup>, Nevan J. Krogan<sup>1,2</sup> and Jonathan S. Weissman<sup>1,2,3</sup>.

\*These authors contributed equally to this work

1. Department of Cellular and Molecular Pharmacology,
  2. The California Institute for Quantitative Biomedical Research,
  3. Howard Hughes Medical Institute,
  4. Graduate Program in Chemistry and Chemical Biology,
  5. Department of Biochemistry and Biophysics,
- University of California, San Francisco  
1700 4<sup>th</sup> Street  
San Francisco, California 94158, USA

Correspondence should be addressed to Jonathan S. Weissman, [weissman@cmp.ucsf.edu](mailto:weissman@cmp.ucsf.edu)

## **Abstract**

**Functional genomic studies in *S. cerevisiae* have contributed enormously to our understanding of cellular processes. Their full potential, however, has been hampered by the limited reagents to systematically study essential genes and the inability to quantify the small effects of most gene deletions on growth. We describe the construction of a library of hypomorphic alleles of essential genes and a high-throughput growth competition assay to measure fitness with unprecedented sensitivity. These tools dramatically increase the breadth and precision with which quantitative genetic analysis can be performed in yeast. We illustrate the value of these approaches by using genetic interactions to reveal new relationships between chromatin modifying factors and to create a functional map of the proteasome. Finally, by measuring the fitness of strains in the yeast deletion library, we address an outstanding enigma regarding the apparent prevalence of gene dispensability, as we find that most genes do contribute to growth.**

## Introduction

A fundamental challenge of the post-genomic era is to assign functions to genes and to understand how gene networks are organized to execute diverse cellular processes. The budding yeast *Saccharomyces cerevisiae* has served as a premier eukaryotic model system for this task<sup>3,7,9,16,22</sup>, and a critical tool for these efforts has been a library of strains in which each non-essential yeast gene is deleted<sup>16</sup>. This resource has made it possible to assess the contribution of each non-essential gene to any measurable phenotype.

These genomic approaches can be extended by systematically measuring genetic interactions<sup>3</sup>. Genetic interactions describe how the phenotype associated with compromising one gene is modulated by perturbing a second gene. Such effects can be defined quantitatively as the difference between the observed magnitude of a phenotype for a double mutant and that expected if the genes do not interact, as given by:  $\varepsilon = W_{AB} - W_A \times W_B$ , where  $\varepsilon$  is the interaction score and  $W_X$  refers to the phenotype seen in genetic background  $X$ <sup>4,5</sup>. Theoretical considerations and empirical experience have established the utility of genetic interactions in defining functional relationships between genes<sup>5-7,9</sup>. Early studies focused on "synthetic sick/lethal" or "negative" interactions<sup>9</sup>, in which the phenotype associated with perturbing two genes simultaneously is more severe than expected given the phenotypes of the individual gene perturbations ( $\varepsilon < 0$ ). More recently, interactions in which the phenotype of the double-mutant is less severe than expected ( $\varepsilon > 0$ , "alleviating" or "positive" interactions) have proven highly valuable, as they often occur between genes that have closely related functions—for example, between members of the same complex or pathway<sup>7,11</sup>.

Functional genomic studies have been hampered, however, by two shortcomings of the existing technologies. The first of these is the limited precision of many techniques for high-throughput growth measurement. Indeed, current approaches are unable to detect growth defects for a surprisingly large fraction of the non-essential gene deletion strains under standard conditions<sup>16,25</sup>. For example, in rich media, only about 20% of non-essential gene deletions are reported to cause significant growth defects<sup>16,24</sup>, whereas at least 80% of genes are detectably expressed<sup>29</sup>. From an evolutionary standpoint it would be surprising if such a large fraction of expressed genes were truly dispensable (i.e. make no contribution to growth); one possible explanation is that for many gene deletions, growth phenotypes are present but difficult to measure using existing approaches. The technical challenges associated with measuring growth defects also have significant implications for chemical and genetic interaction studies, as the ability to quantitatively measure such interactions is critically dependent upon accurate fitness measurements. This is particularly true for identifying alleviating interactions, which typically requires detecting differences in growth even smaller than the defects seen for single-gene deletion strains.

A second major challenge is our limited ability to systematically probe the functions of the 1,033 essential yeast genes (~18% of open reading frames<sup>102</sup>). Essential genes represent a highly conserved and medically relevant gene subset, with ~40% having human counterparts<sup>19</sup>. Two general approaches have been developed to systematically explore essential gene function. One strategy uses inducible gene inactivation, either by transcriptional shut-off<sup>19</sup> or conditional protein destabilization<sup>21</sup>, and has proven quite effective for examining the immediate consequences of gene loss.

However, these approaches typically produce severe steady-state growth defects, complicating analysis of constitutive phenotypes<sup>19,21</sup>. The second approach, which involves the use of heterozygous diploids to reduce gene dosage, suffers from the opposite problem: most of these strains show no obvious growth defects<sup>24</sup>.

In large part due to these limitations, over 1,100 yeast genes remain completely uncharacterized<sup>102</sup>, with many more that are poorly characterized or likely to have non-canonical functions that have been overlooked. To facilitate high-precision functional analysis of the yeast genome, we constructed a library of hypomorphic alleles (i.e. alleles with reduced gene function) for ~82% of essential yeast genes utilizing the Decreased Abundance by mRNA Perturbation (DAmP) approach<sup>7</sup>. Additionally, we developed a rapid and sensitive flow cytometry-based growth competition assay that enables us to detect growth defects and genetic interactions for a far greater fraction of strains in the deletion library than previously reported. Together these tools enable in-depth functional dissection of pathways and complexes.

## **Results**

### **A library of hypomorphic alleles for essential yeast genes**

We previously described an approach, termed DAmP, for creating hypomorphic alleles of essential genes<sup>7</sup>. In this strategy, a gene's 3' untranslated region (3' UTR) is disrupted with an antibiotic resistance cassette (Fig. 2.1a), thereby destabilizing the corresponding transcript and reducing mRNA levels (typically two- to ten-fold)<sup>7</sup>. Here, we report the construction of DAmP alleles for 842 essential yeast genes (~82% of all essential genes) (Fig. 2.1b; Supplementary Fig. 2.1). Strains were constructed first as diploids to minimize the accumulation of secondary mutations, then sporulated to obtain the haploid DAmP library; both collections will be available through Open Biosystems.

We also present a parallel approach for producing stronger hypomorphic alleles in which the DAmP strategy is combined with a short C-terminally fused degradation tag (degron)<sup>103</sup> that targets the protein for proteasomal degradation (Fig. 2.1c). This approach takes advantage of the tandem affinity purification (TAP)-tagged strain library<sup>29</sup> to efficiently produce degron alleles using a cassette bearing homology to the TAP tag and downstream sequences in these strains without requiring gene-specific primers. Addition of the degron leads to 2.5- to >10-fold reductions in protein abundance beyond those seen in TAP-DAmP strains (Fig. 2.1d), and the majority of strains examined showed a corresponding reduction in growth rate (Supplementary Table 2.1).

### **Development of a novel high-throughput growth assay**

The ability to precisely measure fitness in high-throughput is of critical importance to functional genomic studies but has proven difficult to achieve in practice.

A key issue is that functionally relevant differences in growth can be small and thus only become apparent after the cumulative effects of many generations. Consequently, approaches based on colony size<sup>7,9</sup> or optical density (OD)<sup>25,30</sup> measurements that do not carefully control the growth microenvironment are susceptible to external sources of variation that can obscure true biological differences. Methods using competitive growth can avoid this pitfall by measuring relative growth differences among strains competing in a homogeneous environment (for example, microarray-based monitoring of pooled competitions of the deletion library)<sup>16,22</sup>. However, even in a competition-based format, precise measurements over a broad dynamic range are necessary.

We therefore developed a fitness assay in which flow cytometry is used to monitor growth competitions of fluorescently tagged strains. A Red Fluorescent Protein (RFP)-expressing wild-type strain is co-cultured with a Green Fluorescent Protein (GFP)-expressing strain bearing a mutation of interest, and the relative abundance of each strain is monitored over time by flow cytometry. The rate at which the fraction of mutant cells declines serves as a direct measure of the relative growth rate (Fig. 2.2a-c). Importantly, fluorescent protein expression allows the strains to be clearly distinguished with little to no effect on growth (Supplementary Fig. 2.2). This approach is also robust to variability in GFP expression attributable to a given mutation; in principle, even non-fluorescent mutant cells can be distinguished from wild-type cells on the basis of the latter's RFP expression. Furthermore, the large number (~30,000) of cells measured by flow cytometry enables precise quantification of the co-culture composition over a range of ratios spanning approximately five orders of magnitude. Lastly, we paired this strategy



with a high-throughput flow cytometry platform<sup>104</sup> that allows ~2,000 competition time-courses to be completed, in duplicate, per week.

We evaluated the sensitivity and reproducibility of this technique by measuring the growth of 344 DAmP and deletion strains in triplicate. These replicate measurements showed a high level of agreement ( $r = 0.993$ , Fig. 2.3a), with a median standard deviation for the three measurements of 0.0038 (Fig. 2.3b and Supplementary Data). This reproducibility is maintained across a range of growth rates, with only modest increases in error for sicker strains (Fig. 2.3b). Thus, we can accurately measure growth rates from ~0.5-1.0 (where wild-type growth equals 1.0), a range that includes nearly all yeast single-gene deletions. Strains with more extreme fitness defects can likely be measured with simple experimental modifications, such as the use of a slow-growing RFP competitor strain or additional measurements at early time points. Lastly, in its current form, our growth competitions include multiple entries and exits from saturation in addition to exponential growth (see Methods). Thus, our assay likely detects competitive disadvantages in any of these steps, and direct comparisons with continuous exponential-phase growth data should be made with caution. However, our method could be readily modified for a variety of desired growth formats according to experimental needs.

### **Fitness measurements for deletion and DAmP library strains**

To explore the potential of our growth assay, we introduced the GFP expression cassette into the deletion and DAmP libraries and obtained growth rate measurements for 835 DAmP strains and 4,204 deletion strains. We analyzed the correspondence between full biological replicates (independently derived GFP-marked derivatives of the strains)

to evaluate the uncertainty in our measurements (Supplementary Methods, Supplementary Fig. 2.3 and Supplementary Table 2.2). Importantly, even very small growth defects can be confidently detected (for example, > 85% of strains with measured fitness between 0.985 and 0.990 have true growth defects; Supplementary Table 2.2). Thus, we estimate that 360 DAmP strains (~43% of 835 measured) and 1,875 deletion strains (~45% of 4,204 measured) have observable growth defects in minimal media (Fig. 2.3c-d), a number that considerably exceeds previous estimates (Supplementary Note). Although this finding could in part result from differences in growth conditions, our ability to detect growth defects for a larger number of strains is critically dependent on the greater precision of our technique (Supplementary Fig. 2.4) and cannot simply be explained by differences in growth format. In further support of the sensitivity of our assay, we find several differences in the functional properties of genes that can be deleted with no apparent growth defect compared to those that cause even slight growth defects. For example, the former are enriched for genes with very low expression levels<sup>105</sup> and for genes involved in sporulation and the response to stress<sup>106</sup>, whereas the latter are not (data not shown and Supplementary Figs. 2.5 and 2.6). Finally, a small number of strains exhibit a slight growth advantage in our assay (Supplementary Data). Many of these contain mutations in genes encoding components of the cAMP-dependent Protein Kinase (PKA) pathway, including *TPK2*, *TPK3*, *RAS2*, *GPA2*, *GPB1*, *GPB2*, *PDE2*, *SHR5*, and *IRA1*. This striking enrichment suggests that this nutrient-sensing pathway may help to regulate or limit growth.

### **Chemical sensitivities of DAmP strains**

Analysis of chemical sensitivity in heterozygous diploids can provide important insights into mechanisms of drug action<sup>107,108</sup>. We therefore explored the utility of our DAmP library for such screens. Indeed, for four tested antibiotics, DAmP alleles of the known targets confer greater drug sensitivity than is seen in heterozygous diploids (Fig. 2.4a). We also screened the DAmP library for sensitivity to tunicamycin, which inhibits N-linked glycosylation in the endoplasmic reticulum (ER)<sup>109</sup>. The DAmP allele of *ALG7*, the direct target of tunicamycin, was by far the most sensitive strain in the library (Fig. 2.4b). Other sensitive strains identified are consistent with the role of glycosylation in protein folding and include the ER chaperones Kar2 and Pdi1, members of the COP-I coatomer, and the protein synthesis and degradation machinery (Supplementary Data). Notably, several of these strains exhibited only modest sensitivity to tunicamycin and may not have been detected using milder alleles or a less precise growth assay. Together, these findings suggest that our DAmP library and growth assay represent a highly effective complement to haploinsufficiency as a strategy for discovery of drug targets<sup>107,108</sup>.

### **Measurement of genetic interactions by flow cytometry**

A major challenge for functional genomics is the quantitative measurement of genetic interactions. The identification of positive interactions has been particularly difficult, as this requires detecting differences in growth even smaller than the defects seen for single-gene deletion strains. Additionally, a spectrum of positive genetic interactions exists, in which the phenotype of a given mutation may be partially reduced, completely masked, or even suppressed by the presence of a second mutation, with each

of these outcomes having different biological interpretations<sup>30</sup>. However, the magnitude of the genetic interaction score alone cannot be used to resolve these classes, an issue that was recently addressed by the introduction of a normalization<sup>5</sup> that uses as a reference point the growth rate of the slower of the two single mutants (the growth rate expected for the double mutant if the effect of the first mutation is completely masked by the second; Fig 2.5a). Thus, raw interaction scores ( $\epsilon$ ) are re-scaled such that  $\epsilon_{NORM} = 1.0$  for complete masking or alleviation,  $\epsilon_{NORM} = 0$  for no interaction,  $0 < \epsilon_{NORM} < 1$  for partial alleviation, and  $\epsilon_{NORM} > 1$  for suppression<sup>5</sup>. However, because the absolute differences in growth rates involved are typically very small, in practice this normalization has been difficult to implement<sup>30</sup>.

To determine whether our growth assay permits us to distinguish among these types of positive interactions, we examined three complexes and pathways. First, we focused on the Conserved Oligomeric Golgi (COG) complex, a mediator of vesicular trafficking to the Golgi apparatus<sup>110</sup>. The genes *COG5-COG8* encode four tightly associated subunits of the complex and exhibit a similar pattern of phenotypes and genetic interactions<sup>7</sup>. However, colony size-based genetic interaction measurements failed to detect the alleviating interactions expected between these genes<sup>7</sup>, presumably because loss of the complex only mildly affects growth (Fig. 2.5b). By contrast, we can clearly resolve these positive interactions with our growth assay (Fig. 2.5b). Moreover, the normalized interaction scores are  $\sim 1.0$  and thus reflect the complete alleviation expected when the loss of any subunit fully disrupts complex formation.

Next, we examined interactions between *HTZI*, which encodes the histone variant H2A.Z, and members of the Swr complex (Swr-C), a histone exchange factor that

deposits H2A.Z into chromatin<sup>111</sup>. As previously reported<sup>11</sup>, we detect positive interactions between *htz1Δ* and deletions of genes encoding Swr-C members. Surprisingly, however, the absence of H2A.Z reduces fitness more severely than does loss of the complex responsible for its deposition (Fig. 2.5c). Furthermore, deletion of genes encoding Swr-C components suppresses, rather than simply alleviates, the phenotype of the *htz1Δ* strain (i.e.  $\epsilon_{NORM} > 1$ ). This unexpected suppression suggests a more complex relationship between the functions of H2A.Z and those of the Swr-C.

Our genetic interaction measurements also help to dissect the more intricate functional relationships in the pathways involving Rpd3, a multifaceted histone deacetylase that exists in two distinct physical complexes. Specifically, the core components (Rpd3 and Sin3) associate with different sets of accessory proteins to form both a small complex, Rpd3C(S) and a large complex, Rpd3C(L) (Fig. 2.5d)<sup>112</sup>. As expected, we detect positive interactions between genes encoding members of the same complex. However, a more striking pattern emerges after normalization: the wide range of raw  $\epsilon$  values converges on a value very close to 1.0 (complete alleviation; Fig. 2.5d). Such normalization therefore removes the potentially confounding effects of gene deletions with different growth rates.

One exception to this pattern is the histone methyltransferase Set2, which mediates recruitment of Rpd3C(S) to chromatin via Eaf3<sup>112</sup>. Although the genetic interactions between *set2Δ* and members of Rpd3C(S) are positive, the  $\epsilon_{NORM}$  values are considerably less than 1.0 (0.32-0.55; Fig. 2.5d). This incomplete alleviation argues that Set2 has additional functions independent of Rpd3C(S), consistent with a recent report<sup>113</sup>, and that Rpd3C(S) must similarly have Set2-independent activities. This example

underscores the potential of high-resolution genetic interaction measurements to reveal complexity in pathways not readily accessible by traditional approaches.

### **Functional dissection of the 26S proteasome**

The functional dissection of essential multi-protein complexes represents an important technical challenge. We therefore tested the suitability of our DAmP alleles to provide insights into the proteasome, a 2.5-MDa macromolecular machine with over 30 different subunits that are organized into a 20S core complex and a 19S regulatory particle<sup>114</sup> (Fig. 2.6a). The core complex is comprised of alpha and beta rings, while the regulatory particle contains a lid sub-complex that mediates substrate deubiquitination and a base sub-complex that mediates recognition of ubiquitinated substrates and their ATP-dependent unfolding. The intricate yet modular organization of the proteasome makes it an excellent test case for dissection by our genetic approach. Furthermore, although the role of the proteasome in protein degradation is well appreciated, recent studies suggest additional functions, including the possibility that the regulatory particle can act independently of the core<sup>115</sup>.

We measured growth rates for pairwise combinations of DAmP alleles to construct a genetic interaction map of the proteasome. This map reveals a rich set of interactions, with each sub-complex exhibiting a distinct series of interactions consistent with the known organization of the proteasome (Fig. 2.6b). Furthermore, we observe exceptions to these trends that provide more detailed functional insights. For example, *PRE9* is synthetic with DAmP alleles of other alpha ring subunits rather than alleviating, and indeed Pre9 is unique in that it is the only non-essential component of the core (we

used a deletion rather than DAmP allele of *PRE9*). The basis of this dispensability was recently explained by the observation that, in the absence of Pre9, a second copy of Pre6 substitutes for Pre9 in the alpha ring<sup>116</sup>. Consistent with this, we observe a severe synthetic interaction between *pre9Δ* and *pre6-DAmP* (Supplementary Fig. 2.7a). An additional example of the rich information content of our map is the distinction between the ATPase members of the base (*RPT1-6*), which exhibit positive interactions with alpha subunit genes, and the non-ATPase members *RPN2* and *RPN10*, which do not.

We next explored whether normalized interaction scores could facilitate interpretation of the proteasome interaction map. Indeed, normalization revealed that the positive interactions both between pairs of alpha subunits and between pairs of beta subunits represent instances of complete alleviation (Fig. 2.6c). This result is consistent with the cooperative association of these proteins into rings, where the least abundant ring component likely dictates the amount of functional proteasome formed. More strikingly, normalization revealed suppressive interactions between ATPase components of the base and the alpha ring with which the base interacts. Interestingly, the median raw  $\epsilon$  measured between base and alpha ring genes is smaller than that seen for fully alleviating interactions between alpha ring genes (0.06 versus 0.09), and thus only with high precision measurements is it possible to see that the normalized interaction scores actually indicate the opposite relationship (1.77 versus 0.79; Fig. 2.6b-c). The mechanism by which lowering levels of the base proteins suppresses the growth defects caused by reduced levels of the alpha subunits remains to be explored. One possibility is that lowered levels of the alpha ring permit unregulated activity of the base ATPases that may impact recently defined roles for the regulatory particle in transcription<sup>115</sup>. Finally,

we see similar suppressive interactions between *pre7*-DAmP (a beta subunit) and genes encoding the regulatory particle (Supplementary Fig. 2.7b). A molecular basis for these interactions is not known to us, but may merit further exploration. Thus, our analysis of the proteasome demonstrates that rich functional information can be obtained for essential complexes using DAmP alleles.



## Discussion

Here, we present an integrated set of tools that greatly increases our ability to carry out functional genomics in yeast. Our library of DAmP strains has a number of key features that make it a useful resource for the research community. First, the library was constructed in the same strain background as the deletion library and uses the same linked antibiotic marker (kanamycin-resistance, Kan<sup>R</sup>)<sup>16</sup>. Thus, our strains can be immediately used in parallel with the deletion library in the large array of assays that have been developed for that collection. Second, because the DAmP approach is based on mRNA destabilization by 3' UTR disruption, DAmP strains express unmodified proteins using their natural promoters and thus are likely to be free of complications such as mis-regulation or neomorphic activities. Third, DAmP strains often show stronger chemical sensitivities than heterozygous diploids and therefore may be highly useful for the identification of drug targets<sup>107,108</sup>. Fourth, our library has a similar range of growth defects as the deletion library, and therefore should prove equally valuable for studying essential genes as the deletion library has for non-essential genes. Finally, degon+DAmP strains provide a ready means to obtain stronger hypomorphic alleles when desired.

The second key component of our study is the ability to measure fitness with unprecedented sensitivity. This sensitivity allowed us to identify growth phenotypes for a larger fraction of the deletion library (1,875 of 4,204 strains measured) than previously possible<sup>16,24,25</sup>. In total, our analysis reveals that roughly half (2,322 of 4,817) of non-essential gene deletion strains have measurable growth defects in a single condition, as 447 of the 613 strains not included in our dataset were previously shown to exhibit

growth phenotypes<sup>24,25</sup>. Taking into account the 1,033 essential genes, we now arrive at a new picture of yeast gene dispensability in which the clear majority (57%) of the genome (and an even larger fraction of the expressed genome; Supplementary Fig. 2.5) contributes to growth, even under a single growth format. This finding is important in the context of recent attempts to understand the surprising observation that many genes do not appear to contribute to fitness under standard conditions<sup>16,26,28</sup>. This robustness is partially explained by the presence of duplicate genes<sup>26</sup> and by examples of condition-specific gene requirements<sup>16,27,28</sup>. Nonetheless, given that ~80% of genes are detectably expressed in a given condition<sup>29</sup>, it is difficult to rationalize why resources would be expended to produce these proteins if they do not contribute to fitness in that condition. Our data instead reveal that the majority (and perhaps the vast majority) of expressed genes do contribute to fitness. For many genes this contribution is small in a laboratory context, but on an evolutionary timescale the competitive advantage conferred by such genes is sufficient to ensure their retention.

The power of these quantitative genetic tools is illustrated by our ability to gain new functional insights for several complexes and pathways. We are able to detect complete alleviating interactions for members of the COG complex<sup>7,110</sup> and to distinguish additional types of positive genetic interactions that suggest more complex functional relationships for members of the H2A.Z/Swr-C and Rpd3C(S)/Set2 pathways as well as the proteasome. Such examples of unanticipated interactions even for well-characterized genes may indeed be more common than the idealized view of genes functioning predominantly in simple linear pathways.

The ability to dissect pathways and complexes in fine detail argues strongly for applying this approach more broadly across the large fraction of the yeast genome now amenable to quantitative genetic analysis. Indeed, the throughput of our growth assay is such that all currently known genetic and physical interactions can be explored in high resolution. A comprehensive strategy for yeast functional genomics would therefore be to broadly query the genome for interactions using lower resolution ultra-high throughput techniques<sup>7,22</sup>, then probe interaction-rich subsets more precisely by flow cytometry. Finally, our successful analysis of genetic interactions using DAmP alleles suggests that a comparable strategy using RNA interference may be directly applicable to functional genomics in metazoan systems. Thus, in both yeast and higher eukaryotes, quantitative genetic approaches have the potential to provide a transformative effect on efforts to close the gap between genome and function.

## **Methods**

### **Growth rate measurement by flow cytometry**

We prepared growth competitions in 384-well plates (Nunc) by mixing GFP-labeled mutant cells and RFP (dTomato<sup>117</sup>)-labeled wild-type cells using a Biomek NX liquid handling robot (Beckman Coulter). Co-cultures were inoculated at a ratio of 2.5:1 (mutant:wild-type ) and at OD ~0.1. Plates were sealed with Breathe-Easy gas-permeable membranes (Diversified Biotech) and incubated at 30°C in humidified DTS-4 shakers (Elmi) at 1,200 rpm for 24 hours, at which point time-zero (T0) measurements were made. Subsequent measurements (T1-T3) were made every 24 hours, with each 24-hr interval consisting of growth from OD ~0.05 to OD ~5.0. At each time point, we diluted cells 100-fold into fresh liquid media for growth until the following time point. For cytometry measurements, an aliquot of cells was diluted 12-fold into rich media, incubated with shaking for 90 minutes, washed once with Tris-EDTA, pH 8.0 (TE) buffer, then resuspended in TE for analysis. We counted RFP- and GFP-labeled cells using an LSRII cytometer fitted with a high-throughput sampler (Becton Dickinson). GFP was excited at 488 nm and fluorescence collected through a 505 nm long-pass filter and a HQ515/20 band-pass filter (Chroma Technology). dTomato was excited at 532 nm and fluorescence collected through a 600 nm long-pass filter and a 610/20 band-pass filter (Chroma Technology).

### **Additional Methods**

Details of media, strain construction, and additional procedures are described in the Supplementary Methods.

## **Acknowledgments**

We gratefully acknowledge Joseph Hill and Edwin Rodriguez for help with strain construction; Chen-Shan Chin for flow cytometry software development; John Newman for an introduction to high-throughput flow cytometry; Roger Tsien (University of California, San Diego) for a construct encoding dTomato; Brandon Toyama for assistance with graphics; Charles Boone, Guri Giaever and Corey Nislow for communication of results prior to publication; and Vladimir Denic, Julie Hollien, Jan Ihmels, Nicholas Ingolia, John Newman and members of the Weissman laboratory for helpful discussion and critical reading of the manuscript. This work was supported by funding from the Howard Hughes Medical Institute (J.S.W.), the Fannie and John Hertz Foundation (D.K.B.), the National Science Foundation (D.K.B), and the Larry L. Hillblom Foundation (D.M.C.).

## **Competing Financial Interests**

The authors declare that they have no competing financial interests.

## Figure Legends

### Figure 2.1

#### **Construction of a library of hypomorphic alleles of essential yeast genes using the**

#### **DAmP approach. (a)** Schematic of the strategy employed for constructing DAmP

alleles. The Kan<sup>R</sup> cassette was inserted immediately following the open reading frame of each gene by transformation with a PCR product encoding the Kan<sup>R</sup> cassette flanked at each end with homology to the targeted locus. **(b)** Summary of the number of DAmP diploid and haploid strains obtained. Of the 1,033 essential yeast genes, ~970 were obtained in diploid form and 842 as *MATa* haploids. **(c)** Generation of TAP-tagged DAmP and degron+DAmP alleles from existing TAP-tagged strains by high-efficiency transformation with either of two universal cassettes. **(d)** TAP-degron+DAmP alleles typically reduce protein abundance greater than the DAmP allele alone as determined by Western blots against the TAP tag. 3-phosphoglycerate kinase (Pgk1) was probed to normalize for variation in loading.

### Figure 2.2

#### **A flow cytometry-based technique for high-precision growth rate measurements.**

**(a)** Schematic of the assay. GFP-labeled mutant strains are competed against an RFP-labeled wild-type strain in 384-well format with cultures maintained by serial dilution over a 72-hour time-course. At each time point, samples are removed for analysis by flow-cytometry to determine the ratio of GFP<sup>+</sup> to RFP<sup>+</sup> cells. **(b)** Wild-type and mutant cell populations can be readily resolved by flow cytometry. Rare events that appear to be

GFP<sup>+</sup> and RFP<sup>+</sup> represent instances in which a mutant cell and a wild-type cell are mis-identified by the cytometer as a single cell and are taken into account during analysis (Supplementary Methods). (c) Representative fitness data obtained by flow cytometry. A spectrum of relative growth rates spanning ~0.6 (quite sick) to 1.0 (wild-type growth) can readily be observed with a high degree of linearity in the rate of mutant strain depletion over time. Dashed grey line indicates the approximate limit of sensitivity.

### Figure 2.3

#### **Growth rate measurements of deletion and DAmP yeast strains by the flow**

**cytometry-based technique.** (a) The flow cytometry-based approach achieves excellent agreement between replicate measurements ( $r = 0.993$ ). (b) Error as a function of the relative fitness observed. For groups of 30 strains having similar growth rates, median fitness and median standard deviation of triplicate measurements are shown. Inset: distribution of standard deviations of triplicate measurements for all strains measured; the median standard deviation is 0.0038. (c) Distribution predicted from experimental error (green) and observed distribution of relative growth rates (blue) for 835 DAmP strains, with inset showing the cumulative distribution of growth rates. (d) Distribution predicted from experimental error (green) and observed distribution of relative growth rates (blue) for 4,204 deletion strains, with inset showing the cumulative distribution of growth rates.

### Figure 2.4

**Utility of DAmP strains for identification of drug targets. (a)** Sensitivity of DAmP alleles of known targets to the indicated drugs. For the *CDC21*, *FOL1* and *FOL3* strains, the heterozygous diploid strains were not found to be significantly sensitive to the corresponding agents<sup>108</sup>. Shown for comparison is the sensitivity of the *erg11* heterozygous null diploid (*erg11Δ/ERG11*). Differences in strain sensitivity are measured by the differences in radius relative to an isogenic control (WT): *CDC21* = 2.5X; *FOL1* = 1.9X; *FOL3* = 4.3X; *ERG11* = 2.2X (relative to WT) and 1.4X (relative to the heterozygous diploid; average of 2-4 replicates). **(b)** Distribution of growth defects for DAmP library strains grown in media containing 0.25  $\mu\text{g ml}^{-1}$  tunicamycin. The strain bearing a DAmP allele of *ALG7*, the known target of tunicamycin, is indicated with a red datapoint. Additional sensitive strains are also detected (dashed box; Supplementary Data).

## Figure 2.5

**Highly sensitive growth rate measurements enable resolution of previously inaccessible classes of genetic interactions. (a)** Normalization of genetic interaction scores helps to distinguish different types of positive genetic interactions. Raw interactions scores, given by  $\varepsilon = W_{AB} - W_A \times W_B$ , are normalized using the growth rate of the single-mutant strain with the stronger growth phenotype to yield  $\varepsilon_{\text{NORM}} = \varepsilon / [\min(W_A, W_B) - W_A \times W_B]^5$ . **(b)** Comparison of genetic interactions observed between members of the COG complex when using fitnesses measured by flow cytometry (lower left) versus colony size (upper right; data from reference 7). Values shown for interactions calculated from flow cytometry measurements are the raw genetic interaction



score ( $\epsilon$ ; black) and the normalized interaction score ( $\epsilon_{NORM}$ ; red). Relative growth rates for the single gene deletion strains are indicated in blue. (c) Growth rates measured by flow cytometry for strains with deletions of *HTZ1* and/or genes encoding members of the Swr Complex (Swr-C) reveal unexpected genetic interactions for this pathway. Error bars indicate standard deviation,  $n \geq 3$ . (d) Genetic interactions between genes encoding members of Rpd3C(S) and Rpd3C(L) reflect the organization of these complexes (shown schematically at right; note, both complexes also contain additional members). Additional functions for Set2 and Rpd3C(S) suggested by genetic interaction data are indicated by question marks in the schematic. Raw interaction scores (upper right) and normalized interaction scores (lower left) were calculated as in (a). Note that synthetic ( $\epsilon < 0$ ) interactions are normalized using a different scale (see Supplementary Methods).

## Figure 2.6

**Functional dissection of the 26S proteasome.** (a) Schematic representation of the 26S proteasome. Components of the 20S core are indicated in green ( $\alpha$ ) and maroon ( $\beta$ ); components of the 19S regulatory particle are indicated in blue (base) and orange (lid). (b) Map of raw genetic interaction scores for the 26S proteasome. Interactions between pairs of genes encoding components of the same physical sub-complex are indicated by red boxes. (c) Map of normalized interaction scores for the 26S proteasome. In the lower panel, double-mutants were grouped according to the sub-complex(es) bearing the mutations, with the median score for each group shown (\*data for *PRE9* and *PRE7* were excluded when calculating the medians). Individual scores for interactions with the alpha

ring subunits are shown above. Note, synthetic interactions ( $\varepsilon < 0$ ) are normalized using a different procedure (see Supplementary Methods).

