A Systems Biology Approach Reveals the Role of a Novel Methyltransferase in Response to Chemical Stress and Lipid Homeostasis

Elena Lissina¹,², Brian Young³, Malene L. Urbanus²,⁴, Xue Li Guan⁵,⁶, Jonathan Lowenson³, Shawn Hoon⁷, Anastasia Baryshnikova¹,², Isabelle Riezman⁶, Magali Michaut⁵, Howard Riezman⁶, Leah E. Cowen¹, Markus R. Wenk⁵, Steven G. Clarke³, Guri Giaever¹,²,⁸, Corey Nislow¹,²,⁴*

¹Department of Molecular Genetics, University of Toronto, Toronto, Canada, ²Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Canada, ³Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California Los Angeles, Los Angeles, California, United States of America, ⁴Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada, ⁵Department of Biological Sciences, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, Singapore, ⁶Department of Biochemistry, University of Geneva, Geneva, Switzerland, ⁷Molecular Engineering Lab, Agency for Science, Technology, and Research, Singapore, Singapore, ⁸Department of Pharmacy and Pharmaceutical Sciences, University of Toronto, Toronto, Canada

Abstract

Using small molecule probes to understand gene function is an attractive approach that allows functional characterization of genes that are dispensable in standard laboratory conditions and provides insight into the mode of action of these compounds. Using chemogenomic assays we previously identified yeast Crg1, an uncharacterized SAM-dependent methyltransferase, as a novel interactor of the protein phosphatase inhibitor cantharidin. In this study we used a combinatorial approach that exploits contemporary high-throughput techniques available in Saccharomyces cerevisiae combined with rigorous biological follow-up to characterize the interaction of Crg1 with cantharidin. Biochemical analysis of this enzyme followed by a systematic analysis of the interactome and lipidome of CRG1 mutants revealed that Crg1, a stress-responsive SAM-dependent methyltransferase, methylates cantharidin in vitro. Chemogenomic assays uncovered that lipid-related processes are essential for cantharidin resistance in cells sensitized by deletion of the CRG1 gene. Lipidome-wide analysis of mutants further showed that cantharidin induces alterations in glycerophospholipid and sphingolipid abundance in a Crg1-dependent manner. We propose that Crg1 is a small molecule methyltransferase important for maintaining lipid homeostasis in response to drug perturbation. This approach demonstrates the value of combining chemical genomics with other systems-based methods for characterizing proteins and elucidating previously unknown mechanisms of action of small molecule inhibitors.

Introduction

Methyltransferases are a large class of enzymes comprising 0.6–1.6% of protein coding genes in most sequenced organisms [1]. S-adenosyl methionine (SAM)-dependent methyltransferases regulate a dynamic network of cellular signaling events and are required to maintain intracellular homeostasis in the face of external perturbations by catalyzing the methylation of a wide variety of substrates (proteins, nucleic acids, lipids and small molecules) [2–4]. The characterization and understanding of the roles of most methyltransferases remains challenging, however, due to their dispensability in standard growth conditions. Numerous studies from our lab and others have demonstrated that chemogenomic profiling of the Saccharomyces cerevisiae yeast deletion collection [3] is a powerful approach for the identification and subsequent characterization of genes required for growth in the presence of bioactive compounds [6–15]. Moreover, while most yeast genes (~80%) are dispensable for growth in standard laboratory conditions, the presence of chemical and/or environmental perturbations of the cell, 97% of the yeast genome exhibits a fitness defect that would not otherwise have been revealed [15].

Well-established chemogenomic assays in yeast, such as drug-induced Haploinsufficiency Profiling (HIP), Homozygous Profiling (HOP) and Multicopy Suppression Profiling (MSP) are designed to identify small molecule-gene interactions. For example, HIP assay is used to detect compounds that target essential genes, and HOP and MSP are suitable for identification genetic modifiers of drug resistance [8–10,13]. The combination of these chemogenomic assays allowed us to identify a novel gene, THIR209W, that we subsequently named CRG1 (Cantharidin Resistance Gene 1), due...
Author Summary