Figure 2.1

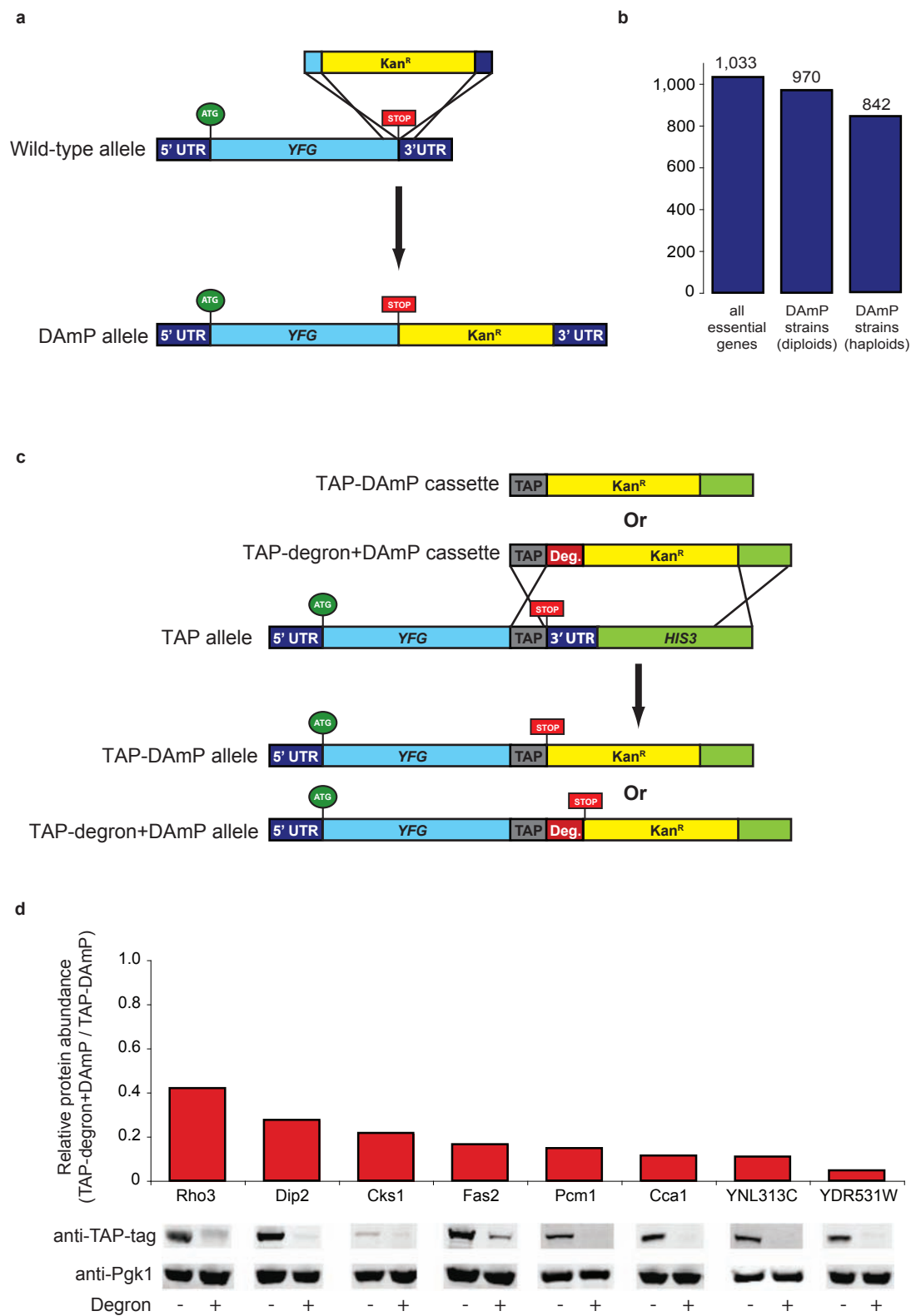


Figure 2.2

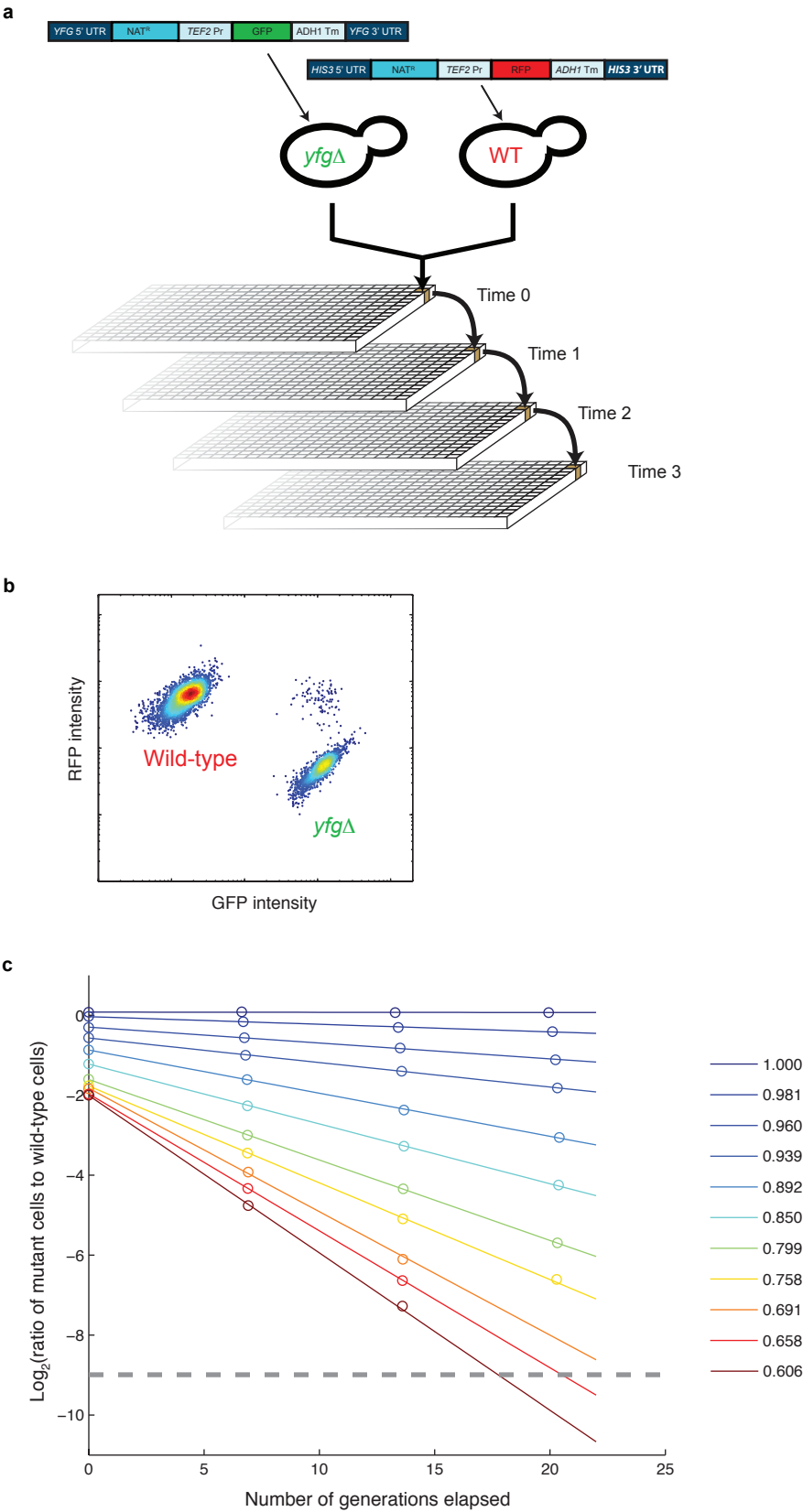


Figure 2.3

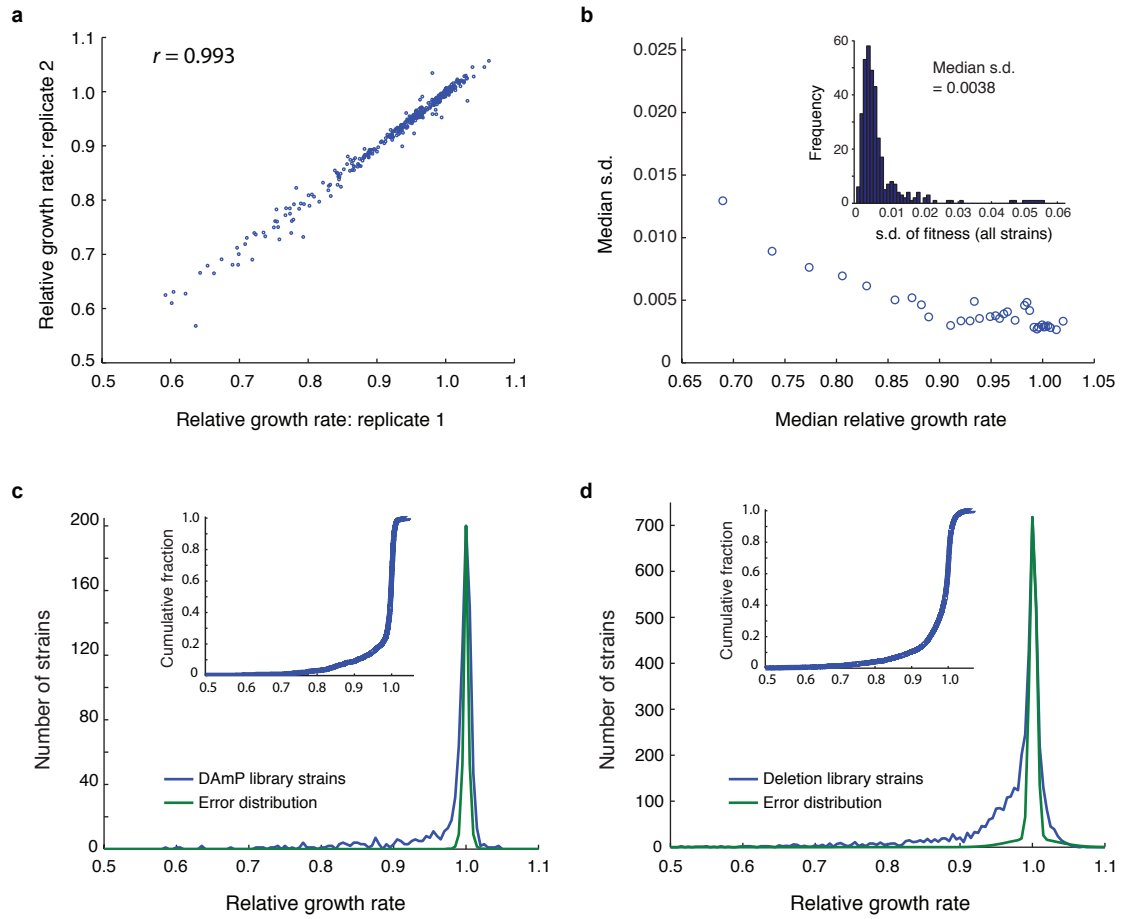


Figure 2.4

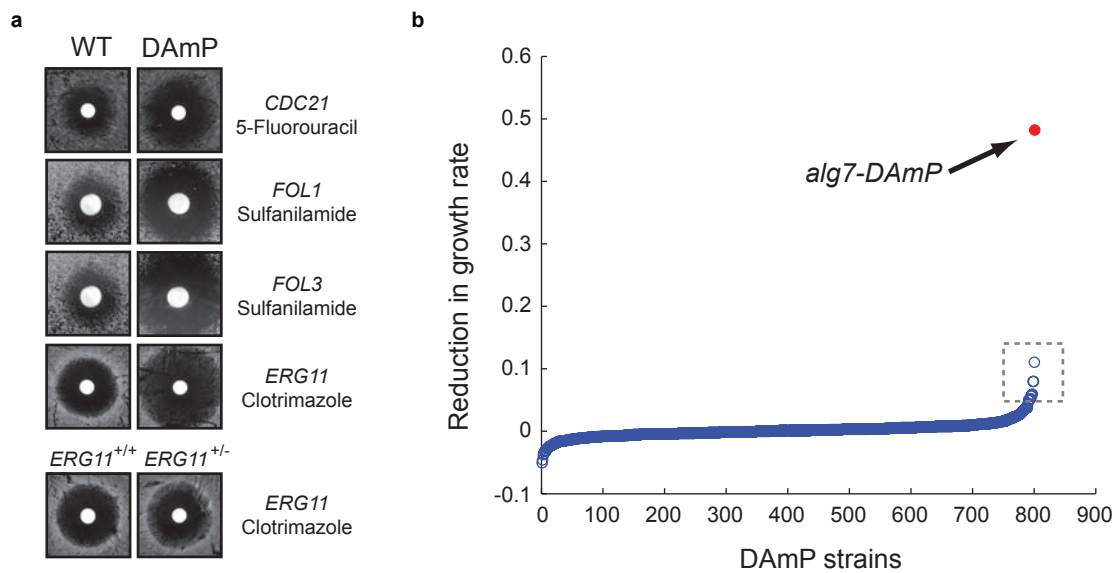


Figure 2.5

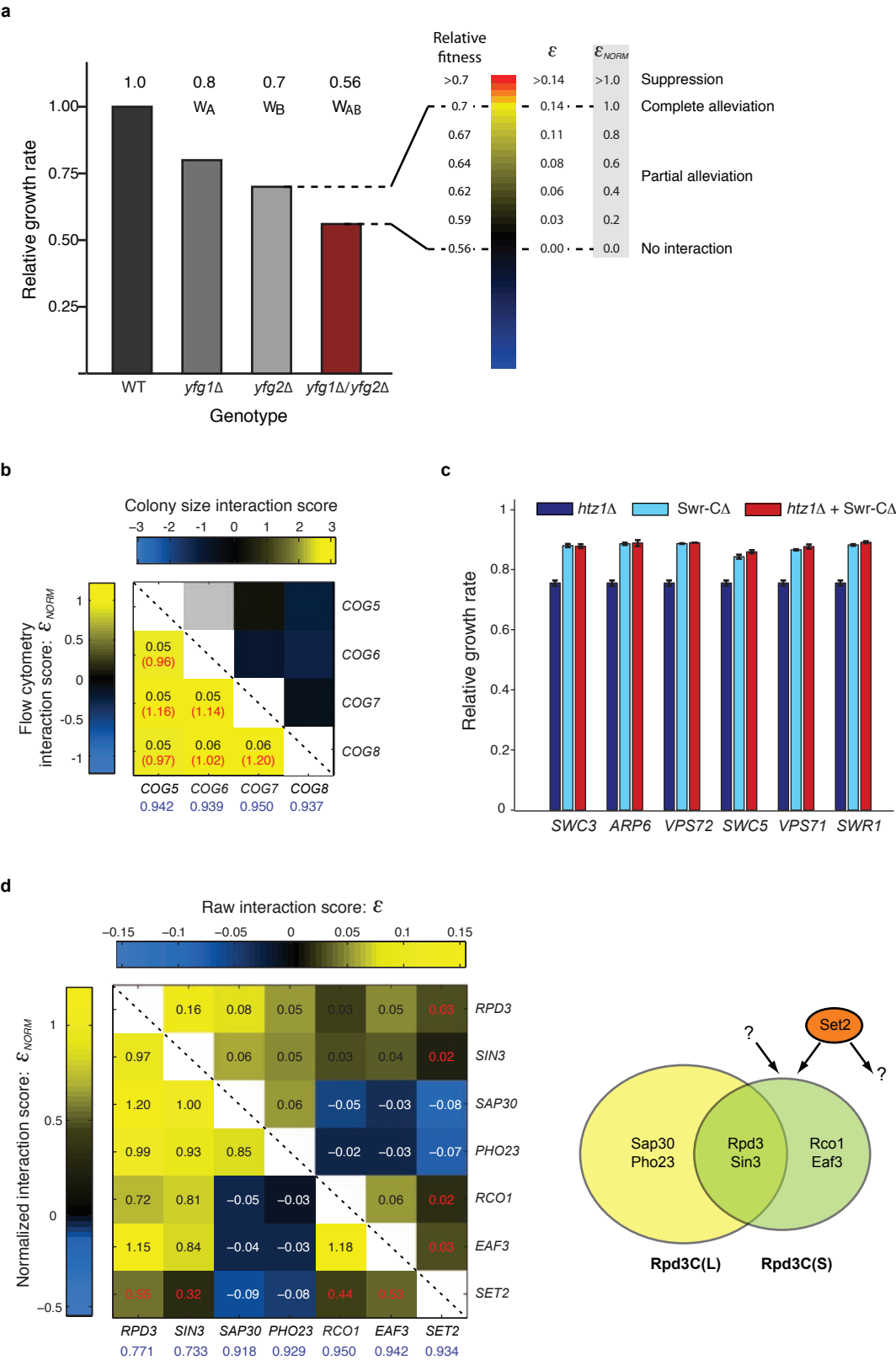
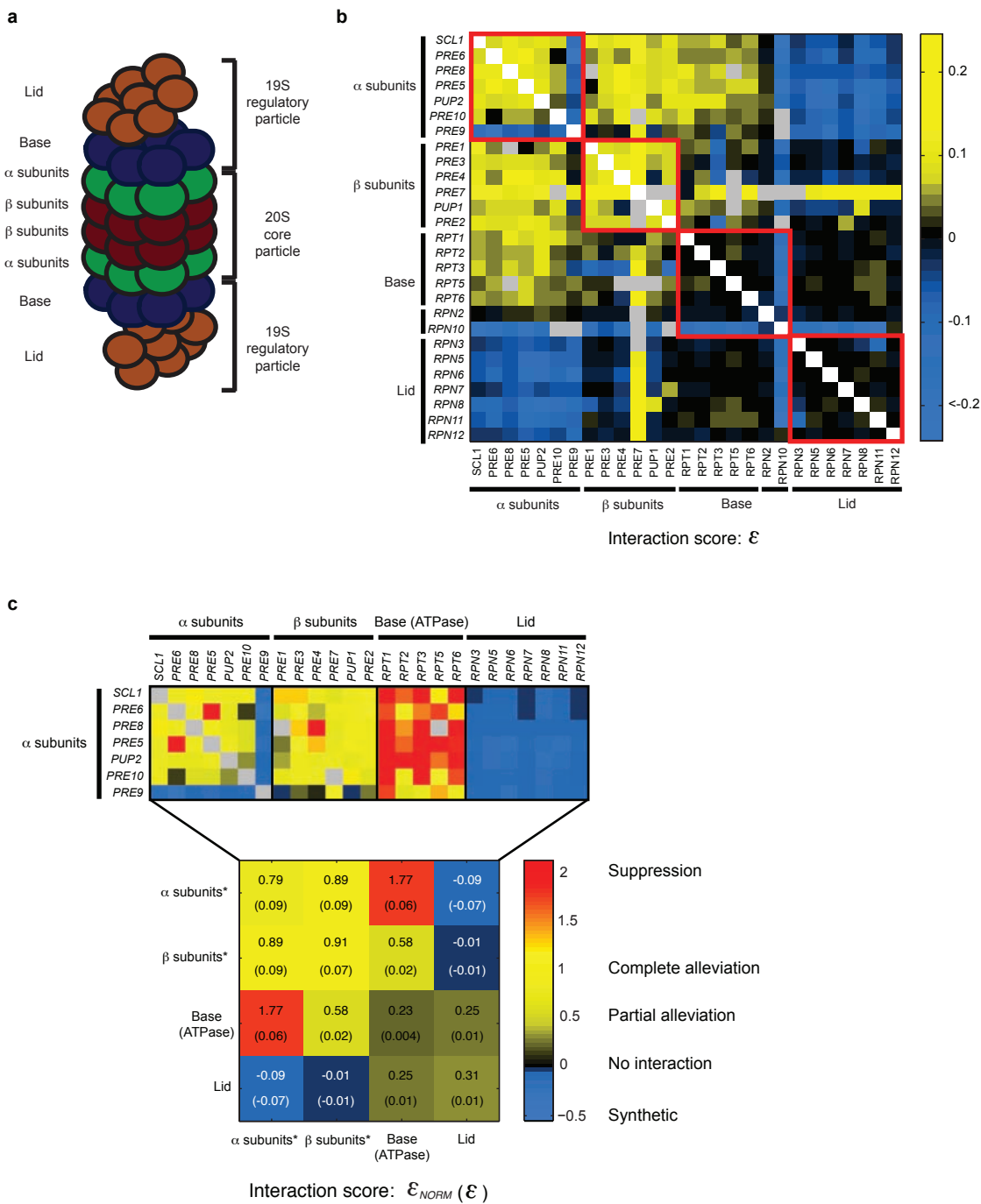
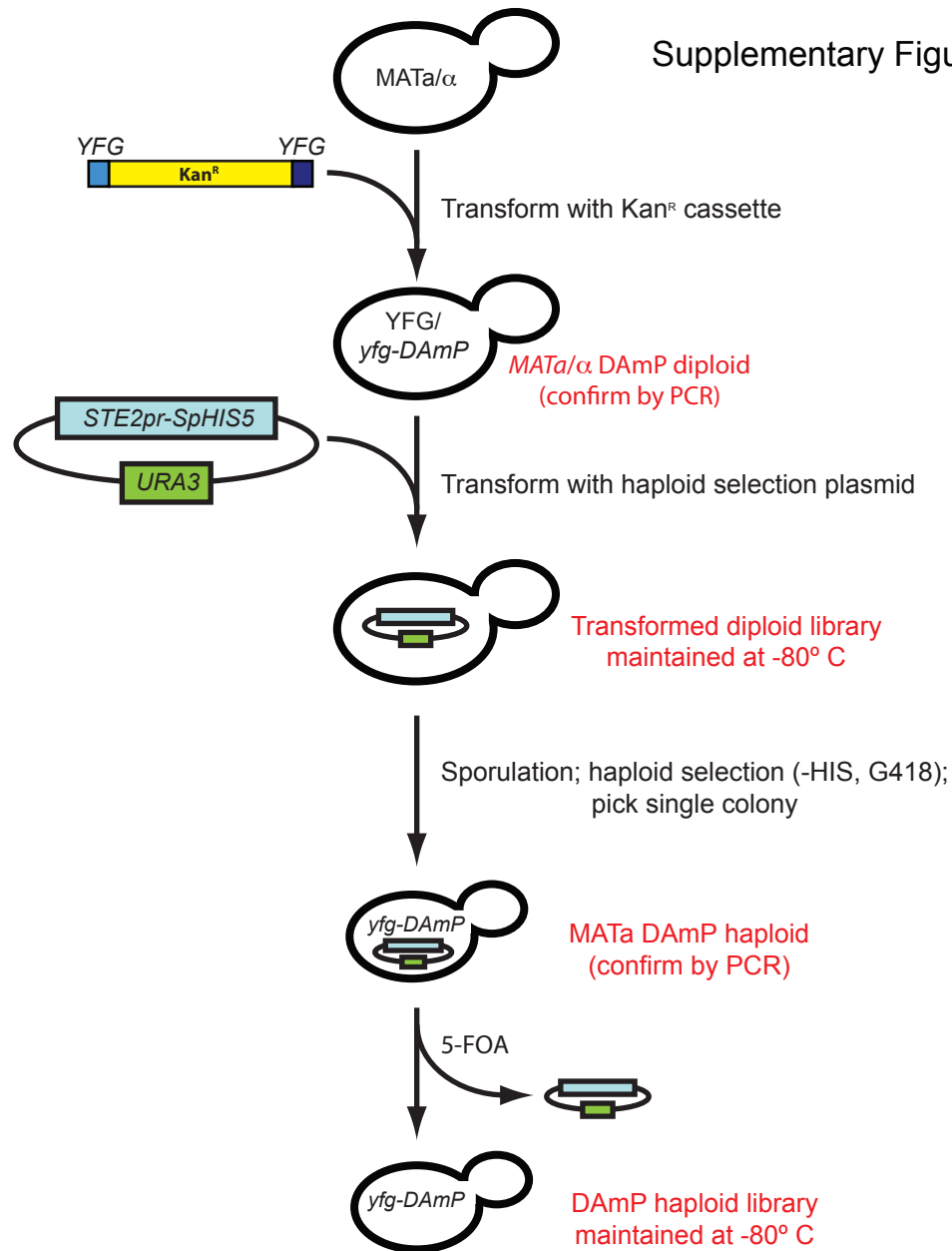


Figure 2.6





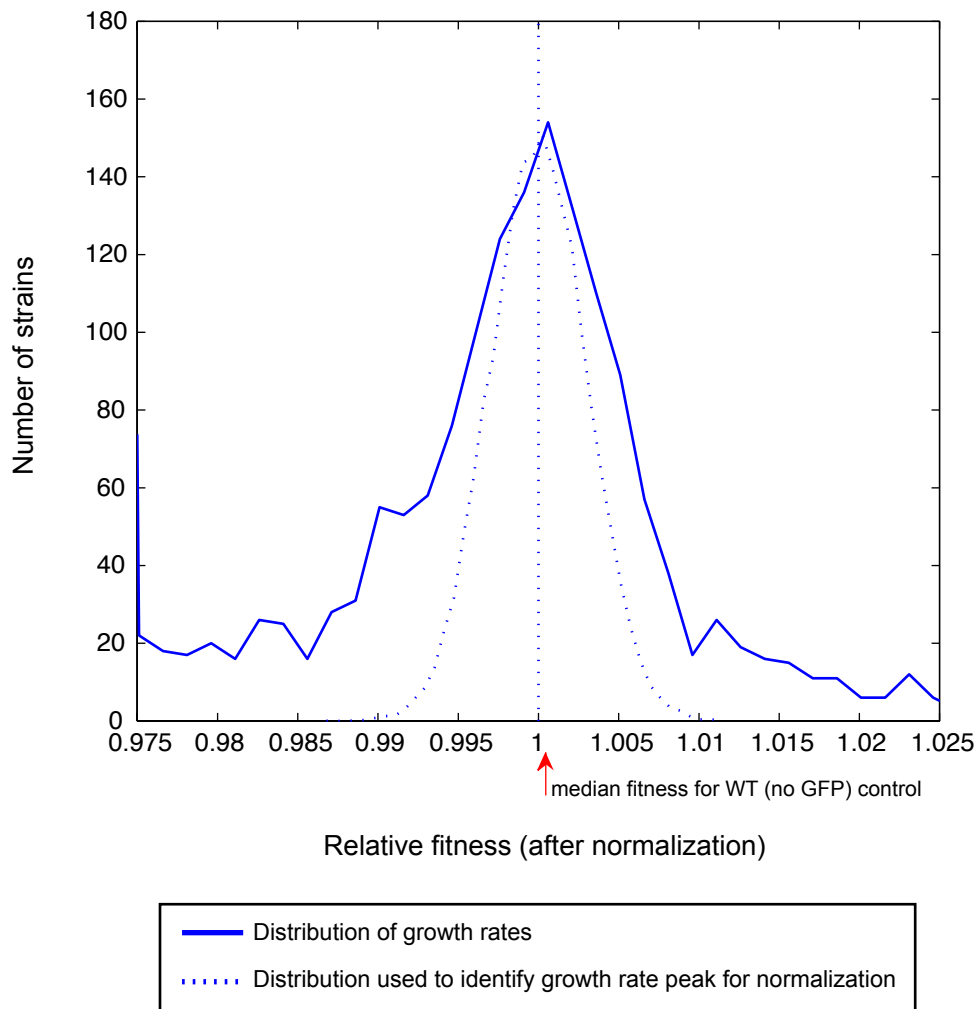
Supplementary Figure 2.1



**Supplementary Figure 2.1. Strategy used for DAmP library construction.**

DAmP alleles were first constructed in diploid strains by transformation of a PCR product bearing 40 nucleotides of homology to the site of integration. DAmP diploids were confirmed by PCR then transformed with a plasmid to enable direct selection of *MATa* haploids following sporulation (through the use of a *S. pombe HIS5* marker driven by the *MATa*-specific *STE2* promoter). Following sporulation, DAmP haploids were reconfirmed by PCR and passed on 5-fluorouracil (5-FOA) to ensure loss of the haploid selection plasmid.

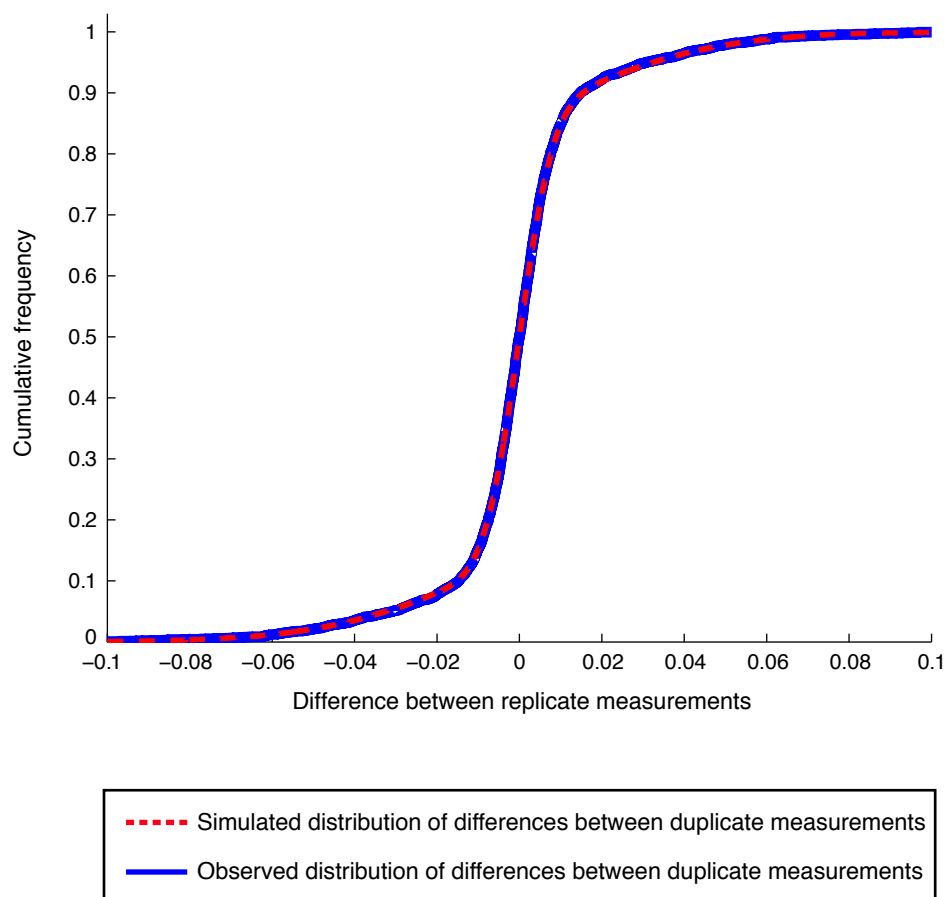
Supplementary Figure 2.2



**Supplementary Figure 2.2. Normalization of fitness measurements.**

Growth rates from each flow cytometry experiment were normalized such that the peak of the distribution of GFP-labeled mutant strains (shown for one representative experiment above; solid line) is equal to a fitness of 1.0. The peak of the distribution of GFP-labeled mutant cells was identified using a Parzen window smoothing strategy (dashed line; see Supplementary Methods). After normalization, the median fitness for a wildtype (WT) strain not expressing GFP is nearly exactly equal to 1.00 (red arrow), confirming that the expression of GFP has very little effect on growth.

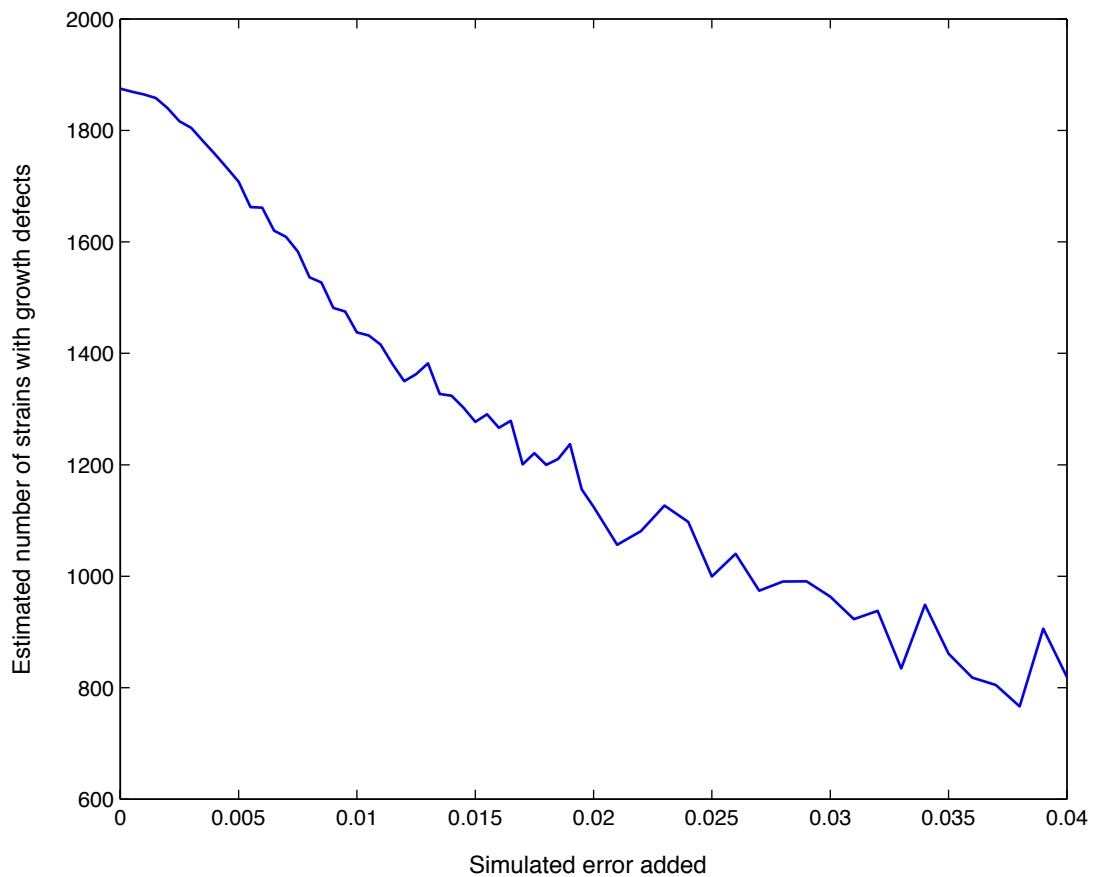
Supplementary Figure 2.3



**Supplementary Figure 2.3: Simulated versus observed distribution of differences between duplicate growth measurements.**

Our error model (dashed red line; see Supplementary Methods) accurately reproduces the observed distribution of differences between duplicate measurements (solid blue line).

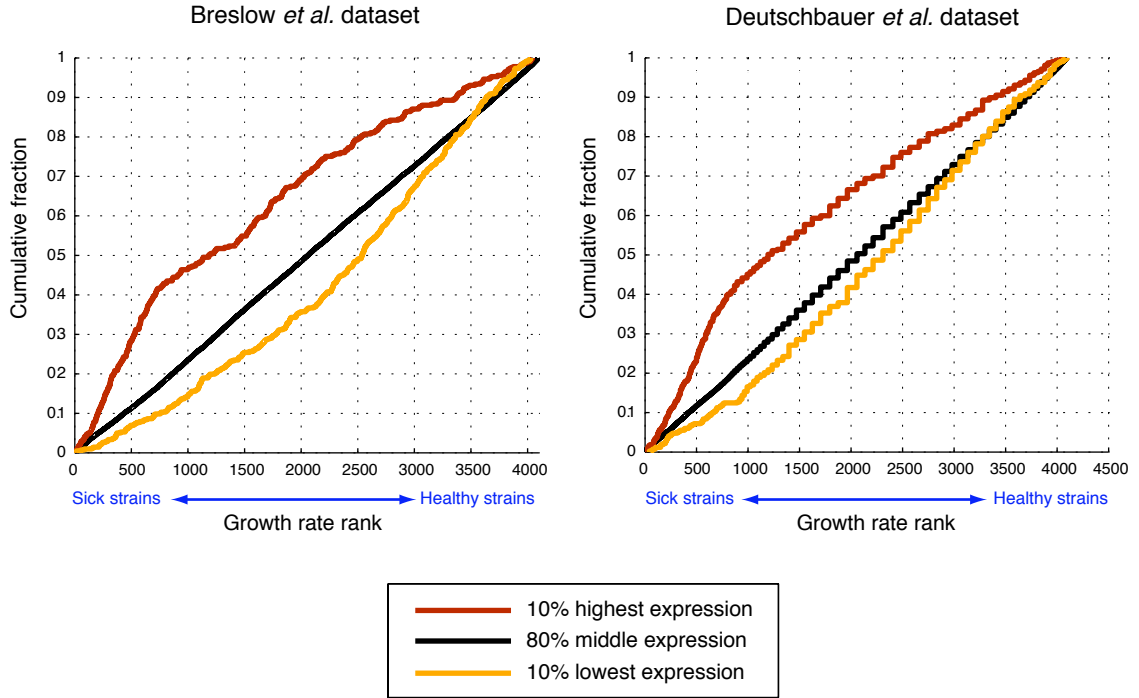
Supplementary Figure 2.4



**Supplementary Figure 2.4. Effect of simulated error on the the apparent number of deletion strains with growth defects.**

Increased measurement error was simulated by the addition of normally distributed noise of a defined magnitude to our growth rate data. The error analysis described previously (see Supplementary Methods) was then re-applied to the simulated data to estimate a number of strains with identifiable growth defects (among the 4,204 strains measured). For each level of error added, 200 simulations were carried out and used to compute a median number of strains with growth defects (plotted above).

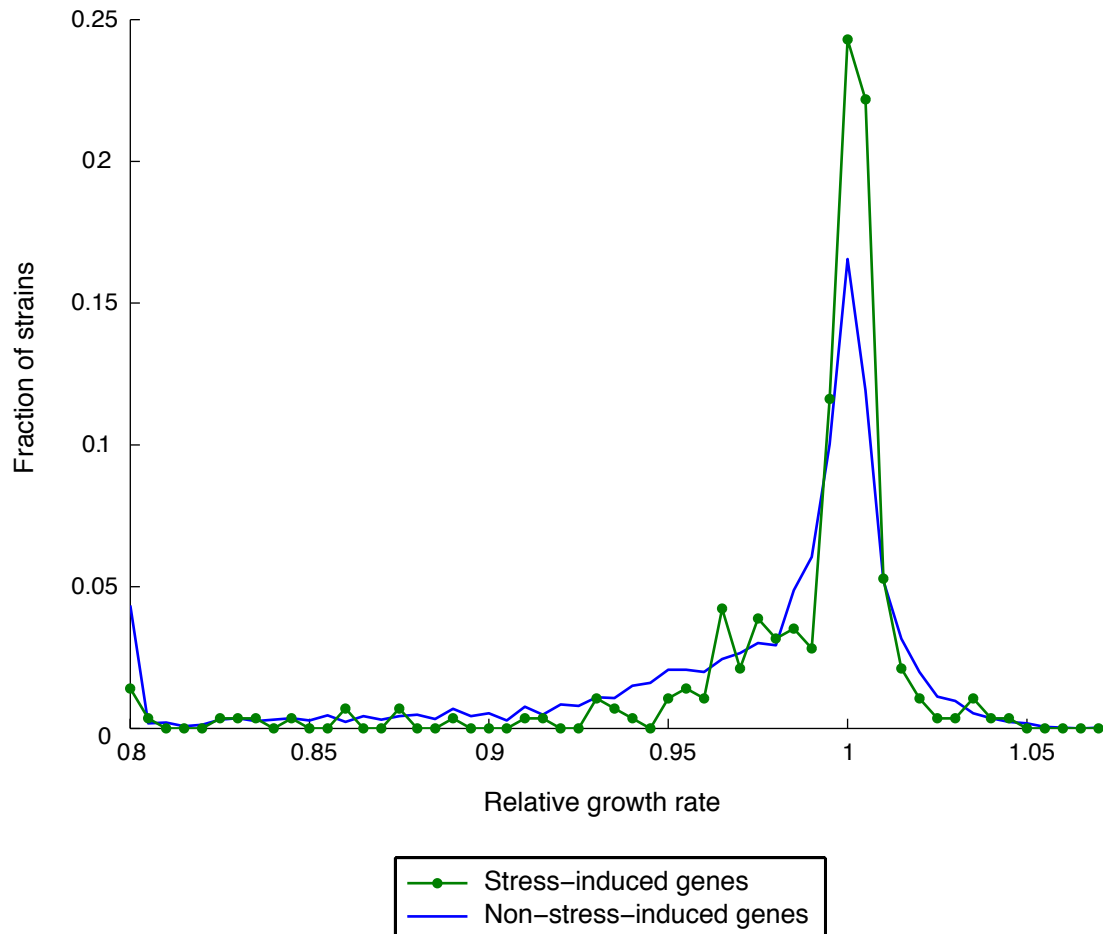
## Supplementary Figure 2.5



### Supplementary Figure 2.5. Effects of gene deletions on growth are correlated with expression levels.

For gene deletions measured in our dataset and in reference 24, genes were ranked according to their growth rate in each dataset. The cumulative distributions of growth rate ranks are plotted for genes with low (bottom 10%), intermediate (10th – 90th percentiles), and high (top 10%) expression levels, as measured by Wang *et al.*<sup>105</sup>. Genes with low expression levels tend not to have growth rate defects in our dataset ( $P = 1\text{E-}7$ , Kolmogorov-Smirnov test), whereas genes with high expression levels are enriched for growth rate defects ( $P = 2\text{E-}16$ , Kolmogorov-Smirnov test). Notably, these effects are also apparent in ranked growth data from reference 24 ( $P = 0.008$  for low expression levels,  $P = 1\text{E-}12$  for high expression levels).

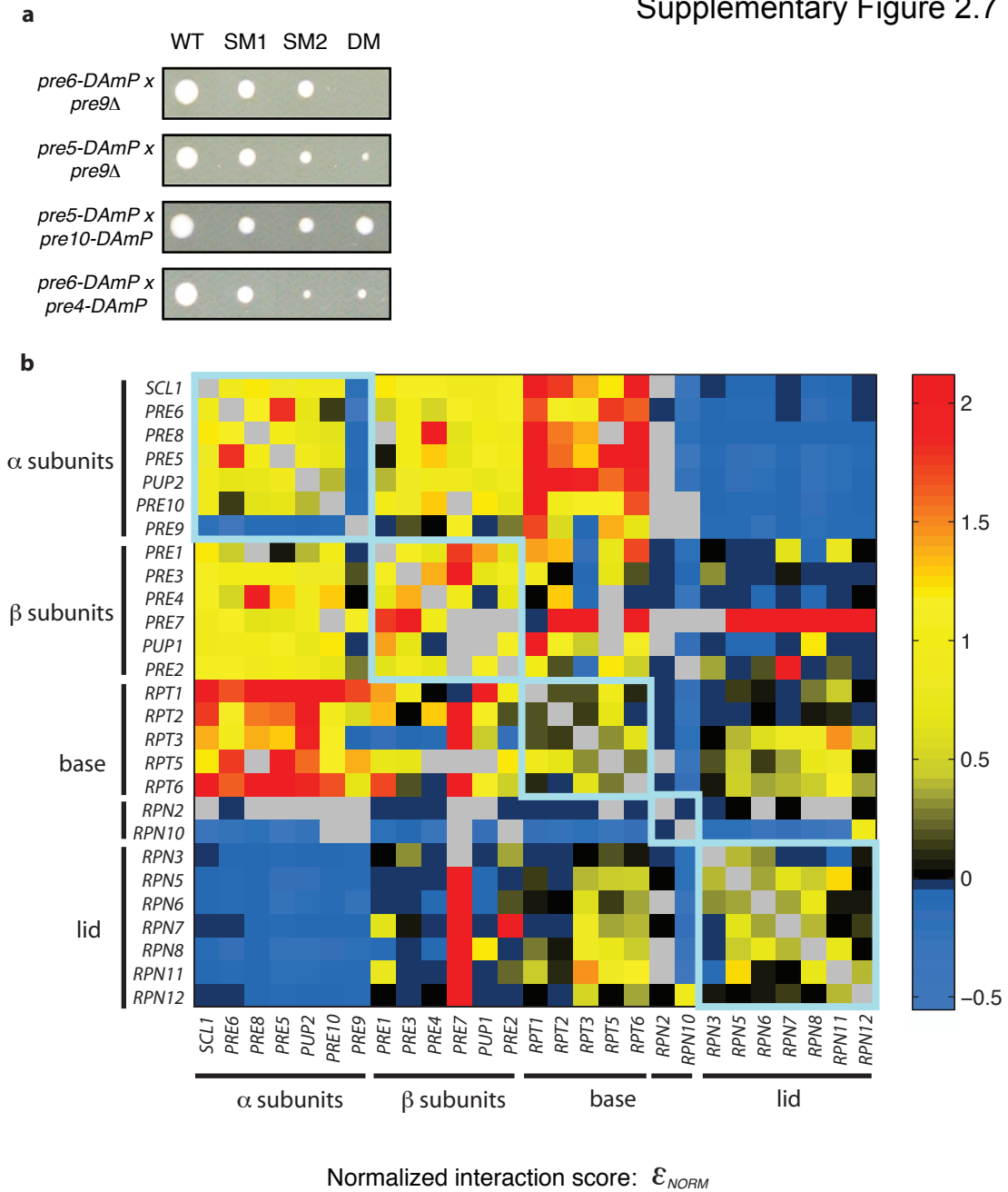
Supplementary Figure 2.6



**Supplementary Figure 2.6. Relationship between growth defect and participation in the environmental stress response.**

Genes needed to respond to stress<sup>106</sup> have an increased likelihood of being dispensable for rapid growth in our assay. Notably, enrichment for stress-responsive genes is seen for fitness values ranging from ~0.99-1.01, but not for genes with even slight growth defects (i.e. growth from 0.97-0.99). This result suggests that small growth defects captured by our assay are functionally relevant.

Supplementary Figure 2.7

**Supplementary Figure 2.7. Genetic interactions between proteasome genes.**

(a) Tetrad analysis confirms negative genetic interactions between *pre9Δ* and DAmP alleles of other alpha subunits, particularly *pre6-DAmP*. In general, however, genes encoding alpha subunits exhibit positive interactions both with each other (for example, between *pre5-DAmP* and *pre10-DAmP*) and with genes encoding beta subunits (for example, *pre6-DAmP* and *pre4-DAmP*). WT = wildtype; SM1 = single mutant 1; SM2 = single mutant 2; DM = double mutant. (b) Normalized genetic interaction scores ( $\mathcal{E}_{NORM}$ ) between proteasome genes.  $\mathcal{E}_{NORM}$  was calculated as described in Supplementary Methods.

**Supplementary Table 2.1. Relative growth rates for TAP, TAP-DAmP and TAP-degron+DAmP alleles.**

Also shown is the fold reduction in protein abundance for the TAP-degron+DAmP strains relative to the corresponding TAP-DAmP strain. Note, growth rates slightly greater than 1.00 likely reflect the fact that SGA markers were absent from the TAP-tagged library derivatives<sup>29</sup>, but present in the wildtype competitor strain.

| <b>GENE</b>           | <b>TAP</b> | <b>TAP-DAmP</b> | <b>TAP-degron+DAmP</b> | <b>Fold protein reduction (deg. vs. DAmP)</b> |
|-----------------------|------------|-----------------|------------------------|-----------------------------------------------|
| <i><b>RHO3</b></i>    | 1.015      | 0.967           | 0.958                  | 2.4                                           |
| <i><b>DIP2</b></i>    | 1.017      | 0.981           | 1.004                  | 3.6                                           |
| <i><b>CKS1</b></i>    | 1.023      | 0.820           | 0.788                  | 4.6                                           |
| <i><b>FAS2</b></i>    | 0.972      | 0.947           | 0.771                  | 6.1                                           |
| <i><b>PCM1</b></i>    | 1.029      | 0.984           | 0.668                  | 6.8                                           |
| <i><b>CCA1</b></i>    | 1.018      | 0.950           | 0.583                  | 8.8                                           |
| <i><b>YNL313C</b></i> | 1.024      | 0.952           | 0.696                  | 9.2                                           |
| <i><b>YDR531W</b></i> | 1.018      | 0.943           | 0.545                  | 21.3                                          |



**Supplementary Table 2.2. Estimated true and false positive rates for identification of strains with growth defects.**

Estimates are shown as a function of observed growth rate (for observed growth rates < 1.0) for deletions strains **(a)** and DAmP strains **(b)**.

**(a) Deletion Strains:**

| Fitness cutoff | Cumulative # strains | Cumulative Fraction positive | Cumulative # positive | Cumulative # negative | Incremental # positive | Incremental # negative | Incremental fraction positive |
|----------------|----------------------|------------------------------|-----------------------|-----------------------|------------------------|------------------------|-------------------------------|
| 0.900          | 438                  | 1.000                        | 438                   | 0                     |                        |                        |                               |
| 0.905          | 455                  | 0.998                        | 454                   | 1                     | 16                     | 0                      | 0.99                          |
| 0.910          | 477                  | 0.998                        | 476                   | 1                     | 22                     | 0                      | 0.98                          |
| 0.915          | 497                  | 0.996                        | 495                   | 2                     | 19                     | 1                      | 0.97                          |
| 0.920          | 529                  | 0.996                        | 527                   | 2                     | 32                     | 1                      | 0.98                          |
| 0.925          | 561                  | 0.993                        | 557                   | 4                     | 30                     | 1                      | 0.96                          |
| 0.930          | 595                  | 0.992                        | 590                   | 5                     | 33                     | 2                      | 0.95                          |
| 0.935          | 645                  | 0.988                        | 637                   | 8                     | 47                     | 2                      | 0.95                          |
| 0.940          | 696                  | 0.984                        | 685                   | 11                    | 48                     | 3                      | 0.94                          |
| 0.945          | 756                  | 0.980                        | 741                   | 15                    | 56                     | 4                      | 0.93                          |
| 0.950          | 835                  | 0.976                        | 815                   | 20                    | 74                     | 5                      | 0.93                          |
| 0.955          | 909                  | 0.971                        | 883                   | 26                    | 68                     | 7                      | 0.91                          |
| 0.960          | 992                  | 0.966                        | 958                   | 34                    | 75                     | 8                      | 0.90                          |
| 0.965          | 1081                 | 0.959                        | 1037                  | 44                    | 79                     | 10                     | 0.89                          |
| 0.970          | 1200                 | 0.954                        | 1145                  | 55                    | 108                    | 11                     | 0.91                          |
| 0.975          | 1309                 | 0.948                        | 1241                  | 68                    | 96                     | 13                     | 0.88                          |
| 0.980          | 1446                 | 0.943                        | 1364                  | 82                    | 123                    | 15                     | 0.89                          |
| 0.985          | 1610                 | 0.939                        | 1511                  | 99                    | 147                    | 16                     | 0.90                          |
| 0.990          | 1820                 | 0.930                        | 1692                  | 128                   | 181                    | 29                     | 0.86                          |
| 0.995          | 2141                 | 0.865                        | 1852                  | 289                   | 160                    | 161                    | 0.50                          |
| 1.000          | 2728                 | 0.687                        | 1875                  | 853                   | 23                     | 563                    | 0.04                          |

**(b) DamP strains:**

| Fitness cutoff | Cumulative # strains | Cumulative Fraction positive | Cumulative # positive | Cumulative # negative | Incremental # positive | Incremental # negative | Incremental fraction positive |
|----------------|----------------------|------------------------------|-----------------------|-----------------------|------------------------|------------------------|-------------------------------|
| 0.900          | 75                   | 1.000                        | 75                    | 0                     |                        |                        |                               |
| 0.905          | 81                   | 1.000                        | 81                    | 0                     | 6                      | 0                      | 1.00                          |
| 0.910          | 87                   | 1.000                        | 87                    | 0                     | 6                      | 0                      | 1.00                          |
| 0.915          | 89                   | 1.000                        | 89                    | 0                     | 2                      | 0                      | 1.00                          |
| 0.920          | 93                   | 1.000                        | 93                    | 0                     | 4                      | 0                      | 1.00                          |
| 0.925          | 97                   | 1.000                        | 97                    | 0                     | 4                      | 0                      | 1.00                          |
| 0.930          | 104                  | 1.000                        | 104                   | 0                     | 7                      | 0                      | 1.00                          |
| 0.935          | 107                  | 1.000                        | 107                   | 0                     | 3                      | 0                      | 1.00                          |
| 0.940          | 113                  | 1.000                        | 113                   | 0                     | 6                      | 0                      | 1.00                          |
| 0.945          | 120                  | 1.000                        | 120                   | 0                     | 7                      | 0                      | 1.00                          |
| 0.950          | 128                  | 1.000                        | 128                   | 0                     | 8                      | 0                      | 1.00                          |
| 0.955          | 135                  | 1.000                        | 135                   | 0                     | 7                      | 0                      | 1.00                          |
| 0.960          | 144                  | 1.000                        | 144                   | 0                     | 9                      | 0                      | 1.00                          |
| 0.965          | 153                  | 1.000                        | 153                   | 0                     | 9                      | 0                      | 1.00                          |
| 0.970          | 160                  | 1.000                        | 160                   | 0                     | 7                      | 0                      | 1.00                          |
| 0.975          | 169                  | 1.000                        | 169                   | 0                     | 9                      | 0                      | 1.00                          |
| 0.980          | 186                  | 1.000                        | 186                   | 0                     | 17                     | 0                      | 0.98                          |
| 0.985          | 209                  | 0.995                        | 208                   | 1                     | 22                     | 1                      | 0.96                          |
| 0.990          | 252                  | 0.976                        | 246                   | 6                     | 38                     | 4                      | 0.90                          |
| 0.995          | 342                  | 0.927                        | 317                   | 25                    | 71                     | 19                     | 0.79                          |
| 1.000          | 526                  | 0.688                        | 362                   | 164                   | 45                     | 139                    | 0.24                          |

## Supplementary Note

We estimated the number of deletion strains previously reported to have growth defects in similar conditions using a variety of methods. First, among the 4204 deletion strains we measured, growth rates for 4098 were also measured in minimal media in reference 24. Using the authors' suggested relative growth cutoff of 0.95, 542 of 4098 gene deletions (~13%) were found to be significantly slow-growing in this study. Second, minimal media growth data have been reported in reference 25 for 4122 of the deletion strains in our study. Using the suggested growth defect cutoff of -0.055 (this study computed growth defects on a different scale), 710 of 4122 gene deletions (~17%) were found to be significantly slow-growing. Combining these two studies, ~16% showed on average a significant growth defect, with 22% showing a growth defect in at least one of the two studies. Finally, to make a more direct comparison that is independent of the choice of cutoffs, we analyzed the replicate data available for growth in rich media published in reference 24 using the error model calculation procedure that we applied to our deletion and DAmP library data. With this analysis, we estimate that a total of ~1060 of 4146 deletion strains (~26%) have reduced fitness in reference 24.

## Supplementary Methods

### Growth media and Strain Construction

Details of growth media used have been described previously<sup>118</sup>. For growth competition experiments, we used minimal media lacking histidine, lysine and arginine and containing canavanine (50 µg/ml; SIGMA), s-Aminoethyl-cysteine (50 µg/ml, SIGMA), G418 (200 µg/ml) and clonNAT (200 µg/ml; Werner BioAgents).

DAmP alleles were constructed in a diploid strain derived from strains BY4741<sup>119</sup> (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 CYH2+/cyh2*) by transformation with a PCR product encoding the kanamycin-resistance (Kan<sup>R</sup>) cassette (see below) flanked at either end by 40 nucleotides of homology to the site of integration. Transformants were selected on YEPD containing 200 µg/ml G418 (GENETICIN; Invitrogen), then grown to saturation in 1 ml liquid YEPD+G418. Lysates were made by boiling cells in 20 mM NaOH for 20 minutes, vortexing briefly, then adding an equal volume of water and centrifuging at 2500 RCF for 2 minutes. Correct insertion of the Kan<sup>R</sup> cassette was confirmed by PCR using gene-specific forward primers and a universal reverse primer complementary to the promoter region of the Kan<sup>R</sup> cassette. Correct diploid strains were transformed with the *URA3*-marked plasmid pRS316-*STE2pr-SpHIS5* encoding the *S. pombe HIS5* gene driven by a *MATa*-specific promoter. Transformed diploids were sporulated and *MATa* DAmP haploids selected on synthetic media lacking histidine and supplemented with cycloheximide (10 µg/ml) and G418 (200 µg/ml). Following a repeated PCR to confirm correct integration of the Kan<sup>R</sup> cassette, DAmP haploids were passaged on 5-Fluorouracil (5-FOA) to ensure loss of the haploid-selection plasmid and then stored at -80°C in 15% glycerol.

To generate the TAP-tagged DAmP and DAmP+Degron strains, we constructed plasmids bearing the TAP sequence either with or without a C-terminal CL1 degron, followed by the Kan<sup>R</sup> cassette (see below). Because the Kan<sup>R</sup> cassette utilizes the same downstream regulatory sequences as the *HIS3* marker contained within the all strains in the TAP library<sup>29</sup> (the *TEF2* terminator), restriction fragments generated from these plasmids can integrate with high efficiency due to homology with both the TAP tag and the *TEF2* terminator. Strains were transformed into existing TAP-tagged strains<sup>29</sup> with restriction fragments and transformants selected on YEPD+G418. Transformants were confirmed by PCR as described above using primers annealing to the TAP tag and the promoter region of the Kan<sup>R</sup> cassette.

To generate constructs suitable for GFP and RFP expression, we inserted a cassette containing the *TEF2* promoter, GFP or RFP (dTomato<sup>117</sup>) and the *ADHI* terminator (cloned from *Ashbya gossypii*) into the *pFA6a-NATMX4* plasmid (see below). This construct was then integrated into either the *his3Δ1* or *leu2Δ0* locus of a *MATα* strain compatible with Synthetic Genetic Array (SGA) methodology (*MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 can1Δ::STE2pr-SpHIS5 lyp1Δ::STE3pr-LEU2*). The resulting strain was then crossed to the deletion and DAmP libraries and SGA-based methods were used to generate haploid GFP-tagged mutant strains for use in growth rate measurements. The RFP-expressing wild-type reference strain used in the growth competitions was constructed in an analogous manner (so that it would be isogenic to the mutant query strains) and contained the Kan<sup>R</sup> cassette at *his3Δ1* and the NAT<sup>R</sup>-dTomato construct at the *leu2Δ0* locus.

GFP-tagged double-mutant deletion/DAmP strains for genetic interaction analysis were generated by crossing BY4741-derived *MATa* single-mutant Kan<sup>R</sup> strains from the deletion/DAmP libraries (or freshly created deletion strains made by standard PCR-based gene disruption) with SGA-compatible *MATα* single-mutant NAT<sup>R</sup> strains that were independently constructed by replacement of the gene of interest with the NAT<sup>R</sup>-GFP cassette described above. Following sporulation, double-mutant haploids were selected using standard SGA methodology. Because growth measurements of strains bearing the appropriate individual gene deletions were also required for genetic interaction calculations (see below), isogenic single-mutant strains were generated by crossing the same Kan<sup>R</sup> and NAT<sup>R</sup>-GFP marked single gene deletion strains with appropriate wild-type strains bearing the Kan<sup>R</sup> or NAT<sup>R</sup> marker integrated at the *his3ΔI* locus.

### **Quantification of protein abundance**

The relative abundance of proteins generated from the TAP-DAmP and TAP-Degron+DAmP alleles was determined by Western blot analysis as previously described<sup>29</sup> using a TAP-specific antibody (rabbit anti-CBPTEV-N peptide, Bethyl Laboratories, Inc.) as well as an antibody against endogenous 3-phosphoglycerate kinase (Pfkfb3; Molecular Probes, Inc.) to control for variation in loading. Bands were detected and quantitated using an Odyssey Infrared Imaging System (Licor Biosciences).

### **Drug sensitivity assays**

For disk analysis, 1/4 inch paper disks (Schleicher & Schuell) were immersed in the various drugs at concentrations as follows: 10 mg/ml 5-FOA (SIGMA); 10 mg/ml

Clotrimazole (SIGMA); 50 mg/ml Sulfanilamide (SIGMA). Disks were placed on SD-complete plates on which  $10^6$  yeast cells of the appropriate genotype were plated. Fitness measurements of DAmP strains in tunicamycin were made using the flow cytometry technique as described above except media was supplemented with 0.25  $\mu\text{g/ml}$  tunicamycin (SIGMA) following the initial T0 cytometry measurement.

### **RNA isolation and Quantitative PCR**

RNA isolation and Quantitative PCR were performed as described previously<sup>7</sup>.

### **Data processing and analysis**

#### *Calculation of the ratio of mutant to wild-type cells for each flow cytometry measurement*

First, unreliable cell measurements were identified and removed. For example, events counted in the first 0.2s and after 5.0s were omitted from analysis, as these time segments occasionally contained aberrant fluorescence values. Additionally, to eliminate non-cellular debris, counted events were excluded if they had undefined forward or side scatter intensities (i.e. equal to 0 or the maximum value of  $2^{18}$ ), or if they fell in the highest or lowest 0.1 percentile of FSC values or 0.2 percentile of SSC values. Typically, ~30,000 cells remained after these filters. Next, each cell measured was assigned to one of four categories: RFP<sup>+</sup>/GFP<sup>-</sup> (wild-type), GFP<sup>+</sup>/RFP<sup>-</sup> (mutant), GFP<sup>+</sup>/RFP<sup>+</sup> (rare events caused by simultaneous counting of a wild-type and mutant cell), and GFP<sup>-</sup>/RFP<sup>-</sup> (very rare non-fluorescent cells). For GFP<sup>-</sup>/RFP<sup>+</sup> cells, it was straightforward to classify them as wild-type on the basis of their reproducibly high RFP levels (analyzed using the PE-Texas Red 'Height' parameter) and low GFP intensity (analyzed using the FITC 'Height'

parameter). GFP-containing mutant cells were also clearly identifiable but occasionally exhibited minor strain-to-strain variability in GFP intensity and RFP-channel auto-fluorescence, and so were counted using a modified strategy that automatically determined appropriate boundaries for a gate drawn around the GFP-expressing mutant cells. A similar strategy was used to identify GFP<sup>+</sup>/RFP<sup>+</sup> cells, and any cells that did not fall into the first three categories were assigned as GFP<sup>-</sup>/RFP<sup>-</sup>. It is worth noting that the existence of rare GFP<sup>+</sup>/RFP<sup>+</sup> cells (typically ~2.5% of all cells) reflects the fact that two cells can occasionally pass through the cytometer simultaneously and be recorded as a single event. Although the possible distorting effects of such events are likely quite small due to their rarity, we corrected for this issue by calculating the number of true individual mutant and wild-type cells from the raw numbers of GFP<sup>+</sup>/RFP<sup>-</sup>, GFP<sup>-</sup>/RFP<sup>+</sup>, and GFP<sup>+</sup>/RFP<sup>+</sup> (assuming that any two cells have an equal probability of being mis-recorded as a single event).

#### *Relative growth rate calculation*

Relative growth rates were calculated from the rate of change of the ratio of mutant to wild-type cells over the course of the competition. Assuming a constant relative growth rate  $x$  for the mutant strain and an initial ratio of mutant to wild-type cells  $r_0$ , then  $r_n$  (the ratio after a given number of generations,  $n$ ) is given by the equation:

$$r_n = r_0 \times 2^{[n \times (x - x_{WT})]},$$

where 1 is the growth rate of the wild-type strain. Thus,  $\log_2(r_n)$  changes linearly during the competition with slope equal to  $x - x_{WT}$ , i.e. the relative growth defect of the mutant strain. We therefore calculated this slope for each strain using the four timepoints at

which we measured the co-cultures by flow cytometry using a weighted linear least-squares fit (with weights for each timepoint determined by the expected counting error given the actual number of GFP and RFP cells recorded). Also, because  $n$  in the above equation represents the number of doublings for the wild-type strain (and not for the overall co-culture), we used the change in the ratio between each pair of timepoints to calculate the number of wild-type doublings from the number of co-culture doublings. In addition, for very sick strains, we commonly found that after an initial large decrease in the ratio of mutant to wild-type cells, by the final timepoint the ratio had leveled out at  $\sim 2^{-10} - 2^{-14}$  ( $\sim 0-40$  mutant cells) and did not continue to change, suggesting that this is our lower limit of detection. We therefore only used the first timepoint with a ratio  $< 2^{-6}$  and excluded all timepoints with measured ratios  $< 2^{-9}$ . In the event that fewer than two timepoints remained, we assigned the mutant strain a relative fitness of “ $<0.50$ ”.

#### *Identification and exclusion of unreliable measurements*

A number of criteria were used to identify and eliminate potentially unreliable measurements. First, strains that did not pass through the strain construction process, as identified by an initial ratio of mutant to wild-type  $< 2^{-6}$ , were removed from subsequent analyses. Second, individual timepoints for a strain in which the RFP or GFP intensity fluctuated significantly during the course of reading the well were removed. Similarly, individual timepoints for a strain were excluded if  $> 2.5\%$  of cells were scored as GFP<sup>-</sup>/RFP<sup>-</sup> (typically the result of a technical problem with the flow cytometer high-throughput sampler causing aberrant fluorescence recordings). Third, strains were removed in cases where a good linear fit of  $\log_2(\text{ratio of mutant to wild-type cells})$  versus



the number of generations elapsed could not be obtained, as determined by the total sum of the square of the residuals or by the presence of an individual residual that was unusually large. Fourth, genes required for mating and for the biosynthesis of histidine, lysine and arginine were excluded from analysis. We note that the number of strains removed using these criteria was limited. Thus, the vast majority of the ~600 deletion strains not included in our final data set are absent not because they were measured and excluded but because they were not present in our deletion library.

#### *Data normalization*

Because for each experiment we inoculated all co-cultures with a wild-type RFP strain that was originally grown from a single colony, it was important to control for the possibility of slight experiment-to-experiment variability in the growth rate of the wild-type strain. We therefore normalized the growth rates measured for each experiment (typically involving the measurement of ~2500 mutant strains) such that the most common or ‘typical’ mutant had relative growth = 1.0000. To find the peak of the distribution of growth rates for each experiment, we used a Parzen window smoothing strategy<sup>120</sup> in which the window was a normal distribution with  $\sigma = 0.0045$ . As illustrated in Supplementary Figure 2.2, this process clearly identifies the peak of the distribution. Furthermore, using the peak of the distribution rather than a property related to the median allowed us to avoid imposing any artificial bias on the number of strains with growth defects and seems reasonable under the assumption that the most common deletion strain will have growth that is indistinguishable from wild-type. In further support of this strategy, measurements of the growth rate of a wild-type control strain not

expressing GFP ranged from 1.0004 to 1.0022 after normalization (Supplementary Fig. 2.2).

#### *Error analysis for growth rate measurements*

Estimates of the number of strains with a growth defect were calculated based on the differences between replicate measurements of identical strains. Specifically, if measurement error is symmetrically distributed about zero, then the distribution of differences between replicate measurements can be used to construct a distribution of error expected for an arbitrary number of measurements. This is possible because the distribution of differences in replicate measurements is equal to the convolution of the overall distribution of measurement errors with itself. For measurements of the deletion library, we modeled the distribution of measurement errors as a sum of two Gaussian distributions with parameters determined by nonlinear regression:  $p(x) = 0.8453 \times N(0, 0.0045) + 0.1457 \times N(0, 0.0334)$ , where  $N(\mu, \sigma)$  is a normal distribution with mean  $\mu$  and standard deviation  $\sigma$ . Importantly, this error model accurately reproduced our observed distribution of differences between replicate measurements (Supplementary Fig. 2.3). We then estimated the distribution of measured growth rates resulting from deletion strains with true growth rate 1.0 by normalizing and translating (by 0.001) the error distribution to match the peak of the histogram of all observed growth rates (Fig. 2.3d). Confidence that a strain with a given measured growth rate grows more slowly than wild-type can then be estimated as the fraction of strains with that approximate growth rate that are not accounted for by the error model. A similar procedure was used for the DAmP strains, except that since each of the DAmP strains was measured twice, there was

no need to estimate the distribution of errors that gave rise to the observed distribution of replicate differences. Instead, the observed histogram of averaged growth rates was compared to an empirically generated error model constructed directly from the set of differences between replicate measurements divided by two. In each case, since measurement error increases for substantially slow-growing strains, only strains with a median growth rate of 0.95 or higher were used to generate the error distributions.

#### *Calculation of genetic interactions scores*

Interaction scores were calculated according to the equations for  $\epsilon$  and  $\epsilon_{\text{NORM}}$  described above (see Results). One exception is that, as originally proposed<sup>5</sup>, a different normalization procedure is used for synthetic interactions, such that synthetic lethality gives a normalized score of -1 ( $\epsilon_{\text{NORM}} = \epsilon / (W_A * W_B$ , for  $\epsilon < 0$ ). For *SET2* and genes encoding components of the Rpd3 and COG complexes, Kan<sup>R</sup>- and NAT<sup>R</sup>-marked deletion strains for each gene were used, thereby providing two independently constructed strains for each double mutant of interest. Fitness data from each separately constructed strain were averaged; these data were typically in very high agreement and used to calculate and normalize genetic interaction scores. This averaging was not performed for proteasome mutant strains, as the separate Kan<sup>R</sup>- and NAT<sup>R</sup>-marked DAmP strains did not necessarily have identical growth rates. Instead, fitnesses and raw interaction scores were separately calculated for each independently constructed strain (i.e. *yfg1-DAmP::Kan<sup>R</sup>* x *yfg2-DAmP::NAT<sup>R</sup>* and *yfg2-DAmP::Kan<sup>R</sup>* x *yfg1-DAmP::NAT<sup>R</sup>*) and then averaged to generate average raw interaction scores. Similarly, because *yfg-DAmP::Kan<sup>R</sup>* and *yfg-DAmP::NAT<sup>R</sup>* do not necessarily have identical growth

rates, it is possible that each construction of the *yfg1-DAmP* / *yfg2-DAmP* strain should be normalized using a different normalization factor. Thus, we averaged these normalization factors and used the resulting value to normalize raw interaction scores. Importantly, this averaging does not distort the normalized interaction scores obtained.

## Sequences for plasmids used in Chapter 2

### pFA6a-KANMX6-DAmP :

GAACGCGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTCGACGGATCCCCGGGTAAATTAAGGCGGCCAG  
ATCTGTTTAGCTTGCCCTCGTCCCCGCCGGGTACCCGGCCAGCGACATGGAGGCCCAGAATACCCTCCTTG  
ACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCAT  
GTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCAAGCAAAAATTACGGCTCCTCGCTGCA  
GACCTGCGAGCAGGGAAACGCTCCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCCTGTAGAGAA  
ATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTCATATACTTCCTTTTAAATCCTTGCTAGGATAC  
AGTTCTCACATCACATCCGAACATAAACAACCATGGGTAAGGAAAAGACTCACGTTTCGAGGCCGCGATTA  
AATTC AACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTGGGCAATCAGGTGCGAC  
AATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCA  
ATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCAT  
TTTATCCGTACTCCTGATGATGCATGGTTACTCACCATGCGATCCCCGGCAAAACAGCATTTCCAGGTATT  
AGAAGAATATCCTGATTAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTTCTGCGCCGGTTGCATTGCA  
TTCTGTTTGTAATTGTCCTTTTAAACAGCGATCGCGTATTTTCGTCTCGCTCAGGCGCAATCACGAATGAAT  
AACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGA  
AATGCATAAGCTTTTGCCATTCTCACCAGATTAGTCGTCATCGTCACTCATGGTGATTTCTCACTTGATAACCTTA  
TTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGAT  
CTTGCCATCCTATGGAAGTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTTCAAAAATATGG  
TATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGTACTGA  
CAATAAAAAGATTCTTGTTTTCAAGAACTTGTCAATTTGTATAGTTTTTTTTATATTGTAGTTGTTCTATTTT  
AATCAAATGTTAGCGTGATTTATATTTTTTTTTCGCCTCGACATCATCTGCCAGATGCGAAGTTAAGTGCG  
CAGAAAGTAATATCATGCGTCAATCGTATGTAATGCTGGTCGCTATACTGCTGTGATTGATACTAACG  
CCGCCATCCAGTGTCGAAAACGAGCTCGAATTCATCGATGATATCAGATCCACTAGTGGCCTATGCGGCCG  
CGGATCTGCCGGTCTCCCTATAGTGAGTCGTATTAATTTTCGATAAGCCAGGTTAACCTGCATTAATGAATC  
GGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG  
CTCGGTCTGTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAG  
GGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTG  
CTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCG  
AAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCCCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGA  
CCCTGCGCGTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCAATGCTCACGC  
TGTAGGTATCTCAGTTTCGGTGTAGGTCGTTTCGCTCAAAGCTGGGCTGTGTGCACGAACCCCCGTTACGCC  
CGACCGCTGCGCCTTATCCGGTAACATATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGG  
CAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGG  
CCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAA  
AAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGC  
AGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTTCTACGGGTCTGACGCTCAGTGG  
AACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAA  
TTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAA  
TCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAG  
ATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACC  
GGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTAT  
CCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTTCGCCAGTTAATAGTTTGCGC  
AACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTACAGCTCCGG  
TTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCCTC  
CGATCGTTGTGCAAGTAAGTTGGCCGCGAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTT  
ACTGTGATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTG  
TATGCGGCGACCGAGTTGCTCTTGCCCGGCGCTCAATACGGGATAATACCGCGCCACATAGCAGAAGTTAA  
AAGTGCTCATCATTTGGAACGTTCTTCGGGGCGAAAACCTCAAGGATCTTACCAGCTGTTGAGATCCAGT  
TCGATGTAACCCACTCGTGACCCCACTGATCTTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGC  
AAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCT  
TCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT  
TAGAAAAATAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCAT  
TATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTTCGGTGATG

ACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGC  
AGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGCTGGCTTAACCTATGCGGCATCAGA  
GCAGATTGTACTGAGAGTGCACCATATGGACATATTGTCGTTAGAACGCGGCTACAATTAATACATAACCT  
TATGTATCATACATACGATTTAGGTGACACTATA

**TAP-DAmP (KAN) :**

GGTCGACGGATCCCCGGG**TTAATTAA**TCCATGGAAGAGAAGATGGAAAAAGAATTTTCATAGCCGTCTCAGC  
AGCCAACCGCTTTAAGAAAATCTCATCTCCGGGGCACTTGATTATGATATTCCAACCTACTGCTAGCGAGA  
ATTTGTATTTTCAGGGAGAATTCGGCCTTGCGCAACACGATGAAGCCGTGGACAACAAATTCAACAAAGAA  
CAACAAAACGCGTTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTCATCCA  
AAGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCTCAGG  
CGCCGAAAGTAGACAACAAATTCAACAAAGAACAACAAAACGCGTTCTATGAGATCTTACATTTACCTAAC  
TTAAACGAAGAACAACGAAACGCCTTCATCCAAAGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTT  
AGCAGAAGCTAAAAAGCTAAATGATGCTCAGGCGCCGAAAGTAGACGCGAATCATCAGTGAacatggaggc  
ccagaataccctccttgacagtccttgacgtgcgagctcaggggcatgatgtgactgtcgcccgtaacatt  
agcccatatacctccatgtataatcatttgcatccatacattttgatggcgacggcggaagcaaaaat  
tacggctcctcgctgcagacctgcgagcagggaaacgctccctcacagacgcgttgaaattgtccccacgc  
cgcgccctgttagagaaatataaaagggttaggatttgccactgaggttcttctttcatataacttctttta  
aaatcttgctaggatacagttctcacatcacatccgaacataaacaacc**atgggtaaggaaaagactcacg**  
**tttcgaggccgcgattaaattccaacatggatgctgatttataatgggtataaatgggctcgcgataatgtc**  
**gggcaatcagggtgcgacaatctatcgattgtatgggaagcccgatgcgccagagttgtttctgaaacatgg**  
**caaaggtagcggttgccaatgatgttacagatgagatggtcagactaaactggctgacggaatttatgcctc**  
**ttccgaccatcaagcattttatccgtactcctgatgatgcattggttactcaccactgcgatccccggcaaa**  
**acagcattccagggtattagaagaatatcctgattcagggtgaaaatattggttgatgcgctggcagtggtcct**  
**gcgccggttgcatcgtattcctggttgtaattgtccttttaacagcgatcgcgtatttcgtctcgtcagg**  
**cgcaatcacgaatgaataacgggttggttgatgcgagtgattttgatgacgagcgtaattggctggcctggt**  
**gaacaagtctggaaagaaatgcataagcttttgccattctcaccggattcagtcgtcactcatggtgattt**  
**ctcacttgataaccttatttttgacgaggggaaattaataggttgatttgatggttgacgagtcggaatcg**  
**cagaccgataaccaggatcttgccatcctatggaactgcctcggtgagttttctccttcatcagaaacggg**  
**ctttttcaaaaatattggtattgataatcctgatgatgaataaattgcagtttcatttgatgctcgatgagtt**  
**ttttcaat**cagtagtgacaataaaaagattcctgtttttcaagaacttgatcatttgatagtttttttatat  
tgtagttgttctatttttaatacaaatgttagcggtgatttatatttttttgcctcgacatcatctgccag  
atgcgaagttaagtgcgcagaaagtaatatcatgcgtcaatcgatgtgaatgctggctgctataactgctg  
tcgattcgataactaacgcgccatccagtgctgaaaaac**gagctc**gaattcatcga

**TAP-DEG+DAmP (KAN) :**

GGTCGACGGATCCCCGGG**TTAATTAA**TCCATGGAAGAGAAGATGGAAAAAGAATTTTCATAGCCGTCTCAGC  
AGCCAACCGCTTTAAGAAAATCTCATCTCCGGGGCACTTGATTATGATATTCCAACCTACTGCTAGCGAGA  
ATTTGTATTTTCAGGGAGAATTCGGCCTTGCGCAACACGATGAAGCCGTGGACAACAAATTCAACAAAGAA  
CAACAAAACGCGTTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTCATCCA  
AAGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCTCAGG  
CGCCGAAAGTAGACAACAAATTCAACAAAGAACAACAAAACGCGTTCTATGAGATCTTACATTTACCTAAC  
TTAAACGAAGAACAACGAAACGCCTTCATCCAAAGTTTAAAGATGACCCAAGCCAAAGCGCTAACCTTTT  
AGCAGAAGCTAAAAAGCTAAATGATGCTCAGGCGCCGAAAGTAGACGCGAATCATCAG**GGTGGTTCTGGTT**  
**CTGCTTGTA**AAAAATTGGTTTTCTTTGTCTCATTTTTGTTATTTCATT**TGTAA**acatggaggccagaata  
ccctccttgacagtccttgacgtgcgagctcaggggcatgatgtgactgtcgcccgtaacatttagccata  
catcccatgtataatcatttgcatccatacattttgatggcgacggcggaagcaaaaattacggctc  
ctcgctgcagacctgcgagcagggaaacgctccctcacagacgcgttgaaattgtccccacgcgcgcccc  
tgtagagaaatataaaagggttaggatttgccactgaggttcttctttcatataacttcttttaaaatcttg  
ctaggatacagttctcacatcacatccgaacataaacaacc**atgggtaaggaaaagactcacg**tttcgagg  
**ccgcgattaaattccaacatggatgctgatttataatgggtataaatgggctcgcgataatgtcgggcaatc**  
**agggtgcgacaatctatcgattgtatgggaagcccgatgcgccagagttgtttctgaaacatggcaaggta**  
**gcggttgccaatgatgttacagatgagatggtcagactaaactggctgacggaatttatgcctcttccgacc**  
**atcaagcattttatccgtactcctgatgatgcattggttactcaccactgcgatccccggcaaaacagcatt**  
**ccagggtattagaagaatatcctgattcagggtgaaaatattggttgatgcgctggcagtggtcctgcgcggt**

tgcattcgattcctgtttgtaattgtccttttaacagcgcgatcgcgatatttcgtctcgcctcaggcgcaatca  
 cgaatgaataacggttttggttgatgagagtgatgtttgatgacgagcgtaatggctggcctgttgaacaagt  
 ctggaaagaaatgcataagcttttgccattctcaccggattcagtcgctcactcatggtgatttctcacttg  
 ataaccttatttttgacgaggggaaattaataggttgattgatgttgacgagtcggaatcgagaccga  
 taccaggatcttgccatcctatggaactgcctcggtgagttttctccttcattacagaaacggctttttca  
 aaaatatggtattgataatcctgatatgaataaattgcagtttcatttgatgctcgatgagtttttctaaat  
 cagtactgacaataaaaaagattcctgtttttcaagaacttgatcatttgatagtttttttatattgtagttg  
 ttctatttttaatacaaatgttagcgtgattttatatttttttcgctcgacatcatctgcccagatgcgaag  
 ttaagtgcgcagaaagtaatatcatgcgtcaatcgatgtgaatgctggtcgctatactgctgctgattcg  
 atactaacgcgcgccatccagtgtcgaaaacgagctcgaattcatcga

Note: The sequence encoding the CL1 degron is highlighted in yellow. Plasmids containing these cassettes were digested with *PacI* and *SacI* (restriction sites underlined) and the resulting fragments used for transformations.