Chemical genetics uses small molecules to perturb biological systems to study gene function. By analogy with genetic lesions, chemical probes act as fast-acting, reversible, and “tunable” conditional alleles. Furthermore, small molecules can target multiple protein targets and target pathways simultaneously to uncover phenotypes that may be masked by genes encoding partially redundant proteins. Finally, potent chemical probes can be useful starting points for the development of human therapeutics. Here, we used cantharidin, a natural toxin, to uncover otherwise “hidden” phenotypes for a methyltransferase that has resisted characterization. This enzyme, Crg1, has no phenotype in standard conditions but is indispensable for survival in the presence of cantharidin. Using this chemical genetic relationship, we characterized novel functions of Crg1, and by combining diverse genomic assays with small molecule perturbation, we characterized the mechanism of cantharidin cytotoxicity. These observations are relevant beyond yeast Crg1 because cantharidin and its analogues have potent anticancer activity, yet its therapeutic use has been limited to topical applications because of its cytotoxicity. Considering that methyltransferases are an extremely abundant and diverse class of cellular proteins, chemical probes such as cantharidin are critical for understanding their cellular roles and defining potential points of therapeutic intervention.

to its requirement for growth in the presence of the small molecule cantharidin [14]. Specifically, both CRG1 heterozygous and homozygous deletion strains exhibited sensitivity to the drug, and the overexpression of CRG1 conferred resistance to the drug. Nonetheless, Crg1 is uncharacterized, except for annotation derived from large-scale analyses [15–17].

Based on its primary sequence, Crg1 is predicted to encode a Class I S-adenosyl-methionine (SAM)-dependent methyltransferase [18]. Crg1 shares close sequence homology with trans-aconitate methyltransferase Tmt1 [BLAST-P] and the BLAST 국제화 values 2.0 × 10^{-9} and 3.0 × 10^{-8}, respectively; Figure S1B). The observed differential drug sensitivity in defined media and rich YPD medium is a common phenomenon for these strains to cantharidin (Figure 1A). In agreement with this gene-dose dependent effect, crg1A/CRG1 heterozygous mutants grew worse than the wild-type strain but better than a crg1A/crg1A homozygous mutant in the presence of cantharidin (500 μM) (Figure S1A).

Because our data suggested that CRG1 responds to cantharidin in a gene dose-dependent manner, we next tested whether the transcription of CRG1 is induced in the presence of the drug. qRT-PCR analysis showed that the relative abundance of CRG1 transcripts increased drastically in the wild-type strain after 60 min of the drug treatment (250 μM) compared to the DMSO control (P-value < 0.02; Figure 1B, left panel). Importantly, a gene-dose dependent effect in the response to cantharidin was also observed for CRG1 transcript levels in crg1A/crg1A heterozygous and CRG1-overexpressing mutants (Figure S2A). In agreement with the qRT-PCR data, we also observed induction of CRG1 at the protein level. GFP-tagged Crg1 protein increased from undetectable levels prior to treatment and accumulated to high levels (restricted to the cytoplasm) following 1 hour of cantharidin treatment (Figure S1B).

Given the known high affinity of cantharidin towards Type 2A protein phosphatases (PP2A) and to a lesser degree towards Type 1 (PP1) [30,31], we tested if CRG1 induction was mediated by chemical inhibition of protein phosphatase function. We phenocopied cantharidin treatment using a panel of protein
phosphatase homozygous deletion strains. Consistent with the results of chemical inhibition of protein phosphatases with cantharidin, we found that the homozygous deletion strains sit4Δ/Δ (PP2A), pp1Δ/Δ (PP2C) and the heterozygous deletion strain glc7Δ/GLC7 (PP1) also resulted in transcriptional upregulation of CRG1 in the absence of cantharidin (Figure 1B, right panel). It is important to note that perturbation of these protein phosphatases accounted for only ~20% of the transcript induction observed by cantharidin. Furthermore, the treatment with calycin A, a structurally distinct PP2 and PP1 inhibitor [35], known to interact with the yeast PP1 GLC7 [14], resulted in an increase of CRG1 transcript level to a similar degree as in glc7Δ/GLC7 mutant (~2.5 fold; Figure S2C). This observation opens up the possibility that cantharidin acts independently of this PPase. This hypothesis is also supported by our observation that overexpression of GLC7 confers resistance to calycin A, but not to cantharidin [14]. These results also suggest that these protein phosphatases are likely to be negative regulators of the cellular pathway regulating CRG1 induction.