**pFA6a-TEF2Pr-eGFP-ADH1-NATMX4 :**

GAACGCGGCCCGCCAGCTGAAGCTTCGTACGCTGCAGGTCGACGGATCCCCGGGTTAATTAAGGCGCGCCAG  
 ATCTGTTTAGCTTGCTTGTCCCCGCCGGGTACCCGGCCAGCGACATGGAGGCCGAGAATACCCCTCCTTG  
 ACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTGCGCCGTACATTTAGCCCATACATCCCCAT  
 GTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCA  
 GACCTGCGAGCAGGGAAACGCTCCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCCTGTAGAGAA  
 ATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTCATATACTTCCTTTTAAATCCTTGCTAGGATAC  
 AGTTCTCACATCACATCCGAACATAAACAACCATGGGTACCACTCTTGACGACACGGCTTACCGGTACCGC  
 ACCAGTGTCCCCGGGGGACGCCGAGGCCATCGAGGCACTGGATGGGTCTTCACCAACGACACCGTCTTCCG  
 CGTCACCGCCACCGGGGACGGCTTACCCCTGCGGGAGGTGCCGGTGGACCCGCCCTGACCAAGGTGTTCC  
 CCGACGACGAATCGGACGACGAATCGGACGACGGGGAGGACGGCGACCCGGACTCCCGGACGTTCTGTCGCG  
 TACGGGGACGACGGCGACCTGGCGGGCTTCGTGGTCTGCTCTGTAATCCGGCTGGAACCGCCGGCTGACCGT  
 CGAGGACATCGAGGTGCGCCCGGAGCACCGGGGGCACGGGGTGGGGCGCGCTTGATGGGGCTCGCGACGG  
 AGTTGCGCCGCGAGCGGGGCGCGGGCACCTCTGGCTGGAGGTACCAACGTCAACGCACCGGCGATCCAC  
 GCGTACCGGCGGATGGGGTTCACCCCTCTGCGGCTGGACACCGCCCTGTACGACGGCACCGCCTCGGACGG  
 CGAGCAGGCGCTCTACATGAGCATGCCCTGCCCTAATCAGTACTGACAATAAAAGATTCTTGTTTTTCAA  
 GAACTTGTCATTTGTATAGTTTTTTTTTATATTGTAGTTGTTCTATTTTAAATCAAATGTTAGCGTGATTTATA  
 TTTTTTTTCGCTCGACATCATCTGCCAGATGCGAAGTTAAGTGCAGAGAAAGTAATATCATGCGTCAAT  
 CGTATGTGAATGCTGGTGCCTATACTGCTGTCGATTGATACTAACGCCGCCATCCAGTGTGAAAACGAG  
 CTCATATATGGGGCCGTATACTTACATATAGTAGATGTCAAGCGTAGGCGCTTCCCCTGCCGGCTGTGAGG  
 GCGCCATAACCAAGGTATCTATAGACCGCAATCAGCAAACTACCTCCGTACATTCATGTTGCACCCACAC  
 ATTTATACACCCAGACCGCGACAAATTACCCATAAGGTTGTTTGTGACGGCGTCTGACAAGAGAACGTGGG  
 AACTTTTTTAGGCTCACCAAAAAGAAAGAAAAATACGAGTTGCTGACAGAAGCCTCAAGAAAAAAAAT  
 TCTTCTTCGACTATGCTGGAGGCAGAGATGATCGAGCCGGTAGTTAACTATATATAGCTAAATTGGTTCCA  
 TCACCTTCTTTTCTGGTGTGCTCCTTCTAGTGCTATTTCTGGCTTTTCTCTATTTTTTTTTTCCATTTTT  
 CTTTCTCTCTTTCTAATATATAAATTCTCTTGCAATTTCTATTTTCTCTCTATCTATTCTACTTGTATAT  
 TCCCTTCAAGGTTTTTTTTTAAGGAGTACTTGTTTTTAGAAATATACGGTCAACGAACATAATTAACATAAA  
 CACTAGTACCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTG  
 ATGTTAATGGGCACAAATTTTCTGTGAGTGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTT  
 AAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCACTTATGGTGT  
 TCAATGCTTTTCAAGATACCCAGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCGAAGGTT  
 ATGTACAGGAAAGAACTATATTTTTTCAAAGATGACGGGAACACAAAGACACGTGCTGAAGTCAAGTTTGAA  
 GGTGATACCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAACATTCTTGGACA  
 CAAATTGGAATACAACATACTACACAATGTATACATCATGGCAGACAAACAAAAGAAATGGAATCAAAG  
 TTAACCTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACT  
 CCAATTGGCGATGGCCCTGTCTTTTACCAGACAACATTACCTGTCCACACAATCTGCCCTTTTCGAAAGA  
 TCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAAACAGCTGCTGGGATTACACATGGCATGG  
 ATGAACATACAAAATAGGGCCTCGAGGCCCGCTATTAAACGCTTTGTAAATGTATAGCTTTTAAATGTGTGATC  
 GCCTGACTTTTGCACGCCGGCGGGCGGCAGCGCGCTGCGTCCAGGCAGCGTCACGGCGCAGGTGGCGGGCT  
 GTGCGAGCTGGCGCGACTGGCGCGACTGTGCTGTACGTGACGGCGACACGGGTGGAGAAAATTTTTTG

CCAACGGCGCGCAGAGCAGTCCTCGACAACGAGCTCGAATTCATCGACCGCGGATCTGCCGGTCTCCCTAT  
 AGTGAGTCGTATTAATTTTCGATAAGCCAGGTTAACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGC  
 GGTTCGCTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTCGGTGCGGCG  
 AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA  
 TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC  
 CGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAG  
 ATACCAGGCGTTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCTGTTCCGACCCTGCCGCTTACCGGATACC  
 TGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTG  
 TAGGTCTGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGG  
 TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGA  
 TTAGCAGAGCAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGA  
 AGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGAAAAAGAGTTGGTAGCTCTTGATC  
 CGGCAAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAG  
 GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGG  
 ATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATC  
 AATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG  
 CGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGC  
 TTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAAT  
 AAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA  
 ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTTCGCCAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTACA  
 GGCATCGTGGTGTACGCTCGTCTGTTTGGTATGGCTTCATTACGCTCCGGTTCCTAACGATCAAGGCGAGT  
 TACATGATCCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTGAGAAGTAAGT  
 TGGCCGAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTATGCCATCCGTAAGA  
 TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTC  
 TTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAGTGCTCATCATTTGAAAAAC  
 GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCA  
 CCAAATGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGC  
 CGCAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCTTTTCAATATTATTGAA  
 GCATTTGTTCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGG  
 GTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACTTA  
 TAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACA  
 TGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCG  
 TCAGCGGGTGTGGCGGGTGTGCGGGCTGGCTTAAGTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCA  
 CCATATGGACATATTGTCTGTTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGAT  
 TTAGGTGACACTATA

**pFA6a-TEF2Pr-dTomato-ADH1-NATMX4 :**

GAACGCGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTTCGACGGATCCCCGGGTTAATTAAGGCGGCCAG  
 ATCTGTTTAGCTTGCTTGTCCCCGCCGGGTACCCGGCCAGCGACATGGAGGCCAGAAATACCTCTCTTG  
 ACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTGCGCCGTACATTTAGCCCATACATCCCCAT  
 GTATAATCATTTGCATCCATACATTTTATGATGGCCGCACGGCGCAAGCAAAAATTACGGCTCCTCGCTGCA  
 GACCTGCGAGCAGGGAACGCTCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCCTGTAGAGAA  
 ATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTCATATACTTCTTTTAAATCTTGCTAGGATAC  
 AGTTCTCACATCACATCCGAACATAAACAACCATGGGTACCACTCTTGACGACACGGCTTACCGGTACCGC  
 ACCAGTGTCCCGGGGACGCCGAGGCCATCGAGGCACTGGATGGGTCTTACCACCGACACCGCTCTTCCG  
 CGTACCCGCCACCGGGGACGGCTTACCCTGCGGGAGGTGCCGGTGACCCGCCCTGACCAAGGTGTTCC  
 CCGACGACGAATCGGACGACGAATCGGACGACGGGAGGACGGCGACCCGGACTCCCGGACGTTCTGTCGCG  
 TACGGGGACGACGGCGACCTGGCGGGCTTCTGGTCTGCTCTGTAATCCGGCTGGAACCGCCGGCTGACCGT  
 CGAGGACATCGAGGTGCCCCGAGACACCGGGGACCGGGGTGCGGCGCGGTTGATGGGGCTCGCGACGG  
 AGTTCGCCCCGCGAGCGGGGCGCGGGCACCTCTGGCTGGAGGTACCAACGTCAACGCACCGGCGATCCAC  
 GCGTACCGGCGGATGGGGTTACCCTCTGCGGCCTGGACACCGCCCTGTACGACGGCACCGCCTCGGACGG  
 CGAGCAGGCGCTCTACATGAGCATGCCCTGCCCTAATCAGTACTGACAATAAAAAGATTCTTGTGTTTCAA  
 GAACTTGTCAATTTGTATAGTTTTTTTTTATATTGTAGTTGTTCTATTTTAAATCAAATGTTAGCGTGATTTATA  
 TTTTTTTTCGCTCGACATCATCTGCCAGATGCGAAGTTAAGTGCAGAGAAAGTAATATCATGCGTCAAT  
 CGTATGTGAATGCTGGTCTGCTATACTGCTGTCGATTGATACTAACGCCGCCATCCAGTGTGAAAACGAG  
 CTCATATATGGGGCCGTATACTTACATATAGTAGATGTCAAGCGTAGGCGCTTCCCCTGCCGGCTGTGAGG



GCGCCATAACCAAGGTATCTATAGACCGCCAATCAGCAAACCTACCTCCGTACATTCATGTTGCACCCACAC  
ATTTATACACCCAGACCGCGACAAATTACCCATAAGGTTGTTTGTGACGGCGTCGTACAAGAGAACGTGGG  
AACTTTTTTAGGCTCACCAAAAAAGAAAGAAAAAATACGAGTTGCTGACAGAAGCCTCAAGAAAAAAAAT  
TCTTCTTCGACTATGCTGGAGGCAGAGATGATCGAGCCGGTAGTTAACTATATATAGCTAAATTGGTTCCA  
TCACCTTCTTTTTCTGGTGTGCTCCTTCTAGTGCTATTTCTGGCTTTTCTCTATTTTTTTTTTTCATTTTT  
CTTTCTCTCTTTCTAATATATAAATTCTCTTGCATTTTCTATTTTTCTCTCTATCTATTCTACTTGTAT  
TCCCTTCAAGGTTTTTTTTTAAGGAGTACTTGTTTTTAGAATATACGGTCAACGAACCTATAATTAATAAA  
CACTAGTACCATGCTGAGCAAGGGCGAGGAGGTCATCAAAGAGTTCATGCGCTTCAAGGTGCGCATGGAGG  
GCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCGGCCCTACGAGGGCACCCAGACC  
GCCAAGCTGAAGGTGACCAAGGGCGGGCCCCCTGCCCTTCGCGCTGGGACATCCTGTCCCCCAGTTTATGTA  
CGGCTCCAAGGCGTACGTGAAGCACCCCGCCGACATCCCCGATTACAAGAAGCTGTCTTCCCCGAGGGCT  
TCAAGTGGGAGCGCGTGATGAACCTTCGAGGACGGCGGTCTGGTGACCGTGACCCGAGCTCCTCCCTGCAG  
GACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAACTTCCCCCGACGGCCCCGTAATGCAGAA  
GAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCGCGACGGCGTGCTGAAGGGCGAGATCC  
ACCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAGAACCATCTACATGGCCAAGAAG  
CCCGTGCAACTGCCCCGCTACTACTACGTGGACACCAAGCTGGACATCACCTCCCAACGAGGACTACAC  
CATCGTGGAACAGTACGAGCGCTCCGAGGGCGGCCACCACCTGTTCTGTACGGCATGGACGAGCTGTACA  
AGTAAGGCCTCGAGGCGCGCTATTAACGCTTTGTAATGTATAGCTTTTAATGTGTGATCGCCTGACTTTTG  
CACGCCGGCGGGCGGCAGCGCGCTGCGTCCAGGCAGCGTCACGGCGCAGGTGGCGGGCTGTGCGAGCTGGC  
GCGACTGGCGCGACTGTGCTGTACGTGACGGCGACACGGGTGGAGAAAATTTTTGGCCAACGGCGCGC  
AGAGCAGTCTCGACAAACGAGCTCGAATTCATCGACCGCGGATCTGCCGGTCTCCCTATAGTGAGTCGTAT  
TAATTTTCGATAAGCCAGGTTAACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATT  
GGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTCTCGGTCTCGGTGCGGCGAGCGGTATCAGC  
TCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG  
GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGAC  
GAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTT  
TCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC  
TCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCAGCTGTAGGTATCTCAGTTCGGTGTAGGTCTGTTTCG  
TCCAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGA  
GGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTT  
GGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAGAGGTTGGTAGCTCTTGATCCGGCAAACAAAC  
CACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAG  
ATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATG  
AGATTATCAAAAAGGATCTTACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTAT  
ATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT  
TTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGC  
CCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGC  
CGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGG  
AAGCTAGAGTAAGTAGTTGCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTG  
TCACGCTCGTCTGTTTTGGTATGGCTTCATTACGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCC  
CATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGAGTGT  
TATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTGTCATGCCATCCGTAAGATGCTTTTCTGTG  
ACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTC  
AATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAGTGCTCATCATTGGAACCGTTCTTCCGGGC  
GAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGACCCCAACTGATCT  
TCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGG  
AATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCTTTTCAATATTATTGAAGCATTTATCAGG  
GTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACA  
TTTTCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCG  
TATCACGAGGCCCTTTCGTCTCGCGCGTTTCCGGTGATGACGGTGAACCTCTGACACATGCAGCTCCCGG  
AGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGT  
GGCGGGTGTGCGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGGACAT  
ATTGTCGTTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACACT  
ATA

## **Chapter 3**

Identification of Orm Family Proteins as Key Mediators of Sphingolipid Homeostasis

## Orm Family Proteins Mediate Sphingolipid Homeostasis

David K. Breslow<sup>1,2,3,4</sup>, Sean R. Collins<sup>1,2,4†</sup>, Bernd Bodenmiller<sup>5†</sup>, Ruedi Aebersold<sup>5,6,7</sup>, Kai Simons<sup>8</sup>, Andrej Shevchenko<sup>8</sup>, Christer S. Ejsing<sup>8,9</sup> & Jonathan S. Weissman<sup>1,2,4</sup>

<sup>1</sup>Department of Cellular and Molecular Pharmacology, <sup>2</sup>Howard Hughes Medical Institute, <sup>3</sup>Graduate Program in Chemistry and Chemical Biology, <sup>4</sup>The California Institute for Quantitative Biomedical Research, University of California, San Francisco, 1700 4th Street, San Francisco, California 94158, USA. <sup>5</sup>Institute of Molecular Systems Biology, ETH Zurich, 8093 Zurich, Switzerland. <sup>6</sup>Institute for Systems Biology, Seattle, WA 98103, USA, <sup>7</sup>Faculty of Science, University of Zurich, 8057 Zurich, Switzerland, <sup>8</sup>Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany. <sup>9</sup>Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark. †Present addresses: Chemical and Systems Biology, Bio-X Program, Stanford University, Stanford, California 94305, USA (S.R.C.); Department of Microbiology and Immunology, Baxter Laboratory in Genetic Pharmacology, Stanford University, 269 Campus Drive, Stanford, California 94305, USA (B.B.).

Despite the essential roles of sphingolipids as both structural components of membranes and critical signalling molecules, we have a limited understanding of how cells sense and regulate their levels. Here we reveal the function in sphingolipid metabolism of the *Saccharomyces cerevisiae* *ORM* genes (known as *ORMDL* genes in humans)—a conserved gene family that includes *ORMDL3*, which has recently been identified as a potential risk factor for childhood asthma. Starting from an unbiased functional genomic approach, we identify Orm proteins as negative regulators of sphingolipid synthesis that form a conserved complex with serine palmitoyltransferase, the first and rate-limiting enzyme in sphingolipid production. We also define a regulatory pathway in which phosphorylation of Orm proteins relieves their inhibitory activity when sphingolipid production is disrupted. Changes in *ORM* gene expression or mutations to their phosphorylation sites cause dysregulation of sphingolipid metabolism. Our work identifies the Orm proteins as critical mediators of sphingolipid homeostasis and raises the possibility that sphingolipid misregulation contributes to the development of childhood asthma.

## Introduction

As cells grow, divide and respond to their environment, they must synthesize lipids to meet metabolic demands while also ensuring the correct balance of a wide array of structurally and functionally diverse lipid species. At the same time, cells need to maintain the correct distribution of lipids within the membrane bilayers of different organelles and the plasma membrane. Regulatory mechanisms that ensure proper levels of some lipid species including sterols<sup>69,70,72</sup> and various glycerolipids<sup>74,77,121</sup> have been identified, with loss of such controls leading to a variety of disease states<sup>69,122</sup>. While remarkable progress has been made in defining the protein machinery responsible for synthesizing<sup>42,43</sup> and transporting sphingolipids<sup>45,46,84,123</sup>, insights into how cells sense and maintain their levels are only now emerging<sup>80,81</sup>. The need for precise regulation of sphingolipids may be particularly acute as, in addition to the structural roles of the terminal products, several biosynthetic intermediates—including sphingosine, ceramide, and their phosphorylated derivatives—are signalling molecules that participate in key physiological and pathological processes<sup>53,124</sup>.

Our investigations into how cells achieve sphingolipid homeostasis stem from analysis of the *ORM/ORMDL* genes, which have been implicated recently in asthma. Asthma has emerged as a major health problem, with rates of childhood disease increasing dramatically over the last three decades<sup>125</sup>. There is a strong heritable component that modulates asthma susceptibility, and many studies have been undertaken to identify genetic risk factors<sup>126</sup>. A breakthrough in these efforts came with the genome-wide association study of Moffatt *et al.*<sup>32</sup>, which identified single nucleotide

polymorphisms (SNPs) at chromosome 17q21 near the *ORMDL3* gene that confer an increased risk of childhood asthma. Subsequent studies have reproduced this finding in multiple ethnic groups<sup>33,34,36</sup>. Additionally, the disease-associated polymorphisms have been shown to modulate expression of *ORMDL3* and the adjacent gene *GSDMB*<sup>32,127</sup>, suggesting that dysregulation of one or both of these genes may contribute to childhood asthma. Thus genetic variants near *ORMDL3* are a widespread risk factor for developing childhood asthma that may raise the incidence of the disease by up to ~20%<sup>35,36</sup>. However, translating these findings into an increased understanding of how *ORMDL3* may contribute to asthma pathogenesis has been hampered by the lack of information on the function of *ORM*-family genes.

Members of the *ORM* gene family encode trans-membrane proteins localized in the Endoplasmic Reticulum (ER) and include two genes in the budding yeast *S. cerevisiae* (*ORM1/2*) and three genes in humans (*ORMDL1/2/3*)<sup>31</sup>. The Orm proteins have no known functional domains, and little is known about their cellular role; however, they are strongly conserved and have been proposed to share a common function<sup>31</sup>. Here we use a combination of global and targeted studies to identify Orm proteins as homeostatic regulators of the first and rate-limiting step in sphingolipid biosynthesis.

## Results

### **Orm1/2 negatively regulate sphingolipid synthesis**

We began our investigation of *S. cerevisiae* *ORM1* and *ORM2* using a global approach to characterize gene function based on large-scale measurements of genetic interactions,

termed epistatic mini-array profiles or E-MAPs<sup>7</sup>. In this strategy, the function of a given gene can be inferred without *a priori* hypotheses by comparing the spectrum of genetic interactions resulting from mutation of that gene with interaction patterns caused by mutations in other genes of defined function. When we compared the genetic interaction profile resulting from deletion of *ORM2* to those obtained for a collection of more than 1400 yeast mutants focused on ER biology (manuscript in preparation and ref. <sup>7</sup>), we observed a strong anti-correlation with the interaction patterns seen for hypomorphic alleles that reduce expression of *LCB1* and *LCB2* (*lcb1-DAmP* and *lcb2-DAmP*<sup>7</sup>), thus suggesting that *ORM2* and *LCB1/2* have opposing cellular roles (Fig. 3.1a and Supplementary Data). We also examined the genetic interactions that result from over-expression of *ORM1* or *ORM2* using the strong promoters *pTDH3* and *pTEF2*, as it was shown that the asthma-associated polymorphisms near *ORMDL3* are associated with increased expression of this gene<sup>32,127</sup>. Over-expression of *ORM1* or *ORM2* produced a pattern of genetic interactions that was highly correlated with those seen for the *lcb1-DAmP* and *lcb2-DAmP* strains, indicating that increased Orm expression phenocopies reduced Lcb1/2 activity (Fig. 3.1a).

*LCB1* and *LCB2* encode serine palmitoyltransferase, an essential hetero-dimeric enzyme that catalyzes the first and rate-limiting step in sphingolipid biosynthesis<sup>42,128</sup>. The synthesis of sphingolipids begins in the ER, where two classes of lipid metabolites, long chain bases (LCBs) and very long chain fatty acids (VLCFAs), are formed (Fig. 3.1d). LCBs are produced by the condensation of serine with fatty acids by serine palmitoyltransferase (Lcb1/2 in yeast and Sptlc1/2/3 in mammals), followed by Tsc10-mediated reduction.<sup>40,42,43</sup> LCBs can then be phosphorylated or N-acylated with

VLCFAs to form long chain base phosphates or ceramides, respectively, with the latter undergoing further modification to generate complex sphingolipids (Fig. 3.1d).

Thus our E-MAP data showing a correlation between increased *ORM* expression and decreased *LCB1/2* function implicate the Orm proteins as negative regulators of sphingolipid synthesis and highlight the reaction carried out by Lcb1/2 as the possible step in the pathway at which Orm1/2 may act (Fig. 3.1a,d). We therefore examined the effects of altering Orm levels on cellular lipid composition using a mass spectrometry-based global lipidomic technique<sup>129</sup>. In agreement with the genetic interaction data, over-expression of *ORM1* or *ORM2* resulted in changes to the lipidome, including reduced LCB and ceramide levels, that closely resembled those seen for the *lcb2-DAmP* mutant (Fig. 3.1b and Supplementary Data). Conversely, cells deleted for *ORM1/2* had highly elevated levels of LCBs and ceramides. In principle, this accumulation of LCBs caused by deletion of *ORM1/2* could result from a failure to consume these species by reaction with VLCFAs to form ceramides, as is seen when *SUR4* is deleted (Fig. 3.1b and ref. <sup>129</sup>). However, in contrast to the *sur4Δ* strain, the increased LCB levels in the *orm1Δ/orm2Δ* strain are also accompanied by increased amounts of the terminal sphingolipid mannosyl-diinositolphosphorylceramide (MIP<sub>2</sub>C). Thus, *ORM1/2* deletion causes increased flux throughout the sphingolipid pathway. This increased flux is also the primary cause of the growth defect seen in *orm1Δ/orm2Δ* cells, as artificially decreasing Lcb1/2 activity, either through the use of myriocin, a drug that specifically inhibits Lcb1/2<sup>86</sup>, or through the *lcb2-DAmP* allele, significantly suppresses the growth defect caused by loss of *ORM1/2* (Fig. 3.1c).



## A complex with Orm1/2, Lcb1/2, and Sac1

We next explored how Orm proteins modulate sphingolipid production by identifying proteins bound by Orm1 and Orm2 *in vivo*. Isolation of functional 3xFlag-tagged forms of Orm1 and Orm2 expressed from their endogenous loci revealed a previously uncharacterized, roughly stoichiometric complex (Fig. 3.2a). Mass spectrometry showed that this complex comprises the serine palmitoyltransferase proteins Lcb1, Lcb2 and Tsc3, in addition to the phosphoinositide phosphatase Sac1<sup>130</sup> (Supplementary Table 3.1). To further characterize physical interactions among these proteins, we utilized a 3xFlag-tagged form of Lcb1 that is able to rescue the inviability seen upon loss of *LCB1* (although this allele does exhibit mild sensitivity to myriocin). Purification of 3xFlag-Lcb1 yielded the same components seen in the Orm1/2 pull-downs (Fig. 3.2b), indicating that the Orm-associated proteins are likely to exist in a single complex. Orm proteins and potentially their binding partners may also form higher-order oligomers, as we detected Orm2 co-immunoprecipitation with 3xFlag-Orm1 (and vice versa) and self-association of differently tagged copies of the same Orm protein (Fig. 3.2a and see later).

The presence of Sac1 in association with Lcb1/2 suggests a new connection between this phosphoinositide phosphatase and sphingolipid biosynthesis. Deletion of *SAC1* leads to elevated LCB levels (Supplementary Fig. 1a and ref. <sup>131</sup>) and resistance to the Lcb1/2 inhibitor myriocin (Supplementary Fig. 3.1b). This resistance was also seen in cells expressing the *sac1-8*<sup>132</sup> catalytically inactive mutant of *SAC1* (Supplementary Fig. 3.1b). Additionally, we found that Sac1 and Orm1/2 bind independently to Lcb1/2 (Fig. 3.2b) and that *SAC1* and *ORM1/2* deletions exhibit synthetic lethality

(Supplementary Fig. 3.1c). These findings together suggest that Sac1 modulates serine palmitoyltransferase activity directly, but in a mode distinct from Orm1/2.

Finally, we showed that the functional complex between Orm proteins and serine palmitoyltransferase is conserved in human cells. Immunoprecipitations from HEK293T cells expressing 3xFlag-tagged Ormdl3 led to a prominent Sptlc1 band detected by Western blot (Fig. 3.3a). Additionally, we found that simultaneous knock-down by RNAi of *ORMDL1/2/3* in HeLa cells causes an approximately three-fold increase in ceramide levels (Fig. 3.3b, Supplementary Fig. 3.2 and Supplementary Data).

Together, these results establish that Orm proteins are components of a novel and conserved protein complex, which we term the SPOTS complex (Serine Palmitoyltransferase, Orm1/2, Tsc3, and Sac1). Additionally, our data suggest that Orm1/2 negatively regulate sphingolipid synthesis by acting directly on Lcb1/2 (for example by altering Lcb1/2 catalytic activity, accessibility of substrates to the enzyme, or its sub-cellular localization).

### **Homeostatic regulation of Orm1/2 activity**

Why would cells include negative regulators of serine palmitoyltransferase, Orm1/2, as core components of this essential enzyme complex? This seemingly paradoxical arrangement prompted us to investigate whether Orm-mediated inhibition of LCB production might be regulated in response to changes in the cellular need for sphingolipid synthesis. We therefore examined how *ORM1/2* deletion affects sensitivity to the Lcb1/2 inhibitor myriocin. Growth in the presence of myriocin is strongly dependent on the

cellular capacity for LCB production, as the *lcb2-DAmP* strain is highly sensitive to myriocin (Fig. 3.4a), and *SAC1* deletion, which leads to increased LCB levels, confers myriocin resistance (Fig. 3.4a). Thus, naively we might expect deletion of *ORM1/2* to also confer pronounced resistance to this drug. However, we observed that wild-type and *orm1Δ/orm2Δ* strains exhibit comparable growth in the presence of myriocin (Fig. 3.4a). Thus, loss of *ORM1/2* strongly impacts cell growth under standard conditions but has little apparent effect when sphingolipid synthesis is disrupted, suggesting that Orm1/2 may be inactivated in response to myriocin treatment.

To further investigate this possibility, we compared the effects of myriocin on the LCB levels of wild-type and *orm1Δ/orm2Δ* strains. As described above, the *ORM1/2* deletion strain exhibits highly elevated LCB levels in the absence of myriocin. However, with increasing doses of myriocin, LCB levels in the *orm1Δ/orm2Δ* strain decrease strongly and approximately converge with those of wild-type (Fig. 3.4b), supporting our growth data indicating that Orm1/2 may be inactivated in response to myriocin treatment (Fig. 3.4a). Interestingly, we also found that wild-type cells maintain roughly constant LCB levels even at intermediate myriocin concentrations (up to  $\sim 40 \text{ ng ml}^{-1}$ ). This robustness is not due to a failure of myriocin to affect Lcb1/2 at these concentrations, as LCB levels in the *orm1Δ/orm2Δ* strain are reduced strongly by the same drug concentrations (Fig. 3.4b; for further evidence see Fig. 3.4d). Rather, these results suggest that progressive inactivation of Orm1/2 may provide cells with a homeostatic mechanism to maintain nearly constant sphingolipid output in the face of increasing Lcb1/2 inhibition. Consistent with this hypothesis, LCB levels in the wild-type strain begin to decline only at myriocin concentrations where Orm-mediated inhibition of LCB

synthesis appears to be fully relieved, as evidenced by convergence of wild-type and *orm1Δ/orm2Δ* LCB levels (Fig. 3.4b).

### **Orm1/2 regulation by phosphorylation**

We next investigated the mechanism by which Orm1/2 activity might be decreased in response to myriocin treatment. Growth in myriocin did not alter Orm1/2 expression levels (Fig. 3.4c) or abolish their ability to interact with Lcb1/2 and Sac1 (Supplementary Fig. 3.3). However, we observed a notable reduction in localization of green fluorescent protein (GFP)–Orm2 to the cortical but not peri-nuclear ER after myriocin treatment (Supplementary Fig. 3.4), raising the possibility that changes in sub-cellular localization contribute to regulation of SPOTS complex activity. We also found a strong reduction in Orm protein co-immunoprecipitation in cells treated with myriocin (Fig. 3.4c), both for Orm1-Orm2 interaction and for self-association of Orm1 and of Orm2. A weak reduction in Orm-Lcb1 binding was also observed in response to myriocin (Fig. 3.4c). These findings suggest a model in which Orm-Lcb1/2 sub-complexes can self-associate into higher-order oligomers in a manner that is inhibited in response to disruptions to sphingolipid synthesis.

We also detected in our Orm1/2 co-immunoprecipitations a slight shift in the electrophoretic mobility of Orm1 upon myriocin treatment, indicating that Orm proteins may be post-translationally modified (see Fig. 3.4c, Flag-Orm1 and HA-Orm1 inputs). Analysis of 3xFlag-Orm1 and 3xFlag-Orm2 in SDS/PAGE gels that incorporate a phosphate-binding agent to improve the separation of phosphorylated species<sup>133</sup> resolved multiple phosphorylated forms for both Orm1 and Orm2 that collapsed to faster-

migrating species upon phosphatase treatment (Fig 3.4d). Importantly, growth in myriocin induced a dose-dependent shift to more highly phosphorylated forms, and this response occurred over the same concentrations of myriocin that led to homeostatic inactivation of Orm1/2, as determined by LCB measurements (compare Fig. 3.4b and Fig. 3.4d). We also observed increased Orm1/2 phosphorylation after shutting off expression of downstream sphingolipid synthetic enzymes such as Tsc10 or Lag1 (in a *lac1Δ* background; Supplementary Fig. 3.5), further indicating that phosphorylation of Orm proteins is a homeostatic response to disruption of sphingolipid production (see Fig. 3.5c). Finally, we did not observe substantial changes in Orm1/2 phosphorylation upon shutting off expression of *AUR1* or *CSG2* (Supplementary Fig. 3.5), thus providing preliminary evidence that a metabolite upstream of these enzymes, such as ceramide, is a sphingolipid intermediate whose levels are sensed to regulate Orm1/2 activity.

To test our phosphorylation-mediated feedback model directly, we identified residues of Orm1 and Orm2 that are phosphorylated. Through a combination of targeted mutagenesis and mass spectrometry, we found several sites of Orm1 and Orm2 phosphorylation in the N-terminal regions of these proteins (Fig. 3.5a and Supplementary Fig. 3.6). Importantly, mutation of these sites to alanine blocks formation of the slower-migrating phosphorylated species seen in phosphate-affinity gels (Fig. 3.5a). This loss of phosphorylation is unlikely to be due to these mutations resulting in non-functional Orm proteins, as the phospho-mutants are expressed at levels comparable to wild-type and rescue the slow growth of the *orm1Δ/orm2Δ* strain (Supplementary Fig. 3.7 and data not shown). Furthermore, immunoprecipitations of phospho-mutant Orm1 and Orm2 revealed that these proteins not only maintain the ability to interact with Lcb1 and each

other, but that blocking phosphorylation prevents the change in higher-order assembly of the SPOTS complex normally seen in response to myriocin (Fig. 3.5b). Thus Orm1/2 phosphorylation appears to be the event that triggers dynamic regulation of SPOTS complex oligomerization.

We next investigated the *in vivo* role of Orm1/2 phosphorylation. Consistent with a basal level of Orm1/2 phosphorylation that is important for steady-state LCB production, we observed a substantial (two-fold) reduction in LCB levels in the *ORM1/2* phospho-mutant strain under standard growth conditions (Fig. 3.5d). Furthermore, we found that this strain is highly sensitive to myriocin treatment (Fig. 3.5e). This result thus confirms a key prediction of our homeostatic model and indicates that although cells may possess additional mechanisms to regulate sphingolipid biosynthesis, such pathways cannot compensate for disruption of the critical feedback control provided by Orm1/2. Thus, Orm proteins are dynamic negative regulators of serine palmitoyltransferase whose inhibitory activity is dependent on adequate levels of downstream sphingolipids (Fig. 3.5c).

## Discussion

Our studies identify Orm proteins as homeostatic regulators of sphingolipid metabolism and demonstrate that alteration of these controls leads to misregulation of sphingolipid production (Fig. 3.5c). Specifically, loss of the Orm1/2-mediated brake on serine palmitoyltransferase activity results in toxic accumulation of sphingolipids, while mutations that prevent this brake from being removed render cells unable to survive disruptions to sphingolipid synthesis.

A phosphorylation-based feedback loop provides a mechanistic basis for homeostatic regulation of sphingolipid production and exhibits a number of features that make it well-suited to precisely control sphingolipid levels. First, phosphorylation of a direct regulator of serine palmitoyltransferase may enable cells to respond rapidly to changing environments. Second, Orm1/2 exhibit partial phosphorylation under standard growth conditions, which may position the Orm1/2-based sphingolipid rheostat for maximal sensitivity to changes in lipid metabolism. Additionally, multi-site phosphorylation of Orm1 and Orm2 has the potential to generate several distinct and stable phospho-states, which may provide cells with a graded mechanism to finely adjust lipid production to match cellular needs. Finally, multiple phosphorylation sites may also render Orm1/2 responsive to multiple physiologic or metabolic cues.

Our identification of the SPOTS complex reveals a novel mechanism by which cells may be able to coordinate a variety of important secretory pathway processes. While a link between sphingolipids and phosphoinositides has been appreciated for some time<sup>40,131,134</sup>, we provide evidence that the phosphoinositide phosphatase Sac1 directly

modulates sphingolipid metabolism, as it is associated physically with serine palmitoyltransferase. These results are of particular interest given the role of phosphoinositides, including the Sac1 substrate phosphatidylinositol-4-phosphate (PI4P), in the trafficking of both proteins and sphingolipids from the ER and Golgi apparatus<sup>45,46,130</sup>. Additionally, changes in Sac1 localization from the ER to the Golgi have been shown in both yeast and mammals to regulate protein flux through the secretory pathway in response to nutrient levels<sup>135</sup>. We therefore suggest that the SPOTS complex may represent a dynamic coordinating center that couples changes in sphingolipid and nutrient levels to the activity and localization of key enzymes of lipid metabolism and trafficking. This model is consistent with our finding that Orm proteins self-associate in a regulated fashion and with evidence that mammalian serine palmitoyltransferase is part of a high-molecular-mass complex<sup>136</sup>.

Finally, our characterization of *ORM* gene function provides a framework for investigating whether alterations in *ORMDL3* activity and sphingolipid metabolism play a role in asthma pathogenesis. A number of studies support a role for sphingolipids such as ceramide and sphingosine-1-phosphate in asthma-associated inflammatory processes, including mast cell degranulation, airway hyper-responsiveness, and immune cell trafficking<sup>137-141</sup>. Our findings now link this growing body of evidence to the identification of SNPs near *ORMDL3* that are associated with childhood asthma<sup>32</sup>. Specifically, our observation that altered Orm levels strongly impact sphingolipid metabolism raises the testable hypothesis that misregulation of sphingolipids may have a direct and causative role in the development of asthma. A broader potential role for sphingolipids in inflammatory disease is also suggested by reports that SNPs near



*ORMDL3* are associated with Crohn's disease<sup>142</sup>, type I diabetes<sup>143</sup> and primary biliary cirrhosis<sup>144</sup>. Finally, the existence of a rich set of pharmacologic tools targeting the sphingolipid pathway<sup>86</sup> raises the possibility of therapeutic intervention to counteract the potential effects of changes in *ORMDL* gene activity.

## Methods Summary

**Phosphorylation analysis by phosphate-affinity SDS/PAGE.** Lysates for phosphorylation analysis were prepared by pelleting and immediately resuspending logarithmically growing yeast in SDS lysis buffer (50mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol) with protease inhibitors (Complete EDTA-free protease inhibitor tablets; Roche) at 65°C. SDS/PAGE gels with phosphate affinity reagent<sup>133</sup> (synthesized according to standard synthetic procedures and kindly provided by D. Fiedler) were prepared with a 7.5% acrylamide resolving gel with 25  $\mu$ M MnCl<sub>2</sub> and 50 $\mu$ M phosphate affinity reagent (375mM Tris, pH 8.8, 0.1% SDS). Gels were run at 70V for 2.5 hr, rinsed twice for 5 min in transfer buffer with 1mM EDTA, and rinsed twice more for 5 min in transfer buffer without EDTA prior to transfer to nitrocellulose membranes. Western blots were performed as described below.

**Western blots.** Proteins were transferred from SDS/PAGE gels to nitrocellulose membranes and probed for proteins of interest using standard Western blot procedures. Antibodies used were as follows: rabbit anti-Flag (Sigma Aldrich), mouse anti-Flag (M2; Sigma Aldrich), mouse anti-HA antibodies 12CA5 (Roche) and HA.11 (Covance Inc.), mouse anti-yPgk1 (Molecular Probes), rabbit anti-yLcb1 (raised against peptide antigen SYIKKSHHDDPYRTTC; Abgent, Inc), mouse anti-hSptlc1 (Becton Dickinson), and rabbit anti-hOrmdl1/2/3 (raised against peptide antigen KGTPFETPDQGKARLC; Abgent, Inc.). Western blots were scanned using an Odyssey fluorescent scanner (Li-Cor Biosciences).

**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

## **Acknowledgments**

We acknowledge A. Falick, S. Zhou and D. King for identification of proteins by mass spectrometry; M. Schuldiner and S. Wang for E-MAP data collection; D. Fiedler and K. Shokat for providing the phosphate-binding acrylamide reagent; E. Griffis and M. D'Ambrosio for assistance with fluorescence microscopy; N. Ingolia for a codon-optimized mCherry-tagging plasmid; E. Burchard and J. Galanter for discussions of asthma genetics; I. Poser and A. Hyman for advice and providing the HeLa-Kyoto cell line; and M. Bassik, G. Brar, V. Denic, A. Frost, N. Ingolia, E. Quan, B. Toyama and other members of the Weissman laboratory for discussions. This work was supported by funding from Deutsche Forschungsgemeinschaft SFB/TR 13 projects A1 (K.S.) and D1 (A.S.), EUFP6 PRISM (K.S.), ETH Zurich (R.A.), the National Heart, Lung, and Blood Institute, National Institute of Health (N01-HV-28179) (R.A.), SystemsX.ch, the Swiss initiative for systems biology (R.A.), the Boehringer Ingelheim Fonds and the Swiss National Science Foundation (B.B.), the Howard Hughes Medical Institute (J.S.W.), the University of California, San Francisco Strategic Asthma Basic Research Center (J.S.W.), the National Science Foundation Graduate Research Fellowship Program (D.K.B.) and the Fannie and John Hertz Foundation (D.K.B.).

## **Author Contributions**

D.K.B. designed, carried out, and interpreted experiments. S.R.C. oversaw E-MAP data collection and analysis. B.B. carried out and analyzed protein mass spectrometry experiments to identify sites of phosphorylation under the supervision of R.A. C.S.E. performed and analysed lipidomic measurements with the support of A.S. and K.S. J.S.W designed and interpreted experiments. D.K.B. and J.S.W. prepared the manuscript.

### **Author Information**

Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints). The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.S.W. ([weissman@cmp.ucsf.edu](mailto:weissman@cmp.ucsf.edu)).

## Figure Legends.

### Figure 3.1. Orm1/2 are negative regulators of sphingolipid synthesis.

**a**, Genetic interaction (E-MAP) profiles were generated for strains harbouring *ORM2* deletion, *ORM1* over-expression, and *ORM2* over-expression alleles (*orm2Δ*, *pTDH3-ORM1* & *pTEF2-ORM1*, and *pTDH3-ORM2* & *pTEF2-ORM2*, respectively).

Correlations between the E-MAP profiles of these strains and those of more than 1400 other yeast mutants are shown in histograms (see **Methods** and **Supplementary Data**).

**b**, Lipids were extracted from the indicated strains and analyzed using a global mass spectrometry approach<sup>129</sup>. Amounts of the indicated metabolites are shown relative to wild-type (WT; average  $\pm$  s.d.,  $n = 4$ ; see **Methods** and **Supplementary Data**). **c**,

Logarithmic-phase growth rates for the indicated strains were measured in standard media or media supplemented with myriocin at 150 ng ml<sup>-1</sup>. Growth rates (average  $\pm$  s.d.,  $n \geq 3$ ) are shown normalized to wild-type (WT); asterisks denote statistical

significance ( $p < 0.05$ ). **d**, Schematic of the sphingolipid biosynthesis pathway in *S. cerevisiae*, with selected metabolites and genes indicated. Abbreviations are as follows:

FA-CoA, fatty acid co-enzyme A; VLCFA-CoA, very long-chain fatty acid co-enzyme A; LCBs, long-chain bases; LCB-Ps, long-chain base phosphates; IPC, inositolphosphorylceramide; MIPC, mannosyl-inositolphosphorylceramide; MIP<sub>2</sub>C, mannosyl-diinositolphosphorylceramide.

### Figure 3.2. Orm/Ormdl proteins form a complex with serine palmitoyltransferase.

**a**, Colloidal-stained gels are shown for proteins eluted after affinity purifications from strains expressing 3xFlag-Orm1 or 3xFlag-Orm2, or from an untagged control strain. Immunoprecipitated proteins were identified by mass spectrometry (see **Supplementary Table 1**; asterisk indicates the partially obscured protein Tsc3, whose presence was confirmed by mass spectrometry). **b**, Affinity purifications of 3xFlag-Lcb1 from wild-type (WT), *sac1Δ*, and *orm1Δorm2Δ* strains were performed as in **a**. Asterisks indicate bands that are lost in deletion backgrounds.

**Figure 3.3. *ORM* gene function is conserved in human cells.**

**a**, HEK293T cells were transfected with either an empty vector or a vector encoding Ormdl3 fused to the 3xFlag epitope (N- or C-terminal fusion). Immunoprecipitations with anti-Flag agarose were analyzed by Western blot against human serine palmitoyltransferase ( $\alpha$ -hSptlc1) and against the Flag epitope ( $\alpha$ -Flag). **b**, HeLa cells were transfected with short interfering RNAs (siRNAs) directed against the indicated genes. After 72 hr, cells were harvested and their lipids were analyzed by mass spectrometry. Ceramide levels normalized to phosphatidylcholine (PC) are shown (mol ceramide per mol PC, normalized relative to untreated cells; average  $\pm$  s.d.,  $n = 3$ ).

**Figure 3.4. Orm1/2 are regulated in response to disruption of sphingolipid synthesis.**

**a**, Serial dilutions of the indicated strains were spotted on plates containing 0, 200 or 400 ng ml<sup>-1</sup> myriocin and imaged after growth for 24–48 h. **b**, LCBs were extracted and analysed from wild-type and *ORM1/2* deletion strains grown in media supplemented with

the indicated concentrations of myriocin. The combined amounts of C18 dihydrosphingosine and C18 phytosphingosine are shown (relative to wild-type LCB levels in the absence of myriocin; average of  $n \geq 2$  experiments). **c**, Native immunoprecipitations of 3×Flag-tagged Orm1 and Orm2 were performed from strains grown in standard media or media supplemented with 150 ng ml<sup>-1</sup> myriocin and analysed by western blot. The indicated strains also expressed 3×HA-Orm1/2 from their endogenous loci (diploid strains were used to examine self-association of Orm1 and Orm2). **d**, Lysates were prepared from yeast expressing 3xFlag-Orm1/2 after growth in media containing the indicated concentrations of myriocin. Western blots show phosphorylated forms of 3xFlag-Orm1 (P-Orm1) and 3xFlag-Orm2 (P-Orm2) after separation on phosphate-affinity gels<sup>133</sup>. An immunoprecipitated (IP) sample treated with calf intestine phosphatase (CIP) shows the position of un-phosphorylated Orm1/2.

**Figure 3.5. Mutation of phosphorylated Orm1/2 residues impairs sphingolipid homeostasis.**

**a**, Lysates were prepared from strains expressing wild-type (WT) or phospho-site mutant 3xFlag-Orm1 or 3xFlag-Orm2 after growth in standard media or media supplemented with 150 ng ml<sup>-1</sup> myriocin. Western blots are shown after protein separation on phosphate-affinity gels. Residues mutated to alanine in the indicated phospho-mutants are highlighted in red below. **b**, Native immunoprecipitations of 3xFlag-tagged wild-type or phospho-mutant Orm1 and Orm2 were performed from strains expressing wild-type or phospho-mutant 3xHA-Orm1/2 after growth in standard media or media supplemented with 100 ng ml<sup>-1</sup> myriocin. Western blots were analyzed as in **Fig. 3.4c**. **c**, Model for

homeostatic regulation of Orm1/2, in which Orm proteins act as negative regulators of serine palmitoyltransferase (Lcb1/Lcb2/Tsc3), whose inhibitory activity is dependent on levels of downstream sphingolipids. P-Orm1/2 denotes phosphorylated Orm1/2; see **Fig. 3.1d** for abbreviations used. **d**, LCBs were extracted and analyzed from wild-type (WT) and 3xFlag-tagged *ORM1/2* double phospho-mutant strains grown in standard media. Data were analyzed as in **Fig. 3.4b** (average  $\pm$  s.d.,  $n = 3$ ). **e**, Serial dilutions of wild-type (WT), *ORM1/2* deletion, and 3xFlag-tagged *ORM1/2* double-phospho-mutant strains were spotted on plates with 0 or 280 ng ml<sup>-1</sup> myriocin and imaged after growth for 24-36 hr.



Figure 3.1

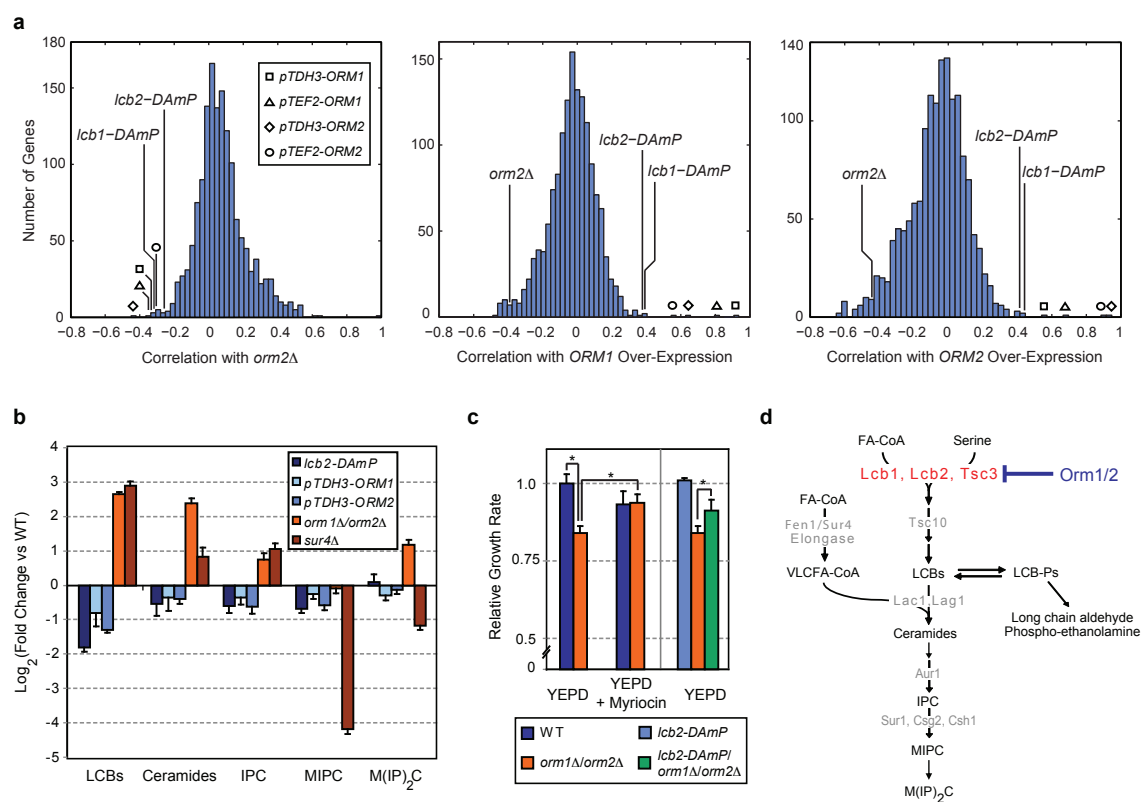


Figure 3.2

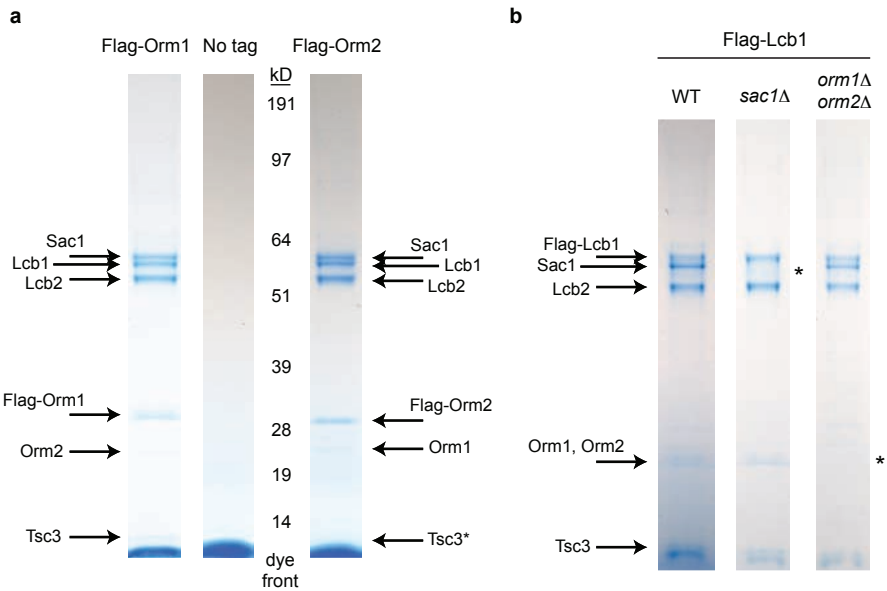


Figure 3.3

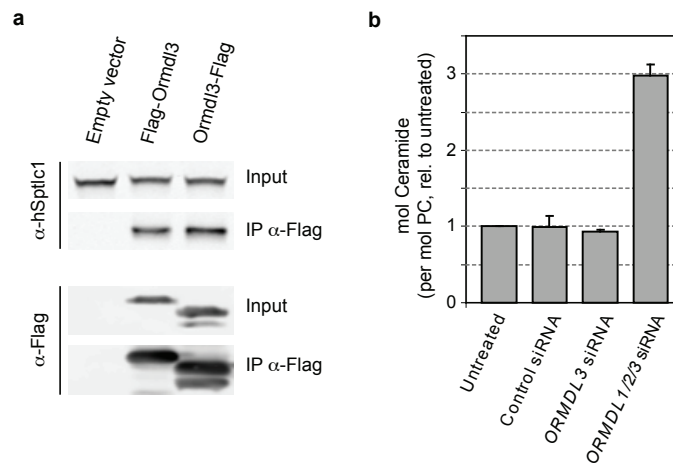


Figure 3.4

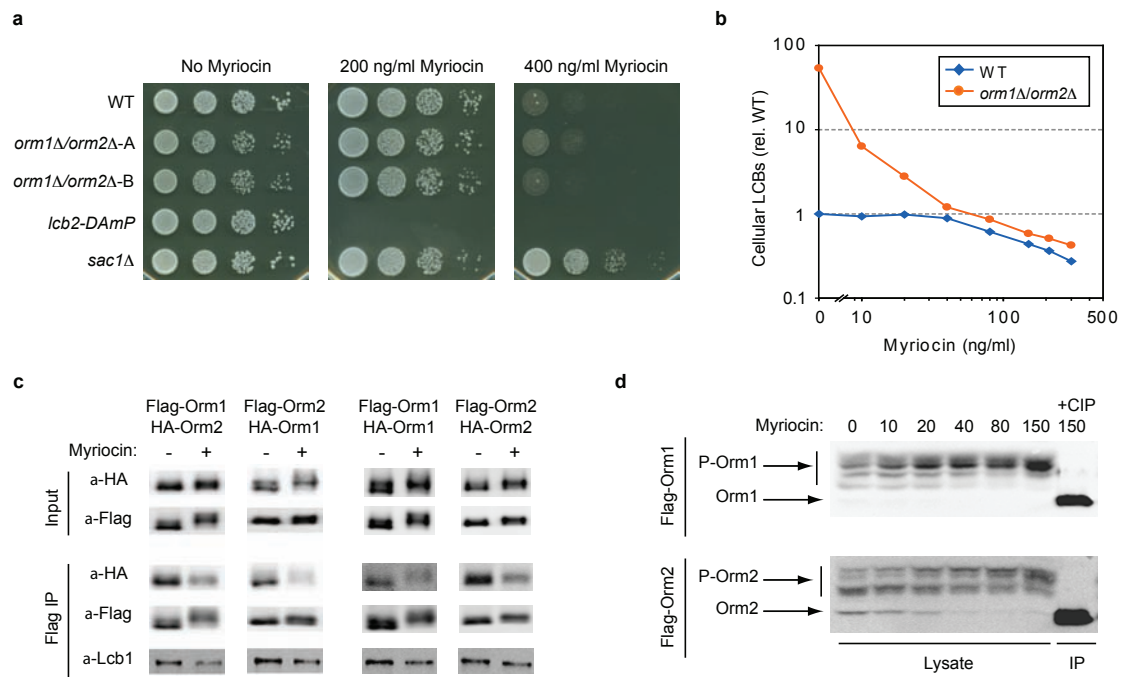
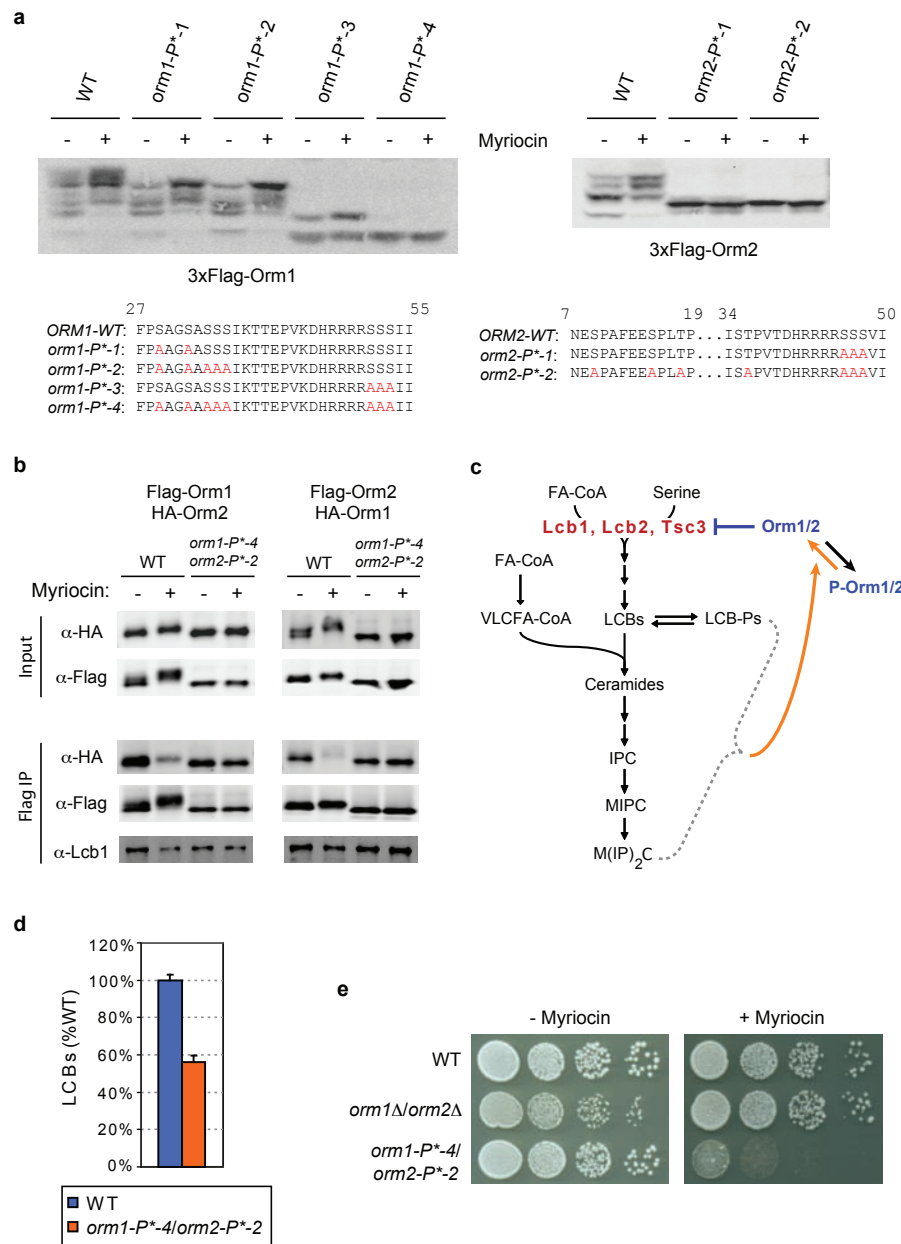
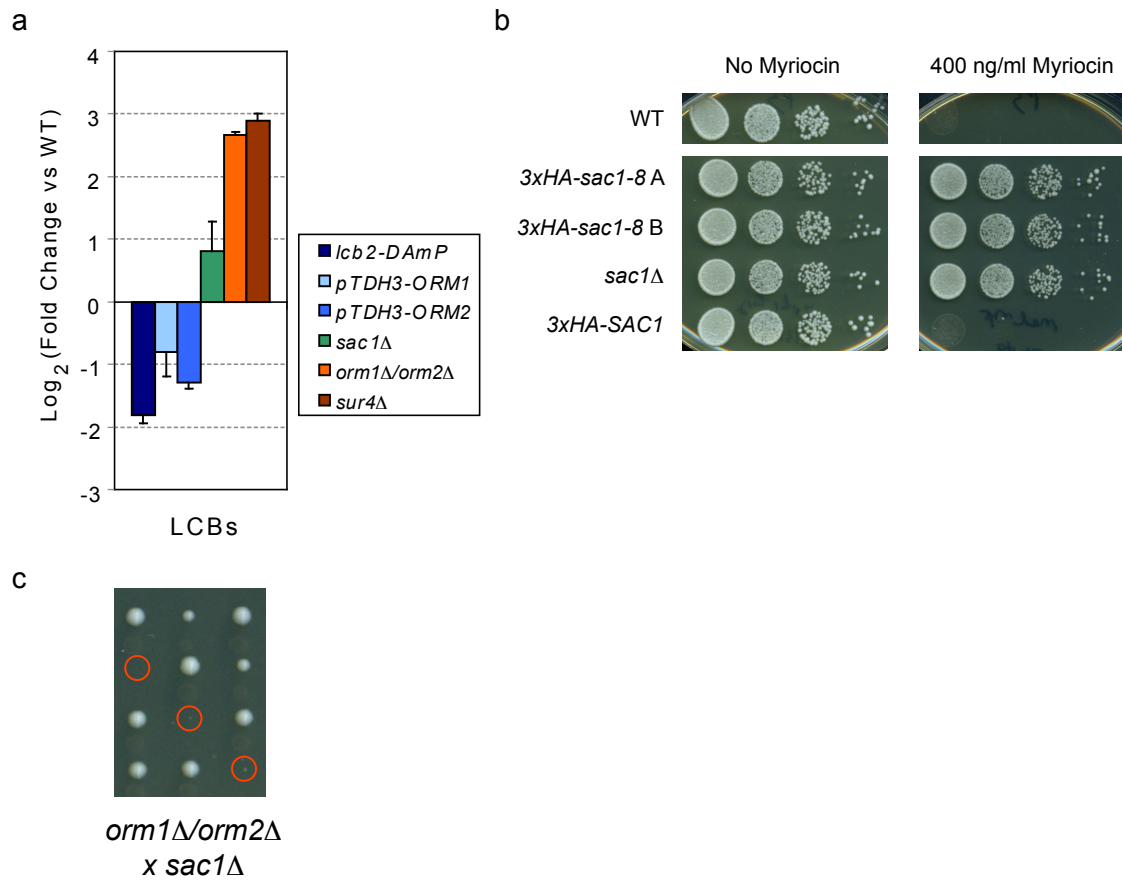


Figure 3.5



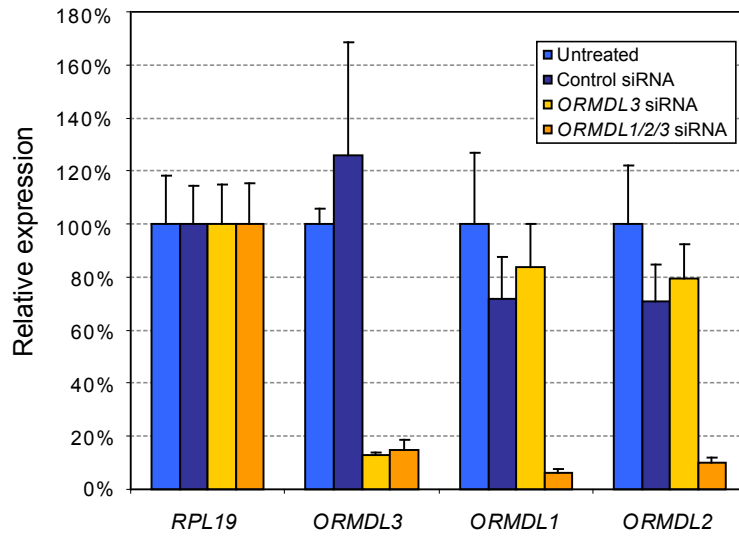
# Supplementary Figure 3.1



## Supplementary Figure 3.1. A functional role for Sac1 in sphingolipid metabolism.

**a**, Lipidomic analysis of LCB levels in the indicated strains are shown (average  $\pm$  s.d.,  $n = 3$ ). **b**, Serial ten-fold dilutions of the indicated strains were spotted on plates with 0 or 400 ng/ml myriocin and imaged after 24-48 hr of growth. The *sac1-8* allele of *SAC1* is a catalytically inactive *SAC1* mutant described previously (ref. 132). **c**, *ORM1/ORM2* and *SAC1* deletion mutants exhibit synthetic lethality. Representative tetrads from a *orm1Δ/orm2Δ*  $\times$  *sac1Δ* cross are shown, with circles indicating spores with the *orm1Δ/orm2Δ/sac1Δ* genotype that fail to grow.

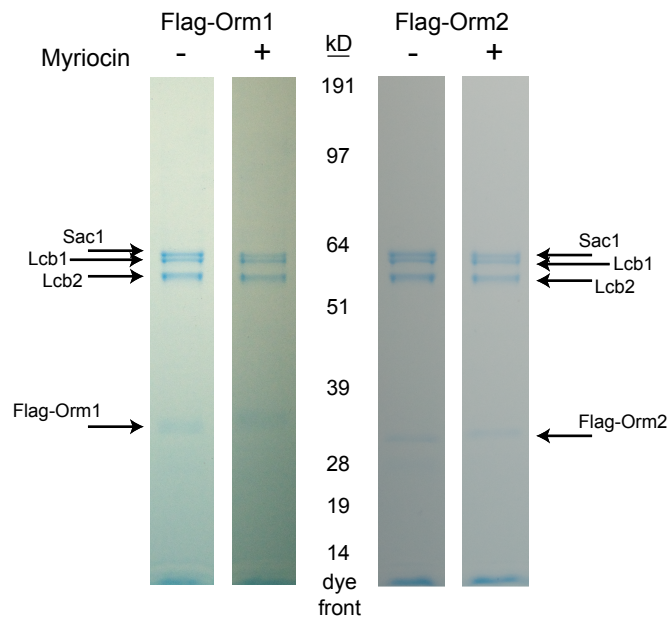
Supplementary Figure 3.2



**Supplementary Figure 3.2. *ORMDL* gene expression after RNAi treatment**

Gene expression was quantified for the indicated genes after transfection of HeLa cells with indicated siRNAs. RT-PCR was performed with primers for the indicated genes using *RPL19* as a reference. Data are normalized to expression levels in untreated cells (average  $\pm$  s.d., n = 3).