We also observed that cantharidin-induced transcription of CRG1 follows a temporal pattern characteristic of diverse environmental stress responses [36], following a peak at 60 min of treatment the transcript levels began to decrease at 120 min (~40 fold, P-value <0.01; Figure 1B). Indeed, a comprehensive genome-wide analysis of diverse environmental stresses from publicly available expression data [36] revealed that the transcript profile of CRG1 in diverse stress conditions correlates highly (r = 0.8) with a well-characterized stress-responsive gene, the heat shock protein SSE2 (Figure S2D), suggesting that CRG1 is also transcriptionally activated by other stress conditions in addition to cantharidin.
Because CRG1 is annotated (based on its amino acid sequence) as a putative SAM-dependent methyltransferase [10], we next asked whether its methyltransferase domain is required for cantharidin tolerance by mutating amino acids (D144A, D67A, E105A-D108A) within the conserved motifs (Figure 1C). These amino acids have previously been shown to be critical for activity of other methyltransferases [37]. Overexpression of these crg1 site-specific mutants in a crg1Δ/Δ strain failed to confer cantharidin resistance while, in contrast, mutation of a non-conserved residue (G96A) in the methyltransferase domain showed resistance equivalent to wild-type CRG1 (Figure 1C). The observed decrease in resistance to cantharidin was not due to reduced expression of the mutated Crg1 proteins (Figure S2E), suggesting that the methyltransferase domain of Crg1 is both functional and important for cellular survival in the presence of the drug.

To identify other potential cellular factors important for Crg1-mediated cantharidin resistance, we profiled the complete yeast transcriptome using whole-genome tiling microarrays. Transcriptional changes in wild type, crg1Δ/Δ and crg1Δ/Δ overexpressing crg1Δ/Δ strains were analyzed after 1 hour of exposure to the drug. To ensure that the transcriptome datasets for the different strains are comparable, the IC₅₀ for wild type (250 μM) was applied to all strains. Even at this high dose, the hypersensitive crg1Δ/Δ strain is viable, after 1 hour of exposure (Figure S3A). When applied for an extended period, this dose is, in fact, inhibitory for growth of crg1Δ/Δ strains (Figure 1A and Figure S1A). Furthermore, the treatment of crg1Δ/Δ strain with a lower dose (30 μM, the IC₅₀ for this mutant) resulted in quantitative difference in the transcriptome profile rather than in any qualitative differences, suggesting that the transcriptional changes are consistent across a range of concentrations. In particular, this observation was relevant to downregulated genes (Figure S3B and S3C). It is also worth noting that the expression of most genes was not affected by crg1 mutation. To uncover cantharidin-specific genes in our transcriptome analysis, we eliminated Environmental Stress Response (ESR) genes known to be activated by a large number of stresses, such as genes required for vacuole biogenesis, response to stress, ribosome biogenesis, and RNA processing [36]. We also eliminated those genes that did not demonstrate at least two-fold difference in the presence of cantharidin or if their differential expression failed to show statistical significance. To detect genes and biological processes that are differentially expressed among the strains and treatments, the enrichment of genes for Gene Ontology (GO) term Biological process in the transcriptomes of wild type, crg1Δ/Δ and CRG1-overexpressing crg1Δ/Δ strains in the presence and absence of cantharidin were compared (Table S1). We also clustered genes according to their expression pattern. The clustering and GO term comparative analysis revealed that significantly downregulated genes (log₂ (drug/DMSO) < -1, P-value < 0.05) in crg1Δ/Δ mutant and the wild type were enriched for the genes of amino acid process (multiple-testing corrected P-value < 1.0 × 10⁻⁷ and P-value < 7.0 × 10⁻⁶, respectively), while the transcriptional profile of cantharidin-resistant CRG1-overexpressing crg1Δ/Δ strain did not demonstrate a similar enrichment (Figure 1D and Figure S3D). Of particular interest, most of the genes that compose methionine biosynthetic process (MET6, MET7, MMP1, STR3, ADE3, SAM1, SAM2, SAH1, MET22, MET31) were differentially expressed between cantharidin-resistant CRG1-overexpressing crg1Δ/Δ, wild type and cantharidin-sensitive crg1Δ/Δ strain in the presence of cantharidin (P-value < 0.05; Table S2; Figure 1E). One noteworthy example is STR3, a cystathionine beta-lyase, the gene that demonstrated the most differential expression in the strains. STR3 was significantly induced by cantharidin in CRG1-overexpressing crg1Δ/Δ strain (log₂ (drug/DMSO) = 4.7, P-value < 0.022) and downregulated in the wild type (log₂ = -0.6) and crg1Δ/Δ (log₂ = -0.35). The observed differential expression of STR3 was further confirmed by qRT-PCR (Figure S3F). STR3 is of interest because it functions in methionine biosynthesis by converting cystathionine into homocysteine, a precursor for methionine, which is a substrate for the generation of SAM. SAM is required as a methyl donor for methylation reactions (Figure 1E). To further explore the role of the CRG1 SAM-dependent methyltransferase, we treated wild-type cells with a combination of cantharidin and S-adenosyl homocysteine (SAH), a non-specific methyltransferase inhibitor. We found that wild-type strains were more sensitive to the cantharidin/SAH combination compared to either single agent (Figure S3F). These observations confirm the requirement of SAM-dependent methyltransferase activity in response to cantharidin, and suggest that Crg1 is a functional methyltransferase that catalyzes a SAM-dependent methylation reaction important for cantharidin resistance.

Crg1 Methylates Cantharidin In Vitro

Given that CRG1 provides cantharidin resistance in a gene dose-dependent manner and because of its close sequence homology to TMT1, a small molecule methyltransferase that catalyzes the formation of methyl esters (Figure 2A), we hypothesized that Crg1 might methylate cantharidin because this drug bears some structural similarity to the substrates of Tmt1 (Figure 2B). To test this possibility, we performed in vitro biochemical assays with purified Crg1, cantharidin, and S-adenosyl-[methyl-¹⁴C]methionine (Figure 3A). These in vitro reactions were separated via reverse phase liquid chromatography and the radioactivity of the collected fractions was quantified with a scintillation counter. We detected a unique peak of radioactivity eluting in the 18-20 min fraction (Figure 3B). The appearance of this peak was both cantharidin and Crg1-dependent, suggesting that it could correspond to methylated cantharidin.

To confirm that the novel activity was catalyzed by Crg1 rather than by a co-purifying protein, we repeated the methylation reactions with mutant forms of Crg1 containing amino acid substitutions at critical residues within the methyltransferase domain. As described earlier, the D144A and E105A-D108A mutations abolished resistance to cantharidin (Figure 1C), so we assessed whether these mutated proteins were able to methylate the drug molecule. We prepared in vitro reactions containing varying concentrations of cantharidin and quantified the amount of acid-labile volatile radioactivity because methyl esters are known to readily hydrolyze in both strongly acidic and basic conditions to yield methanol [19,38]. Unlike the reactions performed with wild-type Crg1, addition of cantharidin to the reactions with mutant forms of the enzyme showed no increase in acid-labile radioactivity (Figure 3C), strongly suggesting that a functional methyltransferase domain in Crg1 is required for cantharidin methylation.

To definitively determine whether cantharidin is a substrate of Crg1, we prepared and analyzed unlabeled reactions containing purified Crg1, cantharidin, and SAM by liquid chromatography-mass spectrometry. We first looked at the extracted ion chromatogram expected for unreacted cantharidin (C₁₀H₁₃O₄⁺; m/z = 197.0814±100 ppm) and found a large peak in cantharin-containing reactions with an elution time of 18.6-18.8 min (Figure S4B). The combined spectra of this peak in the complete reaction mixture contained several species: m/z = 197.0942 and 215.1065, corresponding to the m/z for cantharidin and hydrated cantharidin (C₁₂H₁₅O₄⁺; m/z = 215.0919), respectively (Figure 3D). Because these species co-elute at 18.6-18.8 min in the complete reaction and the control reactions containing cantharidin (Figure S4B and S4C)
and because sterically hindered anhydrides do not favor hydrolysis [39], a likely explanation is that cantharidin forms a co-eluting water adduct during ionization.

Next, we analyzed the full reaction to determine whether Crg1 methylates cantharidin. Specifically, we analyzed the extracted ion chromatogram expected for methyl cantharidin (C_{11}H_{15}O_{4}^{+}; m/z = 211.0970±100 ppm). We identified a peak eluting after cantharidin at 19.2 min corresponding to the mass of methyl cantharidin in the complete reaction mixture (Figure S4D). Importantly, this species was absent in each of our control reactions lacking cantharidin, SAM, or Crg1 (Figure S4D). This is strong evidence that cantharidin is indeed methylated by Crg1. Finally we analyzed the combined spectra of the 19.2-min peak (Figure 3E). In addition to the m/z = 211.1105 species, we observed m/z = 229.1215 and m/z = 197.0930 species, corresponding to hydrated methyl cantharidin (C_{11}H_{17}O_{4}^{+}; m/z = 229.1076) and cantharidin, respectively. Importantly, all of these species co-elute (Figure S4B, S4D and S4E).