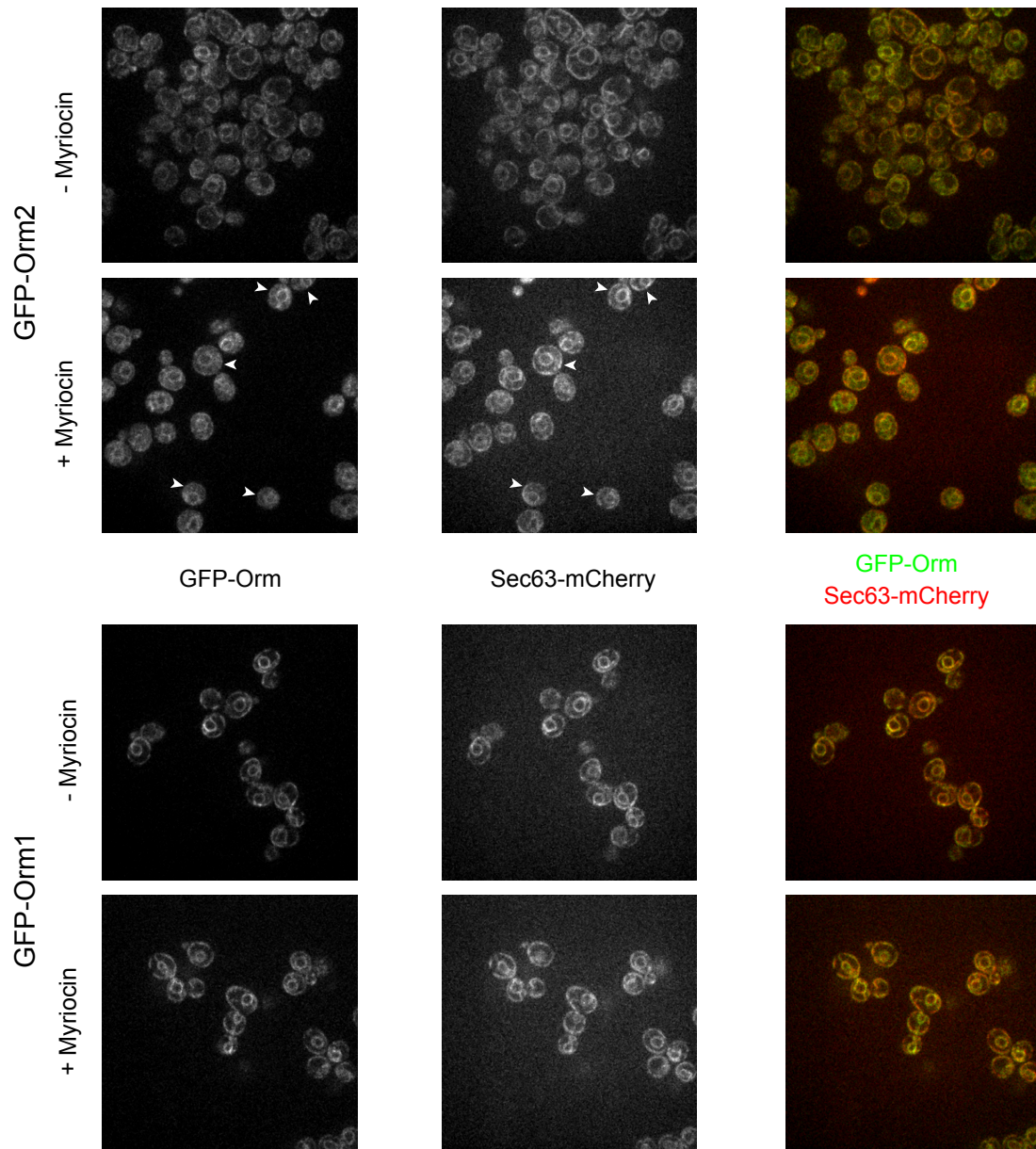
### Supplementary Figure 3.3



**Supplementary Figure 3.3. Myriocin treatment does not prevent formation of the Orm1/2-Lcb1/2-Sac1 complex.** Colloidal-stained SDS/PAGE gels are shown for native affinity purifications from strains expressing 3xFlag-Orm1 or 3xFlag-Orm2. Prior to harvest, the indicated strains were grown for 12-16 hr in standard rich media or media supplemented with 150 ng/ml myriocin.

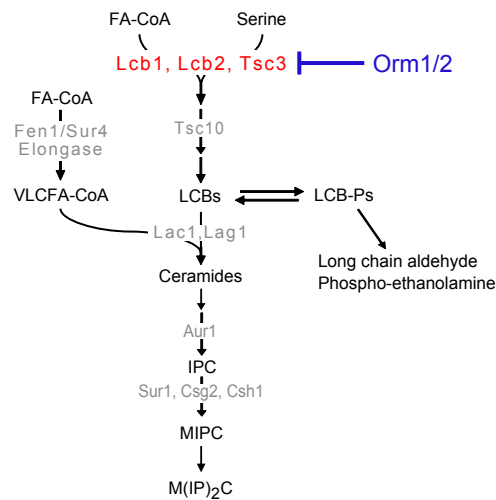
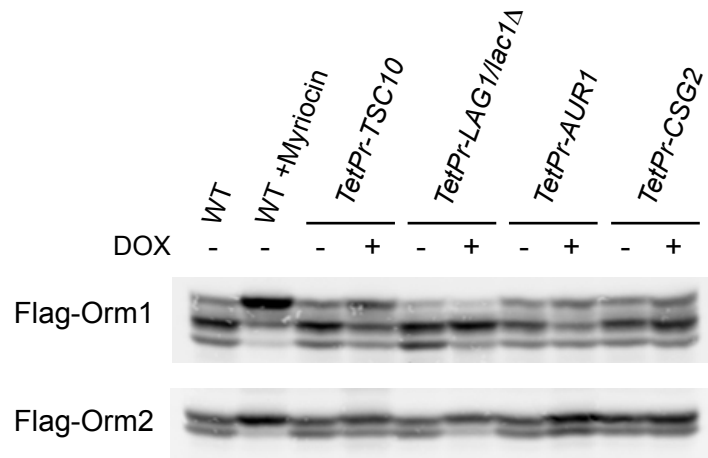


Supplementary Figure 3.4



**Supplementary Figure 3.4. Orm2 localization changes in response to myriocin treatment.** GFP-Orm2 and GFP-Orm1 were visualized in strains grown in rich media with or without myriocin. Sec63-mCherry was used as a marker for ER localization. Arrowheads in images of GFP-Orm2 indicate a reduction in cortical ER localization in response to myriocin treatment. Median filtering was applied to reduce image noise.

Supplementary Figure 3.5

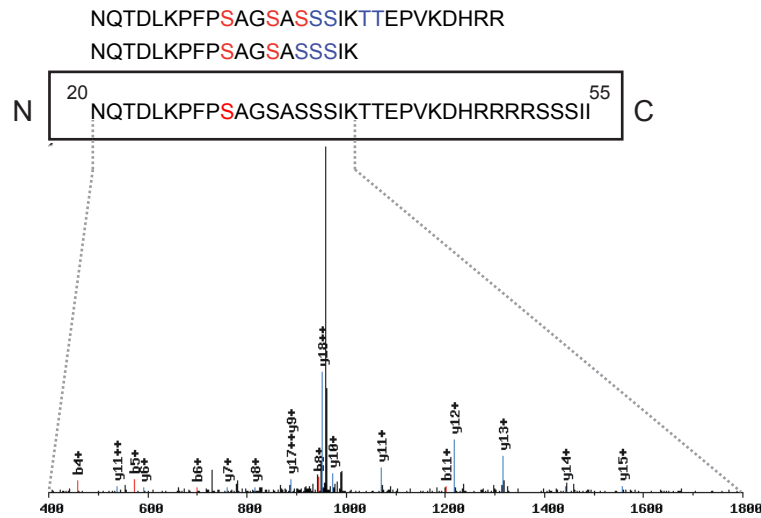


**Supplementary Figure 3.5. Disruptions to sphingolipid synthesis that act downstream of Lcb1/2 but upstream of Aur1 induce Orm1/2 phosphorylation.**

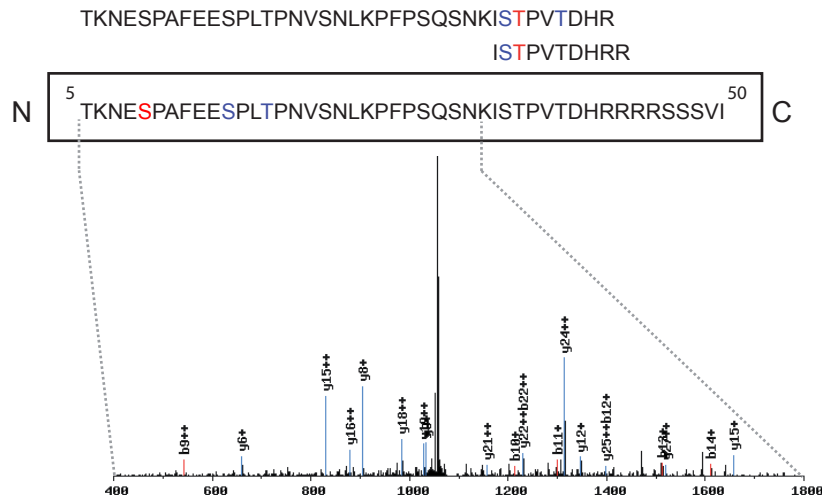
Tetracycline-repressible promoters were inserted in front of the indicated genes in strains expressing 3xFlag-Orm1 or 3xFlag-Orm2 (note: *pTet-LAG1* was combined with deletion of *LAC1*). Lysates were prepared before and after gene expression shut-off (treatment with 5 µg/ml doxycycline for 14-16 hr) and analyzed by Western blot against the Flag epitope after separation on phosphate-affinity SDS/PAGE gels. Note: gel-to-gel differences in banding pattern are due to variability in phosphate-affinity gel resolution.

# Supplementary Figure 3.6

Orm1

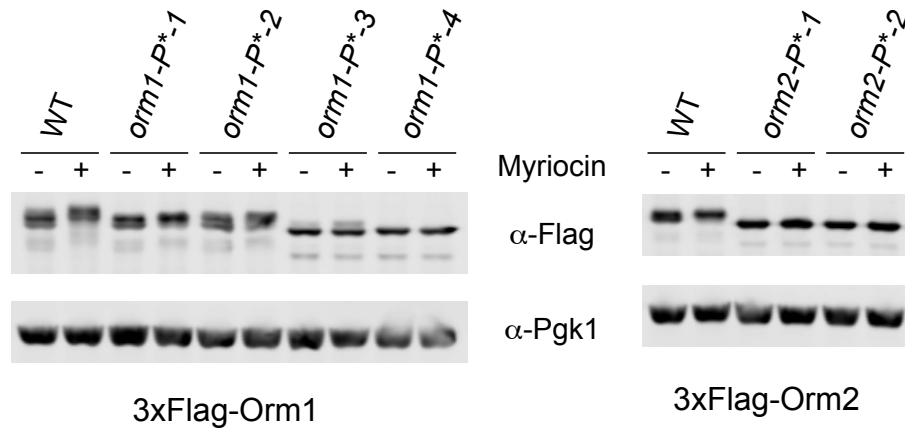


Orm2



**Supplementary Figure 3.6. Mass spectrometry identifies phosphorylated residues on Orm1 and Orm2.** Phospho-peptides from immuno-precipitated 3xFlag-Orm1 and 3xFlag-Orm2 proteins were analyzed using an orbitrap mass spectrometer. For both Orm1 and Orm2, three phospho-peptides were identified. All Orm2 phospho-peptides were singly phosphorylated, whereas singly, doubly and triply phosphorylated peptides were identified for Orm1. The most probable phosphorylation site assignments are highlighted in red, and alternative assignments (with a lower probability) are highlighted in blue. For the boxed peptides NQTDLPFPSSAGSASSSIK (Orm1) and TKNESPAFEESPLTPNVSNLKPFPSSQSNK (Orm2), annotated tandem mass spectra are shown, where s\* indicates the site of phosphorylation.

Supplementary Figure 3.7



**Supplementary Figure 3.7. Wild-type and phospho-mutant forms of Orm1 and Orm2 show similar expression levels.** Lysates from strains expressing 3xFlag-tagged wildtype (WT) or phospho-mutant alleles of *ORM1* and *ORM2* were prepared after growth for 12-16 hr in 0 or 150 ng/ml myriocin. Western blots of these lysates were probed against the Flag epitope and against Pgk1 as a loading control.

|            | <b>IP 3xFlag-Orm1</b> |                     | <b>IP 3xFlag-Orm2</b> |                     |
|------------|-----------------------|---------------------|-----------------------|---------------------|
| Protein ID | # of peptides         | % Sequence Coverage | # of peptides         | % Sequence Coverage |
| Sac1       | 42                    | 74.6                | 33                    | 62.1                |
| Lcb1       | 23                    | 40.9                | 26                    | 43.5                |
| Lcb2       | 23                    | 41.0                | 22                    | 37.1                |
| Orm1       | 10                    | 49.1                | 4                     | 19.4                |
| Orm2       | 6                     | 25.0                | 8                     | 31.9                |
| Tsc3       | 3                     | 30.0                | 1                     | 17.5                |

**Supplementary Table 3.1. Orm-associated proteins identified by mass spectrometry.**

Proteins found in immunoprecipitations of 3xFlag-Orm1 and 3xFlag-Orm2 were analyzed by mass spectrometry. Peptide coverage information for identified proteins corresponding to the bands indicated in **Fig. 3.2a** are shown above.

## Supplementary detailed methods

**Growth media, plasmids, and strain construction.** All yeast experiments were conducted in strain BY4741 (ATCC)<sup>119</sup>, a related strain suitable for E-MAP experiments<sup>7</sup>, and derivatives thereof. Strains were grown in YEPD media or media supplemented with myriocin (Sigma Aldrich; at least 12 hr growth in myriocin in all cases), with the exception of samples prepared for lipidome analysis, which were grown in synthetic defined media.

Deletion, DAmP, over-expression (*pTEF2* and *pTHD3*), mCherry fusion, and tetracycline-repressible promoter strains were constructed by standard PCR-based methods (see **Supplementary Information**). Strains expressing N-terminally epitope-tagged or GFP-tagged forms of wild-type and mutant Orm1, Orm2, Lcb1, and Sac1 were made by integration of DNA fragments encoding these gene variants into strains in which these genes had been deleted with the *URA3* counter-selectable marker. For the essential gene *LCB1*, a diploid strain was used for integration of the tagged allele containing the 3xFlag epitope inserted between codons 9 and 10, as previously reported<sup>145</sup>. *ORM1* and *ORM2* mutants, as well as the previously described *sac1-8* mutation<sup>132</sup>, were created by standard PCR mutagenesis.

HEK293T cells (ATCC) and HeLa-Kyoto cells (a gift from A. Hyman) were grown in DMEM high-glucose media supplemented with penicillin, streptomycin, glutamine, and 10% heat-inactivated fetal calf serum. *ORMDL3* was cloned from HEK293T cDNA into expression plasmids containing N- or C-terminal 3xFlag epitopes (p3XFLAG-CMV-10 and p3XFLAG-CMV-14; Sigma Aldrich).

**E-MAP data collection and analysis.** E-MAP data were collected and analyzed as described previously<sup>7</sup> (manuscript in preparation). Genetic interaction patterns were compared for pairs of mutants by first identifying those genetic backgrounds for which E-MAP data was available for both mutants of interest. The similarity of the genetic interaction scores for the two mutants was then calculated by computing the cosine correlation between their interaction scores across these shared mutant backgrounds. For a given mutant of interest such as *orm2Δ*, these pair-wise comparisons were carried out with all other mutant strains having at least 200 shared genetic interaction data points. For the *ORM1* and *ORM2* over-expression mutants, an average of the *pTDH3* and *pTEF2* interaction profiles was used as the query profile for correlation calculations.

**Lipidome analysis by mass spectrometry.** Lipidome analyses were conducted as described previously<sup>129</sup> with minor modifications based on an existing method<sup>146</sup> for LCB analysis. Samples were harvested from 20ml cultures of yeast cells growing logarithmically in synthetic defined media or from  $\sim 4\text{-}6 \times 10^6$  HeLa cells grown as described above. Cells were washed in water or 155mM ammonium bicarbonate at 4°C, and cell pellets were then frozen immediately in liquid nitrogen. Yeast data are from two independent samples each analyzed twice; HeLa cell data are from samples analyzed in triplicate.

**Growth rate measurements.** Logarithmic growth rates were measured by OD<sub>600</sub> from  $n \geq 3$  replicate samples. Saturated cultures of the relevant strains were diluted into fresh media and grown for >7 hr prior to growth analysis. Myriocin-treated cultures were grown for 3 hr in the presence of drug prior to growth rate analysis.

**Native affinity purifications of solubilized membrane proteins (large-scale, yeast).**

ER-derived microsomes were prepared from yeast cells grown in YEPD (~4000 OD<sub>600</sub> units harvested at OD<sub>600</sub> ~1.4). Cells were harvested, washed in water at 4°C, resuspended in lysis buffer (50mM Hepes-KOH, pH 6.8, 150mM KOAc, 2mM MgOAc, 1mM CaCl<sub>2</sub>, 200mM sorbitol) supplemented with protease inhibitors (Complete EDTA-free cocktail; Roche) and frozen in a drop-by-drop fashion in liquid nitrogen. Frozen cells were then pulverized in a ball mill (6x 3 min at 30Hz; Retsch), thawed in lysis buffer at 4°C, and dounced 10 times to homogeneity. After clarification by two consecutive centrifugations at 1000 x g, microsomes were pelleted at 44000 x g, resuspended in 0.5ml lysis buffer, and then diluted with 14ml IP buffer (50mM Hepes-KOH, pH 6.8, 150mM KOAc, 2mM MgOAc, 1mM CaCl<sub>2</sub>, 15% glycerol) with 2% digitonin (Calbiochem) supplemented with protease inhibitors. After nutating for 1.5 hr at 4°C, un-solubilized material was removed by centrifugation at 44000 x g, and the remaining supernatant was added to anti-Flag resin (150µl bed volume; Sigma Aldrich). Immunoprecipitations were nutated for 3 hr at 4°C and then washed 4x10ml with IP buffer with 0.1% digitonin. Bound proteins were eluted twice with 150µl IP buffer with 0.25% digitonin and 2mg ml<sup>-1</sup> 3xFlag peptide (Sigma Aldrich). Eluates were combined, separated on SDS/PAGE gels, and stained with a colloidal blue staining kit (Invitrogen). Samples were prepared for phospho-site mapping using the same protocol, except that phosphatase inhibitors (Halt phosphatase inhibitor cocktail; Pierce) were included in the buffers used.

**Native affinity purifications of solubilized membrane proteins (small-scale, yeast).**

Lysates for small-scale immunoprecipitations were prepared from logarithmically



growing yeast cells (~25 OD<sub>600</sub> units) grown in YEPD. Cultures were harvested, washed in water at 4°C, and resuspended in 500µl IP buffer (see above) with 0.1% digitonin and supplemented with protease and phosphatase inhibitors. Cell lysates were prepared by bead-beating at 4°C, followed by addition of 500µl IP buffer with 1.9% digitonin to raise the final digitonin concentration to 1%. Membrane proteins were solubilized by nutating lysates at 4°C for 40 min. Unsolubilized material was next removed by centrifugation at 100,000 x g, and clarified lysates were incubated with anti-Flag resin (25µl bed volume) for 2.5 hr. The anti-Flag resin was then washed four times with 1ml IP Buffer containing 0.1% digitonin, and bound proteins were eluted by addition of 30µl IP Buffer with 0.25% digitonin and 2mg ml<sup>-1</sup> 3xFlag peptide.

**Native affinity purifications of solubilized membrane proteins (small-scale, human cells).** HEK293T cells grown in DMEM high-glucose media supplemented with penicillin, streptomycin, glutamine, and 10% heat-inactivated fetal calf serum were transfected using Eugene6 transfection reagent (Roche) according to the manufacturer's instructions. After approximately 48 hr growth, transfected cells were harvested by trypsinization and washed with PBS. Cell pellets were then resuspended at 4°C in 1ml IP buffer (see above) with 2% digitonin and protease inhibitors and nutated for 45 min to solubilize membrane proteins. Insoluble material was removed by centrifugation at 100,000 x g, and the clarified supernatant was incubated with anti-Flag resin (25µl bed volume) for 2.5 hr. The remainder of the immunoprecipitation was done in the same fashion as the yeast small-scale affinity purifications (see above).

**Denaturing immunoprecipitations and phosphatase treatment.** A 50ml yeast culture in logarithmic phase growth was harvested and resuspended in 10ml 5% trichloroacetic

acid and incubated for 10 min at 4°C. Cell pellets were washed once in 1ml 5% TCA, washed twice in 1ml acetone, and then air-dried. 200µl acid-washed glass beads and 150µl SDS lysis buffer (50mM Tris-Cl, pH 7.8, 1mM EDTA, 1% SDS, 2M Urea) supplemented with protease inhibitors were added to the dried pellets, which were then lysed by bead-beating (3x 60s at 4°C). Lysates were diluted by addition of 1350µl Tween IP buffer (100mM Tris-Cl, pH 7.8, 200mM NaCl, 0.5% Tween-20, also supplemented with protease inhibitors) and clarified at 20000 x g. Supernatants were then added to anti-Flag agarose (25µl bed volume) and immunoprecipitated for 2.5 hr at 4°C. The anti-Flag resin was then washed 2x 1ml with Tween IP buffer, 1x 1ml with Tween/Urea wash buffer (100mM Tris-Cl, pH 7.8, 100mM NaCl, 0.5% Tween-20, 2M Urea), and 2x 1ml in phosphatase buffer (NEBuffer 3; New England Biolabs), with 0.1% Tween-20). The washed resin was then resuspended in 54µl phosphatase buffer + 6µl calf intestine phosphatase (New England Biolabs) and incubated for 1 hr at 37°C. After phosphatase treatment, the resin was sequentially washed with 1ml Tween IP buffer and 1ml in SDS/DOC wash buffer (50mM Tris-Cl, pH 7.8, 0.1% SDS, 0.1% Na-deoxycholate). Bound proteins were then eluted by addition of 60µl SDS buffer (100mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol) and incubation for 10 min at 65°C.

**RNAi and plasmid transfection of HeLa cells.** HeLa cells grown in DMEM high-glucose media supplemented with penicillin, streptomycin, glutamine, and 10% heat-inactivated fetal calf serum were transfected using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. After approximately 72 hr growth, transfected cells were harvested by trypsinization for lipidomic and gene expression analysis. For each targeted gene, a pool of four siRNAs was used (ON-

TARGETplus SMARTpool; Dharmacon), with siRNA sequences as follows: *ORMDL1*: 5'-UGGCAAGUUUCUAUACGAA-3', 5'-GGAGUUGGCUUGCUUCAUA-3', 5'-GCUUAUAAGUUAAGGGCA-3', 5'-GGACCAGCUUAACUUAUAA-3'; *ORMDL2*: 5'-UGGUAGGAUUGCUGCAUAU-3', 5'-AGUUUAAUCAGGAUGGGUA-3', 5'-GUGUACUGCUGCCGAAGUU-3', 5'-AGUAUGAUGCUGCGCACUU-3'; *ORMDL3*: 5'-GAACAUGGACCACGCAGUU-3', 5'-ACACUAAGUACGACCAGAU-3', 5'-CGGUACGGCUUCUGGAUUG-3', 5'-UGGGUAGGGAGCUGUCUAA-3'.

**RT-PCR analysis of gene expression in HeLa cells.** RNA was extracted from HeLa cells using the RNeasy kit (Qiagen) after cell homogenization (QIAshredder; Qiagen). Extracted RNA was reverse-transcribed using AMV RT (Promega) and amplified with Taq polymerase (GoTaq; Promega). Real-time SYBR Green fluorescence was monitored using a DNA Engine Opticon System (MJ Research), and the following primers were used: *RPL19*: 5'-ATGTATCACAGCCTGTACCTG-3', 5'-TTCTTGGTCTCTTCCTCCTTG-3'; *ORMDL1*: 5'-CTGACCAGGGTAAAGCAAGG-3', 5'-TCCAAAGATCCGAACACCAT-3'; *ORMDL2*: 5'-CATACGGTGAAAGGGACACC-3', 5'-CGGCAGCAGTACACTTAGCA-3'; *ORMDL3*: 5'-AAGCCCTGCTCACTTGTTTG-3', 5'-CAAAGACCCATCCCACACTT-3'.

**Live-cell yeast fluorescence microscopy.** Yeast cells expressing Sec63-mCherry and GFP-Orm1 or GFP-Orm2 were grown in rich media with or without myriocin and spotted on a concanavalin A-coated 25 x 50 mm coverslip covered with a 22 x 22 mm coverslip. Cells were imaged through the 25 x 50 mm coverslip using a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging Inc.) equipped with a spinning disk confocal

scanhead and laser system (Yokogawa CS10, Yokogawa Electronics; Solamere). Images were collected as Z-stacks with 0.5  $\mu\text{m}$  spacing between frames using  $\mu\text{Manager}$  software and an ImagEM C9100-13 EM-CCD camera (Hamamatsu Photonics).

**Serial-dilution cell spotting assays.** Logarithmically growing yeast were spotted onto agar plates in 10-fold serial dilutions from a starting  $\text{OD}_{600}$  density of 0.3 to a final  $\text{OD}_{600}$  density of  $3 \times 10^{-4}$ . Plates were imaged after growth for 24-48 hr. Plates with myriocin are standard YEPD supplemented with 0.1% tergitol and 2mM citric acid.

**LCB extraction and analysis by HPLC.** LCB extraction and analysis was carried out as described by Lester and Dickson<sup>146</sup>. Myriocin-treated samples were grown for 14-16 hr in the presence of drug prior to extractions. For extractions, 50ml of a logarithmically growing culture was harvested and re-suspended in 10ml 5% TCA at 4°C. Following a 10 min incubation, cells were washed once in 5% TCA and three times in  $\text{H}_2\text{O}$  at 4°C. Cell pellets were then re-suspended in 75 $\mu\text{l}$   $\text{H}_2\text{O}$  followed by addition of 425 $\mu\text{l}$  70.6mM TEA/ethanol. Lipids were extracted by bath sonication (3x 99s, 30W) and incubation at 65°C for 30 min. 150 $\mu\text{l}$  of this extract was derivatized by reaction for 40 min at room temperature with 30 $\mu\text{l}$  of AccQ Fluor reagent (Waters Corp.). Ester-linked lipids were de-acylated by addition of 22.5 $\mu\text{l}$  of 1.5M KOH/methanol. Following 30 min incubation at 37°C, the reaction was neutralized by addition of 22.5 $\mu\text{l}$  1.74M acetic acid/methanol. Samples were analyzed on an Agilent Chemstation HPLC using a reverse-phase C18 column (ZORBAX Eclipse XDB-C18; Agilent).

**Protein identification by mass spectrometry.** Gel slices were trypsinized and mass spectra were acquired on a Bruker Reflex III MALDI-TOF mass spectrometer. Proteins

were identified by searching NCBI database using MS-Fit program on Protein Prospector (University of California, San Francisco, <http://prospector.ucsf.edu>)<sup>147</sup>.

**Identification of phosphorylation sites by mass spectrometry.** Proteins eluted from native affinity purifications of 3xFlag-Orm1 or 3xFlag-Orm2 (see above) were precipitated using ice-cold acetone. After incubation overnight at -20°C, proteins were pelleted at 4°C by centrifugation. Protein pellets were then processed, and the resulting peptide mixtures were analyzed on a hybrid LTQ-Orbitrap mass spectrometer, as described previously<sup>148</sup>. Database searches were performed using the Saccharomyces Genome Database non-redundant database, as described<sup>148</sup>.

**Plasmid sequences.** Plasmid sequences below are derived in part from published constructs described in Gari *et al.*<sup>149</sup> and Shaner *et al.*<sup>117</sup>

#### **pFA6a-NATMX4\_pTEF2\_eGFP\_Adh1-tm-AG**

Primer annealing sequences used for PCR are underlined. TEF2 promoter sequence is in bold font.

```
GAACGCGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTCGACGGATCCCCGGGTTAATTAAGGCGCGCCAG
ATCTGTTTTAGCTTGCCCTTGTCCTCCCGCCGGGTCACCCGGCCAGCGACATGGAGGCCAGAATACCCCTCCTTG
ACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTGCGCCGTACATTTAGCCCATACATCCCAT
GTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCA
GACCTGCGAGCAGGGAAACGCTCCCTCACAGACGCGTTGAATTGTCCCCACGCGCGCCCTGTAGAGAA
ATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTATATACTTCCTTTTAAATCTTGCTAGGATAC
AGTTCTCACATCACATCCGAACATAAACAACCATGGGTACCACTCTTGACGACACGGCTTACCGGTACCGC
ACCAAGTGTCCCGGGGACGCCGAGGCCATCGAGGCACTGGATGGGTCCCTTACCACCGACACCGTCTTCCG
CGTCACCGCCACCGGGGACGGCTTCACCTGCGGGAGGTGCCGGTGGACCCGCCCTGACCAAGGTGTTCC
CCGACGACGAATCGGACGACGAATCGGACGACGGGAGGACGGCGACCCGGAATCCCGGACGTTCTGTCGCG
TACGGGGACGACGGCGACCTGGCGGGCTTCGTGGTCTGCTCGTACTCCGGCTGGAACCGCGCGCTGACCGT
CGAGGACATCGAGGTGCGCCCGGAGCACCGGGGACCGGGTTCGGGCGCGCGTGTGATGGGGCTCGCGACGG
AGTTCTGCGCCGCGAGCGGGGCGCCGGGCACCTCTGGCTGGAGGTACCAACGTCAACGCACCGGCGATCCAC
GCGTACCGGCGGATGGGGTTCACCTCTGCGGCCTGGACACCGCCCTGTACGACGGCACCGCCTCGGACGG
CGAGCAGGCGCTCTACATGAGCATGCCCTGCCCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAA
GAACTTGTCATTTGTATAGTTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATA
TTTTTTTTTCGCCTCGACATCATCTGCCAGATGCGAAGTTAAGTGCAGAAAGTAATATCATGCGTCAAT
CGTATGTGAATGCTGGTGCCTATACTGCTGTCGATTGATACTAACGCCGCCATCCAGTGTGAAAACGAG
CTCATATATGGGGCCGTATACTTACATATAGTAGATGTCAAGCGTAGGCGCTTCCCCTGCCGGCTGTGAGG
```

GCGCCATAACCAAGGTATCTATAGACCGCCAATCAGCAAACCTACCTCCGTACATTCATGTTGCACCCACAC  
ATTTTATACACCCAGACCGCGACAAATTACCCATAAGGTTGTTTGTGACGGCGTCGTACAAGAGAACGTGGG  
AACTTTTTTAGGCTCACCAAAAAAGAAAGAAAAAATACGAGTTGCTGACAGAAGCCTCAAGAAAAAAAAT  
TCTTCTTCGACTATGCTGGAGGCAGAGATGATCGAGCCGGTAGTTAACTATATATAGCTAAATTGGTTCCA  
TCACCTTCTTTTTCTGGTGTGCTCCTTCTAGTGCTATTTCTGGCTTTTTCTATTTTTTTTTTCCATTTTT  
CTTTCTCTCTTTCTAATATATAAATTCTCTTGCATTTTCTATTTTTCTCTCTATCTATTCTACTTGTAT  
TCCCTTCAAGGTTTTTTTTTAAGGAGTACTTGTTTTTAGAAATATACGGTCAACGAACTATAATTAACATAA  
CACTAGTACCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTGAATTAGATGGTG  
ATGTTAATGGGCACAAATTTTCTGTCACTGGAGAGGGTGAAGGTGATGCAACATACGGAACCTTACCCTT  
AAATTTATTTGCACTACTGGAACCTACCTGTTCCATGGCCAACACTTGTCACTACTTTCACTTATGGTGT  
TCAATGCTTTTTCAAGATACCCAGATCATATGAACGGCATGACTTTTTCAAGAGTGCCATGCCGCAAGGTT  
ATGTACAGGAAAGAACTATATTTTTCAAAGATGACGGGAACACTACAAGACACGTGCTGAAGTCAAGTTTGA  
GGTGATACCCTTGTTAATAGAATCGAGTTAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACA  
CAAATTGGAATACAACATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGAATCAAAG  
TTAATTCAAATTAGACACAACATTGAAGATGGAAGCGTTCACTAGCAGACCATTATCAACAAAATACT  
CCAATTGGCGATGGCCCTGTCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTTCGAAAGA  
TCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAAACAGCTGCTGGGATTACACATGGCATGG  
ATGAACATAACAATAGGGCTCGAGGccccgctattaacgctttgtaatgtatagcttttaatgtgtgatc  
gcctgacttttgcacgcggcgccggcgccgagcgcgctgctccaggcgagcgtcacggcgaggtggcgggct  
gtgagagctggcgcgactggcgcgactgtcgctgtcacgtgacggcgaccacgggtggagaaaatttttgg  
ccaacggcgcgagagcagtcctcgacAACGAGCTCGAATTCATCGACCGCGGATCTGCCGCTCTCCCTAT  
AGTGAGTCGTATTAATTTGATAAGCCAGGTTAACTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGC  
GGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTCGGTGCGGCG  
AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA  
TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC  
CGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAG  
ATACCAGGCGTTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCTGTTCCGACCCTGCCGCTTACCGGATACC  
TGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTG  
TAGGTCTGTTCTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTACGCCCCGACCGCTGCGCTTATCCGG  
TAACATCTGTCCTTAGATCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGA  
TTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGA  
AGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAGAGATTGGTAGCTCTTGATC  
CGGCAAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAG  
GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGG  
ATTTTGCTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATC  
AATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG  
CGATCTGTCTATTTCTGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGC  
TTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAAT  
AAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA  
ATTGTTGCCGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACA  
GGCATCGTGGTGTACGCTCGTCTGTTTGGTATGGCTTCATTACGCTCCGGTTCCCAACGATCAAGGCGAGT  
TACATGATCCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTGCAAGTAAGT  
TGGCCGAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTATGCCATCCGTAAGA  
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTC  
TTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAGTGCTCATCATTGGAAAAC  
GTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCA  
CCCACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTTCTGGGTGAGCAAAAACAGGAAGGCCAAATGC  
CGCAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCTTTTTCAATATTATTGAA  
GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGG  
GTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTA  
TAAAAATAGGCGTATCACGAGGCCCTTTCTGCTCGCGCGTTTTCGGTGATGACGGTGAACCTCTGACACA  
TGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCG  
TCAGCGGTGTTGGCGGGTGTGCGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCA  
CCATATGGACATATTGTCGTTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGAT  
TTAGGTGACACTATA

**pFA6a-NATMX4\_pTDH3\_eGFP\_Adh1-tm-AG**

Primer annealing sequences used for PCR are underlined. TDH3 promoter sequence is in bold font.

GAACGCGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTCGACGGATCCCCGGGTTAATTAAGGCGGCCAG  
ATCTGTTTGTAGCTTGCCTTGTCCCCGCCGGGTACCCGGCCAGCGACATGGAGGCCAGAATACCCTCCTTG  
ACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTGCGCCGTACATTTAGCCCATACATCCCCAT  
GTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCA  
GACCTGCGAGCAGGGAAACGCTCCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCCTGTAGAGAA  
ATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTCATATACTTCCTTTTAAAATCTTGCTAGGATAC  
AGTTCTCACATCACATCCGAACATAAACAACCATGGGTACCACTCTTGACGACACGGCTTACCGGTACCGC  
ACCAGTGTCCCGGGGGACGCCGAGGCCATCGAGGCACTGGATGGGTCTTCACCACCGACACCGTCTTCCG  
CGTCACCGCCACCGGGGACGGCTTCACCTGCGGGAGGTGCCGGTGGACCCGCCCCCTGACCAAGGTGTTCC  
CCGACGACGAATCGGACGACGAATCGGACGACGGGAGGACGGCGACCCGGAATCCCGGACGTTTCGTCGCG  
TACGGGGACGACGGCGACCTGGCGGGCTTCGTGGTCGTCTCGTACTCCGGCTGGAACCGCCGGCTGACCGT  
CGAGGACATCGAGGTGCGCCCGGAGCACCGGGGGCACGGGGTCGGGCGCGCGTTGATGGGGTCGCGACGG  
AGTTCGCCCCGCGAGCGGGGCGCCGGGCACCTCTGGCTGGAGGTACCAACGTCAACGCACCGGCGATCCAC  
GCGTACCGGCGGATGGGGTTCACCTCTGCGGCCTGGACACCGCCCTGTACGACGGCACCGCCTCGGACGG  
CGAGCAGGCGCTCTACATGAGCATGCCCTGCCCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAA  
GAACTTGTCATTTGTATAGTTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATA  
TTTTTTTTTCGCTCGACATCATCTGCCAGATGCGAAGTTAAGTGCAGAAAAGTAATATCATGCGTCAAT  
CGTATGTGAATGCTGGTCGCTATACTGCTGTCGATTGATACTAACGCCGCCATCCAGTGTGAAAACGAG  
CTC**CACGCTTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGAATACGTAAATAATTAATA**  
**GTAGTGATTTTCTTAACCTTTATTTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCA**  
**AAATAGGGGGCGGGTTACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCTGGCATCC**  
**ACTAAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAAAAAAAGAATCCCAGCACCAAAATATTG**  
**TTTTCTTCACCAACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAACAG**  
**GCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACACAAGGCAATTGA**  
**CCCACGCATGTATCTATCTCATTCTTACACCTTCTATTACCTTCTGCTCTCTCTGATTTGGAAAAAGCT**  
**GAAAAAAAAGGTTGAAACCAGTTCCTGAAATTATCCCTACTTGACTAATAAGTATATAAAGACGGTAG**  
**GTATTGATTGTAATTCTGTAAATCTATTTCTTAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTT**  
**TAGTTTAAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAACAAAC**ACTAGTACCATGAGTAAAGG  
AGAAGAATTTTTACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTT  
CTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAATACTTACCCTTAAATTTATTTGCACTACTGGA  
AAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCACTTATGGTGTTCAATGCTTTTCAAGATACCC  
AGATCATATGAAACGGCATGACTTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATAT  
TTTTTCAAGATGACGGGAACATAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGA  
ATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAACATTCTTGACACAAATTGGAATACAACTATAA  
CTCACACAATGTATACATCATGGCAGACAAAACAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACA  
ACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTC  
CTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTTGAAAGATCCCAACGAAAAGAGAGACCA  
CATGGTCTCTTCTTGAGTTTGTAAACAGCTGCTGGGATTACACATGGCATGGATGAACATATACAATAGGGCC  
TCGAGGccccgctattaacgcttttgtaatgtatagcttttaatgtgtgatcgccctgacttttgcacgcgcggc  
cggcgcgcgcgcgctgcgtccaggcgcgcgtcacggcgcagggtggcgggctgtgagagctggcgcgactggc  
gcgactgtcgctgtcacgtgacggcgaccacgggtggagaaaatttttggccaacggcgcgagagcagtc  
ctcgacAACGAGCTCGAATTCATCGACCGCGGATCTGCCGGTCTCCCTATAGTGAGTCGTATTAATTTCGA  
TAAGCCAGGTTAACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTGGGCGCTCT  
TCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA  
GGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAA  
AGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAC  
AAATATCGACGCTCAAGTCAGAGTGGCGAAACCCGACAGGACTATAAGATACACGGCTTTCCCTCTGG  
AAAGTCCCTCGTGCCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGG  
GAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTGCTTCGCTCCAAGCTG

GGCTGTGTGCACGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAA  
 CCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG  
 GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGC  
 GCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGG  
 TAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTTGA  
 TCTTTTCTACGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCA  
 AAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTA  
 AACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCAT  
 CCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT  
 GCAATGATACCGCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCAATAAACCCAGCCAGCCGGAAGGGC  
 CGAGCGCAGAAGTGGTCTTCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAG  
 TAAGTAGTTCCGAGTTAATAGTTTGCAGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACAGCTCG  
 TCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTG  
 CAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTGAGAAGTAAGTTGGCCGAGTGTTATCACTCA  
 TGGTTATGGCAGCACTGCATAATTCTCTTACTGTGATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAG  
 TACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGA  
 TAATACCGCGCCACATAGCAGAACTTTAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT  
 CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGACCCAACTGATCTTCAGCATCT  
 TTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGC  
 GACACGGAAATGTTGAATACTCATACTCTTCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTC  
 TCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA  
 AAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAAATAGGCGTATCACGAG  
 GCCCTTTTCGTCTCGCGCGTTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA  
 CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGT  
 CGGGGCTGGCTTAAGTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGGACATATTGTGCTT  
 AGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACACTATA

### pFA6a-NAT-MX4-Tet-Act-pTet

A fusion PCR product of the pFA6a-NATMX4 marker, Tet-Activator, and 4XTet-Operator promoter was generated and cloned into the pCR2.1-TOPO vector (Invitrogen). The sequence of the cloned PCR product is given below (NAT marker is underlined, TetO sites are in bold font):

GGTCGACGGATCCCCGGGTAAATTAAGGCGCGCCAGATCTGTTTAGCTTGCCCTCGTCCCCGCCGGGTACCC  
CGGCCAGCGACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGT  
GACTGTGCGCCGTACATTTAGCCCATACATCCCCATGTATAATCATTGTCATCCATACATTTTGATGGCCG  
CACGGCGCGAAGCAAAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTCACAGACG  
CGTTGAATTGTCCCCACGCCGCGCCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTC  
TTTCATATACTTCCTTTTAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCATG  
GGTACCACTCTTGACGACACGGCTTACCGGTACCGCACCAGTGTCCCGGGGACGCCGAGGCCATCGAGGC  
ACTGGATGGGTCTTCAACACCGACACCGTCTTCCGCGTCACCGCCACCGGGGACGGCTTCAACCTGCGGG  
AGGTGCCGGTGGACCCGCCCTGACCAAGGTGTTCCCCGACGACGAATCGGACGACGAATCGGACGACGGG  
GAGGACGGCGACCCGGACTCCCGGACGTTTCGTGCGGTACGGGGACGACGGCGACCTGGCGGGCTTCGTGGT  
CGTCTCGTACTCCGGCTGGAACCGCCGGCTGACCGTTCGAGGACATCGAGGTGCCCCGGAGCACCGGGGGC  
ACGGGGTGGGGCGCGCTTGATGGGGCTCGCGACGGAGTTCGCCCGCGAGCGGGGCGCCGGGCACCTCTGG  
CTGGAGGTCAACACGTCAACGCACCGGCGATCCACGCGTACCGGCGGATGGGGTTCACCTCTGCGGCCT  
GGACACCGCCCTGTACGACGGCACCGCCTCGGACGGCGAGCAGGCGCTCTACATGAGCATGCCCTGCCCT  
AATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAG  
TTGTTCTATTTTAAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCTCGACATCATCTGCCAGATGCG  
AAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTGCCTATACTGCTGTGAT  
TCGATACTAACGCCGCCATCCAGTGTGAAAACGAGCTCGAATTCTtattacgatcctcgcgccccctacc  
 caccgtactcgtcaattccaagggcatcggttaacatctgctcaaactcgaagtcggccatatccagagcg  
 ccgtagggggcgagtcgtggggggtaaatcccggaacccggggaatccccgtcccccaacatgtccagatc  
 gaaatcgtctagcgcgtcgccatgcgccatcgccacgtcctcgccgtctaagtggagtcgtccccagggc



tgacatcggtcgggggggcggtcgacagtcctgcgcgtgtgtcccgcgaggagaaaggacaggcgaggagcc  
 gccagccccgcctcttcggggggcggtcgctcgccggagatcgagcaggccctcgatggtagaccgcgaatt  
 gtttttcgtacgcgcgcgggtgtacgcggaccactttcacatttaagttgttttctaatccgcatatga  
 tcaattcaaggccgaataagaaggctggctctgcaccttggatgacaaataattcgatagcttgtcgaat  
 aatggcggcatactatcagtagtaggtgtttccctttcttcttagcgacttgatgctcttgatcttccaa  
 tacgcaacctaaagtaaaatgccccacagcgctgagtgcatataatgcatttctctagtgaacaccttggt  
 ggcataaaaaggctaattgattttcgagagtttcatactgtttttctgtaggccgtgtacctaataatgtact  
 tttgctccatcgcatgacttagtaaaagcacatctaaaacttttagcggttattacgtaaaaaatcttgcca  
 gctttcccttcttaaagggcaaaagtgaagtatgggtgcctatctaactctcaatggctaaggcgctcgagca  
 aagcccgccttattttttacatgccaatacaatgtaggctgtctacacctaagcttctgggcgagtttacgg  
 gttgttaaacccttcgattccgacctcattaagcagctctaatagcgctgttaatacactttacttttatctaa  
 tctagacatatgaatttaattcGggggccgcggaggctggatcggtcccgggtgtcttctatggagggtcaaac  
 agcgtggatggcgtctccaggcgatctgacgggtcactaaacgagctctgcttatatagacctccaccgt  
 acagcctaccgcccattttgcgtcaatggggcgagttgttacgacattttggaaagtcccgttgattttg  
 gtgcaaaaacaaactcccattgacgtcaatggggtggagacttgaaatcccgtgagtcacacgcgtatc  
 cagccccattgatgtactgcaaaaaccgcataccatggtaatagcgatgactaatacgtagatgtactgc  
 caagtaggaaagtcccataaggctcatgtactgggcataatgccaggcgggccatttacgctcattgacgtc  
 aatagggggcgtaacttggcatatgatacacttgatgtactgccaagtgggcagtttagcgtaataactcca  
 cccattgacgtcaatggaaagtccctattggcggttactatgggaacatacgtcattattgacgtcaatggg  
 cgggggtcggttgggcgggtcagccaggcgggccatttacgtaagttatgtaacgcggaactccatataatgg  
 gctatgaactaatgaccccgtaattgattactattaataactagtcaataatcaatgtcaacatggcggtta  
 atgttgacatgagccaatataaatgtacatattatgatatggatacaacgtatgcaatgggccaagctcc  
 tcgagtaattcgcgccacttctaaataagcgaatttcttatgatttatgatttttattattaaataagtta  
 taaaaaaaataagtgatatacaaaattttaaagtgaactcttaggttttaaaacgaaaattcttAttccttgagt  
 aactctttcctgtaggtcaggttgccttctcaggtatagTatgaggtcgctcttattgaccacacctctac  
 cggcAGATCCGCTAGGGATAACAGGGTAATATagatcaattcctcgatcgagtttaccact**tcctatcagt**  
**gatagagaaaagtgaagtcgagtttaccactccctatcagtgatagagaaaagtgaagtcgagtttacc**  
**actccctatcagtgatagagaaaagtgaagtcgagtttaccactccctatcagtgatagagaaaagtga**  
**agtcgagtc**cggtaccctatggcatgcatgtgctctgtatgtatataaaactcttggttttcttctttctc  
 taaatattctttccttatacattaggtcctttagtcagtaaaattactataacttctatagacacgcaaacac  
 aaatacacacactaaattaccggatcaattcggg**ATG**

## pNTI8\_mCherry\_URA

Primer annealing sequences used for PCR are underlined. mCherry sequence used for C-terminal tagging is in bold font.

ATATCAGATCCACTAGTGGCCTATGCGGCCGCGGATCTGCCGGTCTCCCTATAGTGAGTCGTATTAATTTT  
 GATAAGCCAGGTTAACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGTATTGGGCGCT  
 CTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCTTCGGCTGCGGCGAGCGGTATCAGCTCACTCA  
 AAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCGAGGAAAGAACATGTGAGCAAAAGGCCAGCA  
 AAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATC  
 ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT  
 GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTC  
 GGGAAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTGCTTCGCTCCAAGC  
 TGGGCTGTGTGCACGAACCCCCGTTTACGCCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCC  
 AACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGT  
 AGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCT  
 GCGCTCTGCTGAAGCCAGTTACCTTCGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAACAAACCACCGCT  
 GGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTT  
 GATCTTTTCTACGGGTCTGACGCTCAGTGGAACGAAACTACGTTAAGGGATTTTGGTCATGAGATTAT  
 CAAAAGGATCTTACCTAGATCCTTTTAAATTAATAAAGTAAAGTTTAAATCAATCTAAAGTATATATAG  
 TAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTT  
 ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGCCCCAGTG  
 CTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGG

GCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAG  
AGTAAGTAGTTTCGCCAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACAGCT  
CGTCGTTTGGTATGGCTTCATTTCAGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTG  
TGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACT  
CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCTATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG  
AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGG  
GATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACT  
CTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCAT  
CTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG  
GCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTG  
TCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCC  
GAAAAGTGCCACCTGACGTCTAAGAAACATTATTATCATGACATTAACCTATAAAAAATAGGCGTATCAGC  
AGGCCCTTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGT  
CACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGT  
GTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGGACATATTGTCTG  
TTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACACTATAGAAC  
GCGGCCGCCAGCTGAAGCTTCGTACGCTGCAGGTGACGGATCGGTGACGGTGCTGGTTTAAATTAACATG  
**TCAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTTCATGAGATTCAAGGTGCACATGGAGGGC**  
**TCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCAGACCCTACGAGGGCACCCAGACCGC**  
**CAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTTCATGTACG**  
**GCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTC**  
**AAGTGGGAGAGAGTGATGAACCTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGA**  
**CGGCGAGTTCATCTACAAGGTGAAGTTGAGAGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGA**  
**AGACCATGGGCTGGGAGGCCTCCTCCGAGAGAATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAG**  
**CAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCC**  
**CGTGACGCTGCCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCA**  
**TTGTGGAACAATATGAAAGAGCTGAAGGTAGACATTCTACTGGTGGTATGGATGAATTGTACAAA****TAA****GGC**  
GCGCCACTTCTAAATAAGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAATA  
AGTGATACAAAATTTTAAAGTGACTCTTAGGTTTTTAAACGAAAATTCTTATTCCTTGAGTAACCTTTTCCT  
TAGGTGAGTTGCTTTCTCAGGTATAGTATGAGGTGCTCTTATTGACCACACCTCTACCGGCAGATCCG  
CTAGGGATAACAGGGTAATATAGATCTGTTTAGCTTGCTCGTCCCCGCCGGGTACCCGGCCAGCGACAT  
GGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTGCGCCGT  
ACATTTAGCCCATACATCCCCATGTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGC  
AAAAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAACGCTCCCCTCACAGACGCGTTGAATTGTCC  
CCACGCCGCGCCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTTCATATACTTC  
CTTTTAAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCATGACAGTCAACACTA  
AGACCTATAGTGAGAGAGCAGAACTCATGCCTCACCAGTAGCACAGCGATTATTTGATTAATGGAAGT  
AAGAAAACCAATTTATGTGCATCAATTGACGTTGATACCACTAAGGAATTCCTTGAATTAATTGATAAATT  
AGGTCCTTATGTATGCTTAATCAAGACTCATATTGATATAATCAATGATTTTTTCTATGAATCCACTATTG  
AACCATTATTAGAACTTTACGTAAACATCAATTTATGATTTTTTGAAGATAGAAAATTTGCTGATATTGGT  
AATACCGTAAAGAAACAATATATTGGTGGAGTTTATAAAATTAGTAGTTGGGCAGATATTACCAATGCTCA  
TGGTGTCACTGGGAATGGAGTGGTTGAAGGATTAAACAGGGAGCTAAAGAAACCACCACCAACCAAGAGC  
CAAGAGGGTTATTGATGTTAGCTGAATTATCATCAGTGGGATCATTAGCATATGGAGAATATTCTCAAAAA  
ACTGTTGAAATTGCTAAATCCGATAAGGAATTTGTTATTGGATTTATTGCCAACGTGATATGGGTGGCCA  
AGAAGAAGGATTTGATTGGCTTATTATGACACCTGGAGTTGGATTAGATGATAAAGGTGATGGATTAGGAC  
AACAATATAGAAGTGTGATGAAGTTGTTAGCATTGGAAGTATGATATTATCATTGTTGGTAGAGGATTGTTT  
GGTAAAGGAAGAGATCCAGATATTGAAGGTAAAGGTATAGAAATGCTGGTTGGAATGCTTATTTGAAAAA  
GACTGGCCAATTATAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTT  
TTTTTATATTGTAGTTGTTCTATTTTAAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCA  
TCTGCCCAGATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTGCCT  
ATACTGCTGTGATTTCGATACTAACGCCGCCATCCAGTGTGCGAAAACGAGCTCGAATTCATCGATG

## **Chapter 4**

Unpublished Data

## Introduction to unpublished data

Subsequent to publication of my initial findings on the function of Orm proteins, I have continued to investigate their role in sphingolipid homeostasis. Specifically, I have focused on elucidating how disruption of sphingolipid synthesis leads to Orm protein phosphorylation. Three principal questions are raised by the findings described in Chapter 3: what sphingolipid species is sensed? what is the sensor for this lipid? and how is this signal transduced to control Orm1/2 phosphorylation? In collaboration with Prof. Jeremy Thorner and Françoise Roelants, a post-doctoral fellow in the Thorner laboratory, we have worked towards identifying the kinase that phosphorylates Orm proteins and the mechanism by which this kinase is regulated by sphingolipid levels.

As described below, several lines of evidence indicate that Ypk1 is the kinase responsible for Orm protein phosphorylation. Ypk1 is a member of the AGC kinase family and is homologous to the mammalian kinase SGK. It has an N-terminal domain of unknown function and a C-terminal kinase domain that phosphorylates substrates on an Arg-X-Arg-X-X-Ser/Thr motif<sup>88</sup>. Loss of *YPK1* causes a significant reduction in yeast growth rate and is lethal in combination with deletion of its paralog *YPK2*<sup>88</sup>. A role for Ypk1 in sphingolipid metabolism is suggested by the finding that over-expression of *YPK1* confers resistance to the serine palmitoyltransferase inhibitor myriocin, whereas deletion of *YPK1* results in hypersensitivity to this drug<sup>91,92</sup>. A specific link between *YPK1* and *ORM* genes is suggested by the isolation of a transposon insertion mutant in *ORM2* in a screen for genes whose disruption suppresses the temperature sensitivity of a *ypk1-1<sup>ts</sup> ypk2Δ* strain<sup>90</sup>.

Several studies have investigated mechanisms by which Ypk1 activity is regulated. First, the N-terminus of Ypk1 may itself have an auto-inhibitory role, as deletion or mutation of the N-terminus leads to Ypk1 hyper-activity<sup>89</sup>. Second, there is preliminary evidence that LCBs can directly activate Ypk1<sup>93</sup>. Lastly, several kinases are thought to phosphorylate and regulate Ypk1, including Pkh1/2, TORC2, and Fpk1/2 (Fig. 4.1a).

Pkh1/2 phosphorylate Ypk1 on its activation loop (also known as the PDK1 site), and this modification is required for Ypk1 activity<sup>88,90,91</sup>. Interestingly, Pkh1/2 activity may also be controlled by sphingolipid levels, either via direct activation by LCBs or via Nce102, a recently identified sensor of plasma membrane sphingolipids<sup>94,95</sup>. TORC2 has also been proposed to phosphorylate Ypk1 on its ‘turn’ and ‘hydrophobic’ motifs (also known as the PDK2 site)<sup>89</sup>. Interestingly, the PDK2 site in Ypk1 has been previously linked to sphingolipid metabolism, as its mutation causes hypersensitivity to myriocin without affecting other known Ypk1-associated phenotypes<sup>91,150</sup>. Ypk1 may be a major TORC2 effector in yeast, as lethality associated with loss of TORC2 activity can be suppressed by *YPK1* hyper-activity<sup>89</sup>. Lastly, Fpk1/2 were recently shown to phosphorylate Ypk1 at Ser51 and Ser71<sup>96</sup>. Ypk1 was also found to phosphorylate Fpk1/2, although this bi-directional regulation makes it difficult to determine the functional consequences of these phosphorylations<sup>96</sup>. It is interesting to note that sphingolipids appear to regulate phosphorylation of Ypk1 at the Fpk1/2 sites. Specifically, myriocin and several other disruptions to the sphingolipid pathway lead to loss of basal phosphorylation at Ser51/71<sup>96</sup>. Because *CSG2* deletion altered Ypk1 phosphorylation at these residues but *IPT1* deletion did not, Roelants *et al.* proposed that

MIPC (the product of Csg2 and substrate for Ipt1) is the lipid that controls S51/71 phosphorylation<sup>96</sup>. Thus, several pathways coordinately regulate Ypk1 activity, and a number of kinases that act on Ypk1 have been linked to sphingolipid metabolism. Thus, a complete understanding of how Orm proteins mediate sphingolipid homeostasis will require a thorough analysis of how Ypk1 is regulated in response to perturbations to sphingolipid synthesis.

## Unpublished results

Prompted by the finding that a transposon insertion in the *ORM2* gene suppresses temperature sensitivity in the *ypk1-I<sup>ts</sup>/ypk2Δ* strain<sup>90</sup>, we have since found that deletion of *ORM1* and *ORM2* together results in even greater suppression of temperature sensitivity (Fig. 4.1b). This observation, in addition to the known effects of *YPK1* mutation on sensitivity to myriocin<sup>91,92</sup>, led us to test whether Ypk1 is the kinase that phosphorylates Orm1/2. We expressed and purified GST fusions to the N-terminus of Orm1 and Orm2 and used these proteins as substrates in an *in vitro* kinase assay. When incubated with immunoprecipitated Ypk1 and radioactive ATP, GST fusions to the N-terminus of Orm1 or Orm2 (Fig. 4.2a,b) are robustly phosphorylated. This phosphorylation is dependent on catalytic activity of Ypk1, as an inhibitor-sensitized Ypk1 variant (Ypk1-L424A) does not phosphorylate Orm1 in the presence of inhibitor (Fig. 4.2a). Mutation of serines within the GST-Orm1 substrate confirms that *in vitro* phosphorylation occurs on the same N-terminal residues that were identified as *in vivo* phosphorylation sites in Chapter 3 (Fig 4.2b). Notably, these sites also match the previously identified Ypk1 consensus motif (Arg-X-Arg-X-X-Ser/Thr)<sup>88</sup>. Thus Ypk1 phosphorylates Orm proteins *in vitro*. We next determined whether Ypk1 is needed for myriocin-induced phosphorylation of Orm1 *in vivo*. In agreement with our *in vitro* findings, deletion of *YPK1* (but not deletion of *YPK2*) prevents the increase in Orm1 phosphorylation seen in response to myriocin treatment, as visualized on phosphate-affinity gels<sup>133</sup> (Fig. 4.2c). Collectively, these data indicate that Ypk1 is the kinase for Orm proteins.

Although Ypk1 mediates Orm protein phosphorylation *in vitro* and *in vivo*, these observations do not indicate how and why Orm phosphorylation is elevated in response

to myriocin treatment. To address this question, we compared *in vitro* activity of Ypk1 in myriocin-treated and untreated cells. Notably, Ypk1 immunoprecipitated from myriocin-treated cells showed a clear increase in activity relative to Ypk1 from untreated cells, thus indicating that increased Orm phosphorylation in myriocin treated cells is likely due to activation of the Ypk1 kinase (Fig. 4.3a; compare WT lanes).

How then is Ypk1 activity induced in myriocin-treated cells? As mentioned above, the Pkh1/2, TORC2 and Fpk1/2 kinases all appear to regulate Ypk1, and it is also possible that a lipid metabolite is directly bound by Ypk1 and modulates its activity. Fortunately, the ability to isolate moderate ( $\mu$ g) quantities of Ypk1 from yeast and preserve its activity state (data not shown) suggests that it should be possible to determine what is altered about Ypk1 when it becomes activated. We have now initiated a variety of mass spectrometry-based approaches to compare Ypk1 from myriocin-treated and untreated cells.

A complementary approach is to test each of the known candidate kinases that act on Ypk1 for a potential role in myriocin-induced regulation. Due to technical challenges, convincing results have only been obtained thus far for Fpk1/2, and these data indicate that Fpk1/2 are dispensable for activation of Ypk1 in response to myriocin. *In vitro*, mutation of the Fpk1/2 sites identified in Ypk1 to alanine (Ypk1-S51/71A) does not alter the effect of myriocin on Ypk1 activity toward GST-Orm substrates (Fig. 4.3a). *In vivo*, the same *ypk1-S51/71A* mutation does not affect Orm1 phosphorylation in the presence or absence of myriocin (Fig. 4.3b). Similar results were also seen in the *fpk1 $\Delta$ /fpk2 $\Delta$*  strain, although higher concentrations of myriocin were used because roughly four-fold more myriocin is needed to slow growth in this strain than in wild-type yeast (Fig. 4.3c and



data not shown; the myriocin resistance upon deletion of *FPK1/2* is likely attributable to Fpk1/2's regulation of the lipid flippases Dnf1/2/3, which may control myriocin entry into cells<sup>96</sup>). Lastly, the lipid-dependence of Fpk1/2-mediated phosphorylation of Ypk1 also suggests that Fpk1/2 are not relevant to myriocin-induced activation of Ypk1. Specifically, MIPC is reported to regulate Fpk1/2-mediated phosphorylation of Ypk1<sup>96</sup>, but Orm1/2 phosphorylation appears to be sensitive to a lipid produced upstream of MIPC (see Supplementary Figure 3.5 and below). This discrepancy is illustrated when MIPC production is reduced by deletion of *CSG2*: this mutation leads to reduced phosphorylation of Ypk1 at S51 and S71, but has a negligible effect on Orm1 phosphorylation (Fig. 4.3d). Thus, Pkh1/2 and TORC2 may be better candidates than Fpk1/2 for kinases that are responsible for myriocin-induced activation of Ypk1. An in depth analysis of the role of these kinases however awaits improved tools to monitor and disrupt phosphorylation of Ypk1 by these kinases.

## Preliminary Conclusions

Here we find that Ypk1 is the kinase that phosphorylates Orm proteins. This conclusion is supported by *in vitro*, *in vivo*, and genetic evidence that Ypk1 phosphorylates Orm proteins on the same residues previously identified as being important for sphingolipid homeostasis. Notably, we have also found that inhibition of sphingolipid synthesis by myriocin treatment, which leads to increased Orm1/2 phosphorylation, appears to do so by causing an increase in Ypk1 activity. Current efforts are now aimed at investigating how Ypk1 is activated in myriocin-treated cells, as addressing this question is likely to clarify our understanding of the feedback pathway in which Orm proteins participate. In particular, it is hoped that characterization of the factors responsible for Ypk1 activation will ultimately reveal a sensor of sphingolipid levels, the nature of the sphingolipid metabolites that are sensed, and the mechanism by which this information is transduced to modulate Orm protein phosphorylation.

## **Methods**

### **Strain construction and yeast growth**

Strain construction, site-directed mutagenesis, and yeast growth were done using standard methods and media, as described in Chapter 3. For *in vivo* analysis of Ypk1 and Orm1 phosphorylation, strains expressing Flag-tagged Orm proteins (see Chapter 3) were modified such that the genomic copy of *YPK1* was deleted by PCR-based gene replacement and wild-type *YPK1* was expressed from a *URA3*-marked plasmid. These strains were then transformed with *G418*-resistance-marked plasmids from which myc-tagged wild-type or *S51/71A YPK1* was expressed using the endogenous *YPK1* promoter. The *URA3*-marked plasmid was then removed by counter-selection on 5-FOA plates, leaving the *G418*-marked plasmid as the sole source of Ypk1. Strains for cell-spotting assays were constructed by deleting *ORM1* in the YPH499 *ypk1-1<sup>ts</sup> ypk2Δ* strain<sup>88</sup> and crossing the resulting strain to an isogenic *orm2Δ* strain.

### **Serial-dilution cell-spotting assays**

Serial dilution and cell spotting were done as described in Chapter 3.

### **Phosphate-affinity SDS-PAGE analysis and Western blotting**

Strains expressing Flag-tagged Orm1 and/or Ypk1-myc were grown in 150 ng ml<sup>-1</sup> myriocin for the indicated times and harvested as described in Chapter 3. Lysates were separated on phosphate-affinity gels<sup>133</sup> and analyzed by Western blotting as in Chapter 3, using anti-Flag (Sigma) and anti-myc monoclonal 9E10 (Roche) antibodies where needed.

### **Purification of GST-Orm1 and GST-Orm2 *in vitro* kinase assay substrates**

GST-Orm1(1-85) and GST-Orm2(1-80) bacterial expression vectors were created by amplifying *ORM* genes from yeast genomic DNA; *ORM* PCR products were cloned into pGEX4T-1 (Amersham) between SalI and NotI for Orm1 and between EcoRI and NotI for Orm2. Expression and purification of the GST fusions was carried out as described previously<sup>96</sup>. Where indicated, immunoblotting against the RxRxxS-P motif was done using an antibody specific for this phosphopeptide sequence (Cell Signaling Technology #9614).

### ***In vitro* Ypk1 kinase assay**

For *in vitro* kinase assays, cultures (40 ml) of BY4741<sup>119</sup> cells expressing from the *GALI* promoter either Ypk1-myc (pAM54), catalytically-inactive Ypk1-K376A-myc (pAM49), or Ypk1-S51A/S71A-myc were grown in SC +Raf-Suc to an OD<sub>600</sub> = 0.6, induced by addition of galactose (2% final concentration), and incubated with shaking at 30°C for 1 h. Where indicated, myriocin (1.25 µM) was then added and incubation continued for an additional 2 h. Immunoprecipitation of Ypk1 and kinase assays were performed as described previously<sup>96</sup>. For Fig 4.2a, wild-type and analog-sensitive (AS) L424A-Ypk1 were generously provided by Yidi Sun and David Drubin. Briefly, wild-type and AS Ypk1-TEV-myc were expressed from the *GALI* promoter in strain BJ2168 by growth in 2% galactose for 12 h. Ypk1-TEV-myc was then immunoprecipitated with anti-myc resin and released by TEV cleavage for analysis of kinase activity. Where Ypk1-AS activity was analyzed, approximately three times more kinase was used than for Ypk1 wild-type. The AS-specific inhibitor 3MOB-PP1 was added at a concentration of 10 µM during the kinase assay as indicated.

## Figure Legends

### Figure 4.1

**Deletion of *ORM1/2* suppresses *ypk1/2* mutants.** (a) Schematic of Ypk1 and known sites of phosphorylation by upstream kinases Fpk1/2, Pkh1/2, and TORC2. (b) Serial dilutions of the indicated *ypk1-1<sup>ts</sup>* *ypk2Δ* strains were spotted on plates and grown at the indicated temperatures.

### Figure 4.2

**Ypk1 phosphorylates Orm proteins.** (a) Analog-sensitive (AS) or wild-type (WT) Ypk1-myc was immunoprecipitated from strains expressing these proteins from a *GALI*-driven plasmid after growth in SC-Gal media. Ypk1-myc captured on anti-myc resin was incubated with a GST fusion to the N-terminus of Orm1 (GST-Orm1(1-85)) and with  $^{32}\text{P}$ -ATP in the presence or absence of the AS-specific inhibitor 3MOB-PP1 (10  $\mu\text{M}$ ). Reactions were analyzed by autoradiography (top left), Coomassie blue staining (top right), and immunoblotting with antibodies against the RXXRX-S(P) phospho-motif (bottom left) and against the GST protein (bottom right). (b) Ypk1 kinase activity was analyzed as in (a), using wild-type (WT) GST-Orm1(1-85), *S51/52/53A* mutant GST-Orm1(1-85), and wild-type GST-Orm2(1-80) as substrates. Below, the sequence surrounding the Orm1 phosphorylation sites at S51-S53 is indicated, with the mutated residues highlighted in red. (c) Lysates were prepared from wild-type (WT), *ypk1Δ*, and *ypk2Δ* yeast expressing 3xFlag-Orm1 after growth in rich media containing 150 ng ml<sup>-1</sup>

myriocin for the indicated times. Western blots show phosphorylated forms of 3xFlag-Orm1 (P-Orm1) after separation on phosphate-affinity gels<sup>133</sup>.

### Figure 4.3

#### **Ypk1 is activated upon myriocin treatment in an Fpk1/2-independent manner.**

(a) Strains expressing wild-type (WT) or *S51A/S71A* mutant forms of Ypk1-myc from a *GALI*-driven plasmid were grown in SC-Gal media in the presence or absence of 1.25  $\mu$ M myriocin for 2h and harvested for analysis of Ypk1 activity. *In vitro* kinase assays were carried out with GST-Orm1(1-85) and GST-Orm2(1-80) substrates as in **Fig. 4.2a**.

(b) Lysates were prepared from strains expressing 3xFlag-Orm1 and wild-type (WT) or *S51A/S71A* mutant Ypk1-myc as the sole source of Ypk1. Yeast were grown in rich media containing 150 ng ml<sup>-1</sup> myriocin for the indicated times and 3xFlag-Orm1 phosphorylation state was analyzed on phosphate-affinity gels as in **Fig. 4.2c**. (c) Lysates were prepared from wild-type (WT) and *fpk1Δ/fpk2Δ* strains after growth in rich media containing the indicated concentrations of myriocin. Wild-type strains were grown in the presence of myriocin for 6h; *fpk1Δ/fpk2Δ* strains were grown in the presence of myriocin for 8h. Orm1 phosphorylation was analyzed as in **Fig. 4.2c**. (d) Lysates were prepared from wild-type (WT) and *csg2Δ* strains expressing Flag-Orm1 and Ypk1-myc as the sole copies of these proteins. Prior to harvesting, strains were grown in rich media containing 150 ng ml<sup>-1</sup> myriocin for the indicated times. Ypk1-myc and Flag-Orm1 phosphorylation were analyzed on phosphate-affinity gels as in **Fig. 4.2c**. P-S51/71-Ypk1 indicates the position of Ypk1 phosphorylated at Ser51 and Ser71.

Figure 4.1

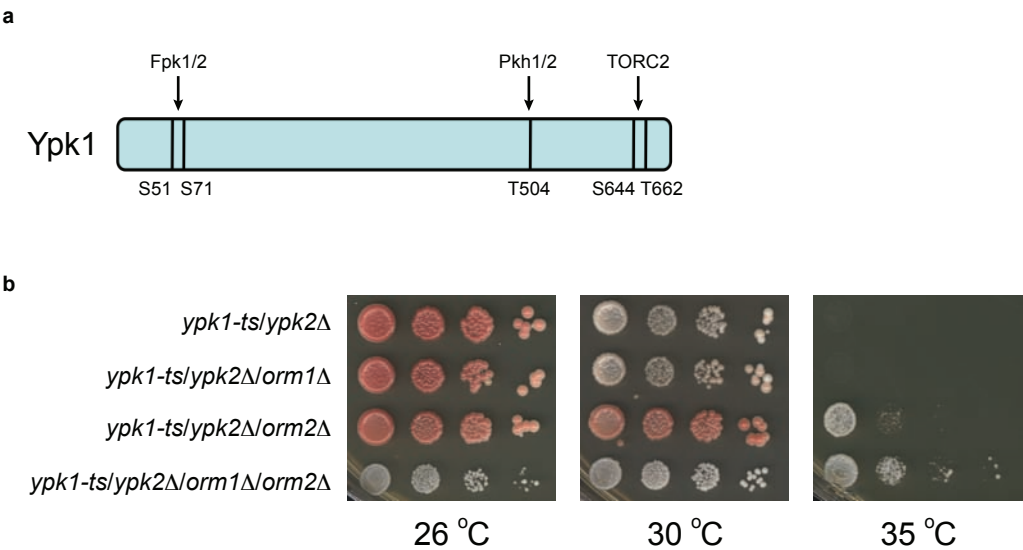


Figure 4.2

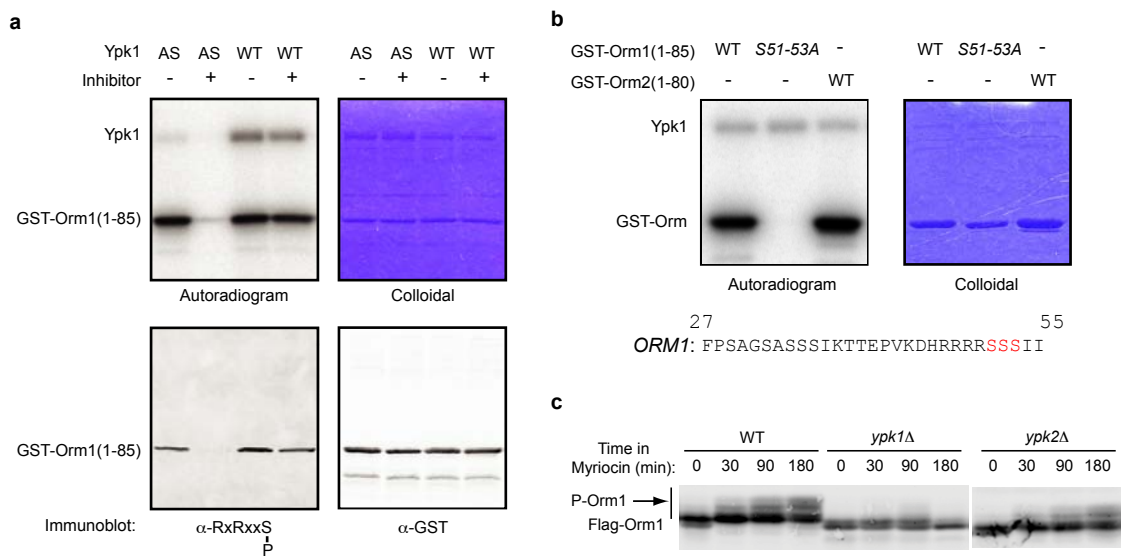
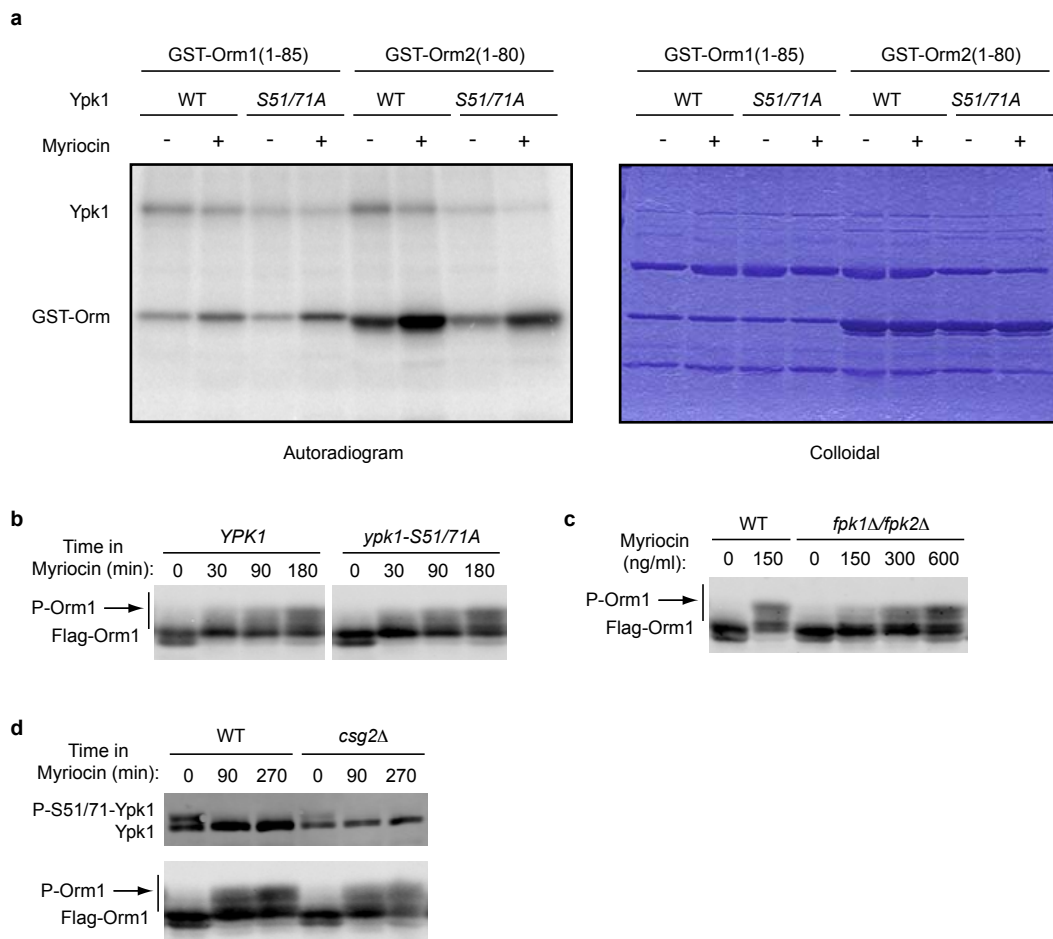




Figure 4.3



## **Chapter 5**

### Summary

## Summary

Large-scale measurement of genetic interactions is a powerful strategy for functional characterization of genes whose cellular roles are not understood. However, current approaches rely on methods for high-throughput growth rate measurement that lack the precision needed to identify small growth phenotypes. Consequently, many genetic interactions have been difficult to identify, classify, and quantify. For the first part of my dissertation, I developed a high-precision method for growth rate measurement based on co-culture competitions between fluorescently marked wild-type and mutant strains. This assay provides substantial improvements in precision and made it possible to find for the first time that most yeast genes do contribute to optimal growth, even under a single experimental condition. From a theoretical perspective, this observation resolves a long-standing puzzle concerning the apparently high level of gene dispensability seen in yeast. From a practical standpoint, having detectable growth phenotypes for a larger fraction of yeast genes expands the number of yeast genes accessible to quantitative genetic analysis. Indeed, with a more accurate growth assay, we were able to detect genetic interactions missed by previous approaches, to distinguish different types of positive genetic interactions, and to use this information to gain new insights even for well-studied genes involved in chromatin modification and in the 26S proteasome.

In principle, this assay should be readily adaptable to a variety of organisms. For other related yeast strains, improved growth measurements should enable evolutionary comparisons of the contributions of homologous genes to growth and of genetic interactions between homologous gene pairs. In metazoan organisms, similar growth

competitions may be used to functionally explore their genomes (for example, in conjunction with RNAi), although it remains to be seen how well suited double RNAi is for genetic interaction analysis. Encouragingly, efforts to utilize RNAi and growth competitions in mammalian cells are already underway<sup>151</sup>.

In the second part of my thesis, I investigated the function of the *ORM* gene family. Beginning from large-scale genetic interaction data, I found that Orm proteins are negative regulators of sphingolipid synthesis that form a conserved complex with serine palmitoyltransferase, the first and rate-limiting enzyme in the pathway. Loss of Orm proteins causes toxic over-production of sphingolipids, whereas over-expression of Orm proteins results in decreased sphingolipid levels. The activity of Orm proteins is regulated in turn by a phosphorylation-based feedback mechanism. When sphingolipid synthesis is disrupted, Orm proteins are inactivated by phosphorylation by the Ypk1 kinase. Phosphorylation relieves their inhibition of serine palmitoyltransferase and thus serves to restore sphingolipid output and ensure sphingolipid homeostasis.

Orm proteins represent, to our knowledge, the first identified negative regulators of a sphingolipid synthetic enzyme and the first molecularly defined mechanism that enables cells to adjust *de novo* sphingolipid synthesis according to metabolic need. A number of features of Orm-mediated feedback regulation may make this pathway well suited to control sphingolipid synthesis. For example, phosphorylation offers a rapid mechanism to respond to changing demand for sphingolipids, and multiple sites of Orm phosphorylation may provide a graded mechanism to tune sphingolipid synthesis.

A number of exciting directions for future investigation are suggested by our findings. The question of how cells are able to sense sphingolipid levels is of primary interest to us currently. This is a longstanding question in the field of sphingolipid research, and we expect that our identification of Orm proteins and their regulation by Ypk1 should be a useful foothold in elucidating the identity of a cellular sphingolipid sensor. A second interesting direction to explore is the possibility that Orm proteins are regulated in response to a wide range of conditions beyond perturbations to sphingolipid synthesis. For example, it will be interesting to see if Orm proteins are regulated during the cell cycle, in response to cellular stress, or in conjunction with other types of lipids. Similarly, it is possible that Orm1 and Orm2 are differentially regulated under certain conditions.

A third set of questions for future investigation are suggested by the finding of serine palmitoyltransferase, Orm proteins, and Sac1 in a coherent, roughly stoichiometric complex. What is the purpose of placing these proteins in a single complex? Sac1 and its substrate (PI4P) are known to control protein and sphingolipid transport through the secretory pathway and are thought to adjust protein secretion in response to nutrient supply<sup>45,46,135</sup>. Our data indicate that Sac1 directly modulates sphingolipid synthesis, but by what mechanism? Additionally, the SPOTS complex reveals a new connection between sphingolipids and phosphoinositides whose physiological significance requires further investigation. One appealing model is that the SPOTS acts as a dynamic coordinating center that couples changes in sphingolipid and nutrient levels to the activity and localization of key enzymes of lipid metabolism and trafficking.

More information is also needed regarding the structural organization of the SPOTS complex and the mechanism of Orm-mediated inhibition of serine palmitoyltransferase. For example, do Orm proteins regulate Lcb1/2 by modulating catalytic activity of the enzyme, by controlling substrate accessibility to the active site, or by an alternate mechanism? How does this regulation occur at a structural level and how is it related to phosphorylation-induced changes in SPOTS complex stoichiometry? Such questions will likely be difficult to address given the membrane-embedded nature of the SPOTS complex, but may be amenable to techniques such as *in vitro* enzymology, X-ray crystallography, and electron microscopy.

Lastly, our preliminary finding that *ORM* gene function is conserved in mammalian cells suggests several directions for future investigation. Most immediately, it will be important to explore how *ORMDL* proteins are regulated in mammalian cells and if they also act to ensure sphingolipid homeostasis. Additionally, the recent identification of a genetic risk factor for childhood asthma linked to the *ORMDL3* locus now raises the testable hypothesis that misregulation of sphingolipid metabolism contributes to asthma development. For example, one could ask whether altering *ORMDL* gene activity has any effect in established mouse models of asthma, whether sphingolipid levels are altered in patients with asthma (some evidence already indicates that this is the case<sup>138,139,152</sup>), or whether other sphingolipid-related genes exhibit a genetic association to asthma (as has recently been reported for *SIPRI*, which encodes the S1P receptor<sup>153</sup>). Lastly, the *ORMDL3* locus has also been linked to a number of other inflammatory diseases including type I diabetes<sup>143</sup>, primary biliary cirrhosis<sup>144,154</sup>, ulcerative colitis<sup>155</sup>, and Crohn's disease<sup>142</sup>. Therefore, it will be important to investigate

whether *ORMDL* genes may have more general roles in inflammation and auto-immunity.

In summary, genetic interactions represent a powerful tool for functional genomics in yeast and for investigating novel gene families of interest. The utility of such approaches is exemplified by their role in our identification of *ORM* genes as mediators of sphingolipid homeostasis. Ongoing technological progress is likely to further increase the value of these approaches and to bring the power of genetic interaction data to higher organisms.

## **Appendix A**

### References



## References

- 1 Mewes, H. W. *et al.* Overview of the yeast genome. *Nature* **387**, 7-65, doi:10.1038/42755 (1997).
- 2 Clayton, R. A., White, O., Ketchum, K. A. & Venter, J. C. The first genome from the third domain of life. *Nature* **387**, 459-462, doi:10.1038/387459a0 (1997).
- 3 Boone, C., Bussey, H. & Andrews, B. J. Exploring genetic interactions and networks with yeast. *Nat Rev Genet* **8**, 437-449 (2007).
- 4 Mani, R., St Onge, R. P., Hartman, J. L. t., Giaever, G. & Roth, F. P. Defining genetic interaction. *Proc Natl Acad Sci U S A* **105**, 3461-3466 (2008).
- 5 Segre, D., Deluna, A., Church, G. M. & Kishony, R. Modular epistasis in yeast metabolism. *Nat Genet* **37**, 77-83 (2005).
- 6 Kelley, R. & Ideker, T. Systematic interpretation of genetic interactions using protein networks. *Nat Biotechnol* **23**, 561-566 (2005).
- 7 Schuldiner, M. *et al.* Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. *Cell* **123**, 507-519 (2005).
- 8 Tong, A. *et al.* Global mapping of the yeast genetic interaction network. *Science* **303**, 808-813 (2004).
- 9 Tong, A. H. *et al.* Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* **294**, 2364-2368 (2001).
- 10 Costanzo, M. *et al.* The genetic landscape of a cell. *Science* **327**, 425-431, doi:10.1126/science.1180823 (2010).

- 11 Collins, S. *et al.* Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map. *Nature* **446**, 806-810, doi:10.1038/nature05649 (2007).
- 12 Pan, X. *et al.* A DNA integrity network in the yeast *Saccharomyces cerevisiae*. *Cell* **124**, 1069-1081 (2006).
- 13 Ye, P. *et al.* Gene function prediction from congruent synthetic lethal interactions in yeast. *Mol Syst Biol* **1**, 2005.0026, doi:10.1038/msb4100034 (2005).
- 14 Denic, V. & Weissman, J. S. A molecular caliper mechanism for determining very long-chain fatty acid length. *Cell* **130**, 663-677, doi:S0092-8674(07)00829-X [pii] 10.1016/j.cell.2007.06.031 (2007).
- 15 Schuldiner, M. *et al.* The GET complex mediates insertion of tail-anchored proteins into the ER membrane. *Cell* **134**, 634-645, doi:S0092-8674(08)00777-0 [pii] 10.1016/j.cell.2008.06.025 (2008).
- 16 Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387-391 (2002).
- 17 Winzeler, E. *et al.* Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**, 901-906 (1999).
- 18 Breslow, D. *et al.* A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nat Methods* **5**, 711-718 (2008).
- 19 Mnaimneh, S. *et al.* Exploration of essential gene functions via titratable promoter alleles. *Cell* **118**, 31-44 (2004).

- 20 Ben-Aroya, S. *et al.* Toward a comprehensive temperature-sensitive mutant repository of the essential genes of *Saccharomyces cerevisiae*. *Mol Cell* **30**, 248-258, doi:S1097-2765(08)00167-6 [pii] 10.1016/j.molcel.2008.02.021 (2008).
- 21 Kanemaki, M., Sanchez-Diaz, A., Gambus, A. & Labib, K. Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo. *Nature* **423**, 720-724 (2003).
- 22 Pan, X. *et al.* A robust toolkit for functional profiling of the yeast genome. *Mol Cell* **16**, 487-496 (2004).
- 23 Decourty, L. *et al.* Linking functionally related genes by sensitive and quantitative characterization of genetic interaction profiles. *Proc Natl Acad Sci U S A* **105**, 5821-5826 (2008).
- 24 Deutschbauer, A. M. *et al.* Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. *Genetics* **169**, 1915-1925 (2005).
- 25 Warringer, J., Ericson, E., Fernandez, L., Nerman, O. & Blomberg, A. High-resolution yeast phenomics resolves different physiological features in the saline response. *Proc Natl Acad Sci U S A* **100**, 15724-15729 (2003).
- 26 Gu, Z. *et al.* Role of duplicate genes in genetic robustness against null mutations. *Nature* **421**, 63-66 (2003).
- 27 Hillenmeyer, M. E. *et al.* The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* **320**, 362-365 (2008).
- 28 Papp, B., Pal, C. & Hurst, L. D. Metabolic network analysis of the causes and evolution of enzyme dispensability in yeast. *Nature* **429**, 661-664 (2004).

- 29 Ghaemmaghami, S. *et al.* Global analysis of protein expression in yeast. *Nature* **425**, 737-741, doi:10.1038/nature02046 (2003).
- 30 St Onge, R. *et al.* Systematic pathway analysis using high-resolution fitness profiling of combinatorial gene deletions. *Nat Genet* **39**, 199-206, doi:10.1038/ng1948 (2007).
- 31 Hjelmqvist, L. *et al.* ORMDL proteins are a conserved new family of endoplasmic reticulum membrane proteins. *Genome Biol* **3**, RESEARCH0027 (2002).
- 32 Moffatt, M. F. *et al.* Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* **448**, 470-473 (2007).
- 33 Bouzigon, E. *et al.* Effect of 17q21 Variants and Smoking Exposure in Early-Onset Asthma. *N Engl J Med* (2008).
- 34 Galanter, J. *et al.* ORMDL3 gene is associated with asthma in three ethnically diverse populations. *Am J Respir Crit Care Med* **177**, 1194-1200 (2008).
- 35 Tavendale, R., Macgregor, D. F., Mukhopadhyay, S. & Palmer, C. N. A polymorphism controlling ORMDL3 expression is associated with asthma that is poorly controlled by current medications. *J Allergy Clin Immunol* **121**, 860-863 (2008).
- 36 Wu, H. *et al.* Genetic variation in ORM1-like 3 (ORMDL3) and gasdermin-like (GSDML) and childhood asthma. *Allergy* **64**, 629-635 (2009).
- 37 Sleiman, P. M. *et al.* Variants of DENND1B associated with asthma in children. *N Engl J Med* **362**, 36-44, doi:NEJMoa0901867 [pii] 10.1056/NEJMoa0901867 (2010).

- 38 Altshuler, D., Daly, M. J. & Lander, E. S. Genetic mapping in human disease. *Science* **322**, 881-888, doi:322/5903/881 [pii] 10.1126/science.1156409 (2008).
- 39 Schadt, E. E. Molecular networks as sensors and drivers of common human diseases. *Nature* **461**, 218-223, doi:nature08454 [pii] 10.1038/nature08454 (2009).
- 40 Beeler, T. *et al.* The *Saccharomyces cerevisiae* TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca<sup>2+</sup>-sensitive *csg2Delta* mutant. *J Biol Chem* **273**, 30688-30694 (1998).
- 41 Desfarges, L. *et al.* Yeast mutants affected in viability upon starvation have a modified phospholipid composition. *Yeast* **9**, 267-277, doi:10.1002/yea.320090306 (1993).
- 42 Cowart, L. A. & Obeid, L. M. Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. *Biochim Biophys Acta* **1771**, 421-431 (2007).
- 43 Dickson, R. C., Sumanasekera, C. & Lester, R. L. Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. *Prog Lipid Res* **45**, 447-465 (2006).
- 44 Funato, K. & Riezman, H. Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast. *J Cell Biol* **155**, 949-959 (2001).
- 45 Hanada, K. *et al.* Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426**, 803-809 (2003).

- 46 D'Angelo, G. *et al.* Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature* **449**, 62-67 (2007).
- 47 Lingwood, D. & Simons, K. Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50, doi:10.1126/science.1174621 (2010).
- 48 Lippincott-Schwartz, J. & Phair, R. D. Lipids and cholesterol as regulators of traffic in the endomembrane system. *Annu Rev Biophys* **39**, 559-578, doi:10.1146/annurev.biophys.093008.131357 (2010).
- 49 Eggeling, C. *et al.* Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* **457**, 1159-1162, doi:10.1038/nature07596 (2009).
- 50 Sharpe, H. J., Stevens, T. J. & Munro, S. A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell* **142**, 158-169, doi:10.1016/j.cell.2010.05.037 (2010).
- 51 Tam, C. *et al.* Exocytosis of acid sphingomyelinase by wounded cells promotes endocytosis and plasma membrane repair. *The Journal of cell biology*, doi:10.1083/jcb.201003053 (2010).
- 52 Trajkovic, K. *et al.* Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* **319**, 1244-1247 (2008).
- 53 Hannun, Y. A. & Obeid, L. M. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* **9**, 139-150 (2008).
- 54 Fyrst, H. & Saba, J. D. An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nat Chem Biol* **6**, 489-497, doi:10.1038/nchembio.392 (2010).

- 55 Kawahara, A. *et al.* The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. *Science* **323**, 524-527, doi:1167449 [pii] 10.1126/science.1167449 (2009).
- 56 Osborne, N. *et al.* The spinster homolog, two of hearts, is required for sphingosine 1-phosphate signaling in zebrafish. *Curr Biol* **18**, 1882-1888, doi:S0960-9822(08)01431-0 [pii] 10.1016/j.cub.2008.10.061 (2008).
- 57 Hait, N. C. *et al.* Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* **325**, 1254-1257, doi:10.1126/science.1176709 (2009).
- 58 Alvarez, S. E. *et al.* Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* **465**, 1084-1088, doi:10.1038/nature09128 (2010).
- 59 Kolesnick, R. & Hannun, Y. Ceramide and apoptosis. *Trends Biochem Sci* **24**, 224-225; author reply 227 (1999).
- 60 Nickels, J. T. & Broach, J. R. A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of *Saccharomyces cerevisiae*. *Genes Dev* **10**, 382-394 (1996).
- 61 Hannun, Y. A. & Obeid, L. M. The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem* **277**, 25847-25850, doi:10.1074/jbc.R200008200 (2002).
- 62 Dickson, R. *et al.* Sphingolipids are potential heat stress signals in *Saccharomyces*. *J Biol Chem* **272**, 30196-30200 (1997).

- 63 Jenkins, G. *et al.* Involvement of yeast sphingolipids in the heat stress response of *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 32566-32572 (1997).
- 64 Wells, G., Dickson, R. & Lester, R. Heat-induced elevation of ceramide in *Saccharomyces cerevisiae* via de novo synthesis. *J Biol Chem* **273**, 7235-7243 (1998).
- 65 Deng, X. *et al.* Ceramide biogenesis is required for radiation-induced apoptosis in the germ line of *C. elegans*. *Science* **322**, 110-115 (2008).
- 66 Cuvillier, O. *et al.* Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800-803, doi:10.1038/381800a0 (1996).
- 67 Spiegel, S. & Milstien, S. Sphingosine 1-phosphate, a key cell signaling molecule. *J Biol Chem* **277**, 25851-25854, doi:10.1074/jbc.R200007200 (2002).
- 68 Menuz, V. *et al.* Protection of *C. elegans* from anoxia by HYL-2 ceramide synthase. *Science* **324**, 381-384 (2009).
- 69 Brown, M. S. & Goldstein, J. L. Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. *J Lipid Res* **50 Suppl**, S15-27 (2009).
- 70 Hampton, R. Y. & Garza, R. M. Protein quality control as a strategy for cellular regulation: lessons from ubiquitin-mediated regulation of the sterol pathway. *Chem Rev* **109**, 1561-1574 (2009).
- 71 Brown, M. & Goldstein, J. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331-340 (1997).
- 72 Burg, J. S. *et al.* Insig regulates HMG-CoA reductase by controlling enzyme phosphorylation in fission yeast. *Cell Metab* **8**, 522-531 (2008).



- 73 Radhakrishnan, A., Sun, L. P., Kwon, H. J., Brown, M. S. & Goldstein, J. L.  
Direct binding of cholesterol to the purified membrane region of SCAP:  
mechanism for a sterol-sensing domain. *Mol Cell* **15**, 259-268,  
doi:10.1016/j.molcel.2004.06.019  
S1097276504003521 [pii] (2004).
- 74 Loewen, C. J. *et al.* Phospholipid metabolism regulated by a transcription factor  
sensing phosphatidic acid. *Science* **304**, 1644-1647 (2004).
- 75 Zhang, Y. M. & Rock, C. O. Membrane lipid homeostasis in bacteria. *Nat Rev  
Microbiol* **6**, 222-233, doi:nrmicro1839 [pii]  
10.1038/nrmicro1839 (2008).
- 76 Miller, D. J., Zhang, Y. M., Subramanian, C., Rock, C. O. & White, S. W.  
Structural basis for the transcriptional regulation of membrane lipid homeostasis.  
*Nat Struct Mol Biol*, doi:nsmb.1847 [pii]  
10.1038/nsmb.1847 (2010).
- 77 Kurat, C. F. *et al.* Cdk1/Cdc28-dependent activation of the major triacylglycerol  
lipase Tgl4 in yeast links lipolysis to cell-cycle progression. *Mol Cell* **33**, 53-63  
(2009).
- 78 Dobrosotskaya, I. Y., Seegmiller, A. C., Brown, M. S., Goldstein, J. L. &  
Rawson, R. B. Regulation of SREBP processing and membrane lipid production  
by phospholipids in *Drosophila*. *Science* **296**, 879-883,  
doi:10.1126/science.1071124 (2002).
- 79 Cowart, L. & Hannun, Y. Selective substrate supply in the regulation of yeast de  
novo sphingolipid synthesis. *J Biol Chem* **282**, 12330-12340 (2007).

- 80 Aronova, S. *et al.* Regulation of ceramide biosynthesis by TOR complex 2. *Cell Metab* **7**, 148-158 (2008).
- 81 Vacaru, A. M. *et al.* Sphingomyelin synthase-related protein SMSr controls ceramide homeostasis in the ER. *J Cell Biol* **185**, 1013-1027 (2009).
- 82 van Echten, G., Birk, R., Brenner-Weiss, G., Schmidt, R. & Sandhoff, K. Modulation of sphingolipid biosynthesis in primary cultured neurons by long chain bases. *J Biol Chem* **265**, 9333-9339 (1990).
- 83 Mandon, E., van Echten, G., Birk, R., Schmidt, R. & Sandhoff, K. Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases. *Eur J Biochem* **198**, 667-674 (1991).
- 84 Kumagai, K., Kawano, M., Shinkai-Ouchi, F., Nishijima, M. & Hanada, K. Interorganelle trafficking of ceramide is regulated by phosphorylation-dependent cooperativity between the PH and START domains of CERT. *J Biol Chem* **282**, 17758-17766 (2007).
- 85 Tomishige, N., Kumagai, K., Kusuda, J., Nishijima, M. & Hanada, K. Casein kinase I $\gamma$ 2 down-regulates trafficking of ceramide in the synthesis of sphingomyelin. *Mol Biol Cell* **20**, 348-357, doi:10.1091/mbc.E08-07-0669 (2009).
- 86 Delgado, A., Casas, J., Llebaria, A., Abad, J. L. & Fabrias, G. Inhibitors of sphingolipid metabolism enzymes. *Biochim Biophys Acta* **1758**, 1957-1977 (2006).
- 87 Dickson, R. Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast. *J Lipid Res* **49**, 909-921 (2008).

- 88 Casamayor, A., Torrance, P. D., Kobayashi, T., Thorner, J. & Alessi, D. R. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol* **9**, 186-197, doi:S0960-9822(99)80088-8 [pii] (1999).
- 89 Kamada, Y. *et al.* Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol Cell Biol* **25**, 7239-7248, doi:10.1128/MCB.25.16.7239-7248.2005 (2005).
- 90 Roelants, F. M., Torrance, P. D., Bezman, N. & Thorner, J. Pkh1 and pkh2 differentially phosphorylate and activate ypk1 and ykr2 and define protein kinase modules required for maintenance of cell wall integrity. *Mol Biol Cell* **13**, 3005-3028, doi:10.1091/mbc.E02-04-0201 (2002).
- 91 Roelants, F. M., Torrance, P. D. & Thorner, J. Differential roles of PDK1- and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Pkc1 and Sch9. *Microbiology (Reading, Engl)* **150**, 3289-3304, doi:10.1099/mic.0.27286-0 (2004).
- 92 Sun, Y. *et al.* Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol* **20**, 4411-4419 (2000).
- 93 Liu, K., Zhang, X., Lester, R. & Dickson, R. The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in *Saccharomyces cerevisiae* including Ypk1, Ypk2, and Sch9. *J Biol Chem* **280**, 22679-22687 (2005).

- 94 Friant, S., Lombardi, R., Schmelzle, T., Hall, M. & Riezman, H. Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *EMBO J* **20**, 6783-6792 (2001).
- 95 Frohlich, F. *et al.* A genome-wide screen for genes affecting eisosomes reveals Nce102 function in sphingolipid signaling. *J Cell Biol* **185**, 1227-1242 (2009).
- 96 Roelants, F. M., Baltz, A. G., Trott, A. E., Fereres, S. & Thorner, J. A protein kinase network regulates the function of aminophospholipid flippases. *Proc Natl Acad Sci USA* **107**, 34-39, doi:10.1073/pnas.0912497106 (2010).
- 97 Bauer, R. *et al.* Schlank, a member of the ceramide synthase family controls growth and body fat in *Drosophila*. *EMBO J* **28**, 3706-3716, doi:emboj2009305 [pii] 10.1038/emboj.2009.305 (2009).
- 98 Scheek, S., Brown, M. S. & Goldstein, J. L. Sphingomyelin depletion in cultured cells blocks proteolysis of sterol regulatory element binding proteins at site 1. *Proc Natl Acad Sci USA* **94**, 11179-11183 (1997).
- 99 Perry, R. J. & Ridgway, N. D. Oxysterol-binding protein and vesicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein. *Mol Biol Cell* **17**, 2604-2616, doi:E06-01-0060 [pii] 10.1091/mbc.E06-01-0060 (2006).
- 100 Guan, X. L. *et al.* Functional interactions between sphingolipids and sterols in biological membranes regulating cell physiology. *Mol Biol Cell* **20**, 2083-2095, doi:10.1091/mbc.E08-11-1126 (2009).

- 101 Vik, A. & Rine, J. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **21**, 6395-6405 (2001).
- 102 Nash, R. *et al.* Expanded protein information at SGD: new pages and proteome browser. *Nucleic Acids Res* **35**, D468-471 (2007).
- 103 Gilon, T., Chomsky, O. & Kulka, R. G. Degradation signals for ubiquitin system proteolysis in *Saccharomyces cerevisiae*. *Embo J* **17**, 2759-2766 (1998).
- 104 Newman, J. R. *et al.* Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature* **441**, 840-846 (2006).
- 105 Wang, Y. *et al.* Precision and functional specificity in mRNA decay. *Proc Natl Acad Sci U S A* **99**, 5860-5865 (2002).
- 106 Gasch, A. P. *et al.* Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11**, 4241-4257 (2000).
- 107 Giaever, G. *et al.* Genomic profiling of drug sensitivities via induced haploinsufficiency. *Nat Genet* **21**, 278-283 (1999).
- 108 Lum, P. Y. *et al.* Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. *Cell* **116**, 121-137 (2004).
- 109 Barnes, G., Hansen, W. J., Holcomb, C. L. & Rine, J. Asparagine-linked glycosylation in *Saccharomyces cerevisiae*: genetic analysis of an early step. *Mol Cell Biol* **4**, 2381-2388 (1984).
- 110 Ungar, D., Oka, T., Krieger, M. & Hughson, F. M. Retrograde transport on the COG railway. *Trends Cell Biol* **16**, 113-120 (2006).
- 111 Zlatanova, J. & Thakar, A. H2A.Z: view from the top. *Structure* **16**, 166-179 (2008).

- 112 Yang, X. J. & Seto, E. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat Rev Mol Cell Biol* **9**, 206-218 (2008).
- 113 Tompa, R. & Madhani, H. D. Histone H3 lysine 36 methylation antagonizes silencing in *Saccharomyces cerevisiae* independently of the Rpd3S histone deacetylase complex. *Genetics* **175**, 585-593 (2007).
- 114 Hanna, J. & Finley, D. A proteasome for all occasions. *FEBS Lett* **581**, 2854-2861 (2007).
- 115 Collins, G. A. & Tansey, W. P. The proteasome: a utility tool for transcription? *Curr Opin Genet Dev* **16**, 197-202 (2006).
- 116 Velichutina, I., Connerly, P. L., Arendt, C. S., Li, X. & Hochstrasser, M. Plasticity in eucaryotic 20S proteasome ring assembly revealed by a subunit deletion in yeast. *Embo J* **23**, 500-510 (2004).
- 117 Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* **22**, 1567-1572 (2004).
- 118 Schuldiner, M., Collins, S. R., Weissman, J. S. & Krogan, N. J. Quantitative genetic analysis in *Saccharomyces cerevisiae* using epistatic miniarray profiles (E-MAPs) and its application to chromatin functions. *Methods* **40**, 344-352, doi:S1046-2023(06)00224-6 [pii] 10.1016/j.ymeth.2006.07.034 (2006).
- 119 Brachmann, C. B. *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene

- disruption and other applications. *Yeast* **14**, 115-132, doi:10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2 [pii]  
10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2  
(1998).
- 120 Duda, R. O., Hart, P. E. & Stork, D. G. *Pattern Classification*. 2nd edn, (John Wiley & Sons, Inc, 2001).
  - 121 Martin, C. E., Oh, C. S. & Jiang, Y. Regulation of long chain unsaturated fatty acid synthesis in yeast. *Biochim Biophys Acta* **1771**, 271-285 (2007).
  - 122 Fischer, J. *et al.* The gene encoding adipose triglyceride lipase (PNPLA2) is mutated in neutral lipid storage disease with myopathy. *Nat Genet* **39**, 28-30 (2007).
  - 123 Klemm, R. W. *et al.* Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. *J Cell Biol* **185**, 601-612 (2009).
  - 124 Lebman, D. A. & Spiegel, S. Cross-talk at the crossroads of sphingosine-1-phosphate, growth factors, and cytokine signaling. *J Lipid Res* **49**, 1388-1394 (2008).
  - 125 Akinbami, L. J., Moorman, J. E., Garbe, P. L. & Sondik, E. J. Status of childhood asthma in the United States, 1980-2007. *Pediatrics* **123 Suppl 3**, S131-145 (2009).
  - 126 Vercelli, D. Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol* **8**, 169-182 (2008).

- 127 Verlaan, D. J. *et al.* Allele-specific chromatin remodeling in the ZPBP2/GSDMB/ORMDL3 locus associated with the risk of asthma and autoimmune disease. *Am J Hum Genet* **85**, 377-393 (2009).
- 128 Hanada, K. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta* **1632**, 16-30 (2003).
- 129 Ejlsing, C. S. *et al.* Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci U S A* **106**, 2136-2141 (2009).
- 130 Strahl, T. & Thorner, J. Synthesis and function of membrane phosphoinositides in budding yeast, *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1771**, 353-404 (2007).
- 131 Brice, S. E., Alford, C. W. & Cowart, L. A. Modulation of sphingolipid metabolism by the phosphatidylinositol-4-phosphate phosphatase Sac1p through regulation of phosphatidylinositol in *Saccharomyces cerevisiae*. *J Biol Chem* **284**, 7588-7596 (2009).
- 132 Kearns, B. G. *et al.* Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature* **387**, 101-105 (1997).
- 133 Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. & Koike, T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* **5**, 749-757 (2006).
- 134 Tabuchi, M., Audhya, A., Parsons, A. B., Boone, C. & Emr, S. D. The phosphatidylinositol 4,5-bisphosphate and TORC2 binding proteins Slm1 and Slm2 function in sphingolipid regulation. *Mol Cell Biol* **26**, 5861-5875 (2006).



- 135 Blagoveshchenskaya, A. & Mayinger, P. SAC1 lipid phosphatase and growth control of the secretory pathway. *Mol Biosyst* **5**, 36-42 (2009).
- 136 Hornemann, T., Wei, Y. & von Eckardstein, A. Is the mammalian serine palmitoyltransferase a high-molecular-mass complex? *Biochem J* **405**, 157-164 (2007).
- 137 Rivera, J., Proia, R. L. & Olivera, A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol* **8**, 753-763 (2008).
- 138 Ammit, A. J. *et al.* Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *Faseb J* **15**, 1212-1214 (2001).
- 139 Masini, E. *et al.* Ceramide: a key signaling molecule in a Guinea pig model of allergic asthmatic response and airway inflammation. *J Pharmacol Exp Ther* **324**, 548-557 (2008).
- 140 Ryan, J. J. & Spiegel, S. The role of sphingosine-1-phosphate and its receptors in asthma. *Drug News Perspect* **21**, 89-96 (2008).
- 141 Idzko, M. *et al.* Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* **116**, 2935-2944 (2006).
- 142 Barrett, J. C. *et al.* Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* **40**, 955-962 (2008).
- 143 Barrett, J. C. *et al.* Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat Genet* (2009).
- 144 Hirschfield, G. M. *et al.* Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. *N Engl J Med* **360**, 2544-2555 (2009).

- 145 Gable, K., Slife, H., Bacikova, D., Monaghan, E. & Dunn, T. M. Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. *J Biol Chem* **275**, 7597-7603 (2000).
- 146 Lester, R. L. & Dickson, R. C. High-performance liquid chromatography analysis of molecular species of sphingolipid-related long chain bases and long chain base phosphates in *Saccharomyces cerevisiae* after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. *Anal Biochem* **298**, 283-292 (2001).
- 147 Jiménez, C. R., L, H., Y., Q. & Burlingame, A. L. *In-Gel Digestion of Proteins for MALDI-MS Fingerprint Mapping* Vol. 16.4 (John Wiley & Sons, Inc., 1998).
- 148 Huber, A. *et al.* Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. *Genes Dev* **23**, 1929-1943 (2009).
- 149 Gari, E., Piedrafita, L., Aldea, M. & Herrero, E. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* **13**, 837-848 (1997).
- 150 Tanoue, D. *et al.* The requirement for the hydrophobic motif phosphorylation of Ypk1 in yeast differs depending on the downstream events, including endocytosis, cell growth, and resistance to a sphingolipid biosynthesis inhibitor, ISP-1. *Arch Biochem Biophys* **437**, 29-41, doi:10.1016/j.abb.2005.02.030 (2005).
- 151 Smogorzewska, A. *et al.* Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* **129**, 289-301, doi:10.1016/j.cell.2007.03.009 (2007).

- 152 Uhlig, S. & Gulbins, E. Sphingolipids in the Lungs. *Am J Respir Crit Care Med*  
(2008).
- 153 Sun, X. *et al.* Functional variants of the sphingosine-1-phosphate receptor 1 gene  
associate with asthma susceptibility. *J Allergy Clin Immunol* **126**, 241-249 e243,  
doi:S0091-6749(10)00733-5 [pii]  
10.1016/j.jaci.2010.04.036 (2010).
- 154 Liu, X. *et al.* Genome-wide meta-analyses identify three loci associated with  
primary biliary cirrhosis. *Nat Genet* **42**, 658-660, doi:ng.627 [pii]  
10.1038/ng.627 (2010).
- 155 McGovern, D. P. *et al.* Genome-wide association identifies multiple ulcerative  
colitis susceptibility loci. *Nat Genet* **42**, 332-337, doi:ng.549 [pii]  
10.1038/ng.549 (2010).

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

David O'Leary 8/22/2010

Author Signature

Date