Based on its close sequence homology to Tmt1, we suspect that Crg1 catalyzes the formation of a cantharidin methyl ester (Figure 2). In solution, this putative methyl ester is likely in equilibrium with its ring-closed methyl anhydride form. If the equilibrium favors the ester form, some fraction of the cantharidin methyl ester could undergo ring-closing elimination reactions during ionization to yield methyl cantharidin and cantharidin products. Likewise, if the equilibrium favors the methyl anhydride, it may possibly undergo in-source fragmentation to give cantharidin, and like cantharidin, it may simply form a water adduct during ionization. Although our mass spectrometry data strongly support our hypothesis that Crg1 methylates cantharidin in vitro, additional analysis is needed to determine the structure of the methylated drug molecule. It is possible that the observed methyl cantharidin and hydrated methyl cantharidin species are ionization products of another methylated cantharidin derivative that is the actual product of the Crg1-catalyzed reaction.

Revealing the Genetic Interactome of CRG1 in the Presence of Cantharidin

To identify cellular processes required for cantharidin resistance and to define the spectrum of genes that compensate for the absence of CRG1 in the presence of cantharidin, we used a chemogenomic approach to analyze genetic interactions between CRG1 and each of the ~4800 non-essential yeast genes both in the presence and absence of the drug. A genetic interaction between two genes occurs when the phenotype of the double deletion mutant shows significant deviation in fitness compared with the expected (multiplicative) effect of combining two single mutants (e.g. sickness or synthetic lethality) [17,40]. When synthetic lethality is observed, it suggests that the genes may have overlapping functions. By analogy, identification of drug-gene interactions will similarly uncover genes that act in parallel with a gene of interest and these interactions can illuminate a compound’s effect on a cell. To perform this experiment, we generated double deletion mutants (with a cg1Δ strain as the query) using Synthetic Genetic Array (SGA) technology [40], pooled all viable double deletion mutants and analyzed their growth in a competitive fitness assay in the presence and absence of cantharidin (Figure 4A) [41]. Six highly reproducible and independent cg1ΔxxxΔ pools (r = 0.72, Figure S5A) were further averaged yielding 70 double deletion mutants (Table S3; Datasets S1 and S2) that showed significant growth defects (log2 (drug/DMSO) < -1, P-value < 0.05) in the presence of an IC_{50} dose of cantharidin (30 μM) in YPD when grown in a pool. Noteworthy, these genes were not sensitive as single deletion mutants (P-value < 0.025; Figure 4B and 4C, Table S3), and, thus, the effect was specific to the double mutant combination. To obtain a general overview of “aggravating” (negative) interactors of CRG1 in the presence of cantharidin, we categorized this set of genes according to their GO term Biological process (Figure 4B). This dataset comprised diverse biological processes, including vesicle-mediated transport (P-value < 0.008), chromosome organization (P-value < 0.001), response to chemical stimulus (P-value < 0.019), lipid metabolic process (P-value < 2.0×10^{-5}), response to stress (P-value < 0.018), and protein modification process (P-value < 0.003).

We also identified the serine/threonine kinase DBF2 as a strong suppressor of CRG1-dependent cantharidin toxicity (log2 (drug/DMSO) = 1.12, P-value < 4.0×10^{-5}; Figure 4B). This interaction was confirmed by evaluating the fitness of individual strains in liquid and on solid SD medium in the presence of 25 μM and 10 μM cantharidin, respectively (lethal doses for cg1Δ strain in these media conditions; Figure 4C and Figure S5B). Furthermore, the alleviating
interaction between *DBF2* and *CRG1* was not observed at 37°C, indicating cantharidin-specific nature of this interaction (Figure S5B). In addition to the well-characterized roles of *Dbf2* in the mitotic exit network [42], this newly uncovered interaction suggests that this kinase may have an opposing function to the protein phosphatases (PP2A and PP1), the primary targets of cantharidin [30,31]. As independent evidence for an interaction with cantharidin, we found that *DBF2* transcript levels were significantly decreased in a *crg1ΔD* mutant in the presence of cantharidin (250 μM) (2-fold, *P*-value, 0.013) compared to DMSO control, and that change was not detected in other strains (Figure S5C). This observation confirms the role of Crg1 in phosphorylation/dephosphorylation homeostasis during cantharidin stress.

**CRG1 Is Important for Lipid Homeostasis during Cantharidin Treatment**

To identify genes specifically required for growth in the presence of cantharidin, we removed the genes that behave as multidrug resistance genes (MDR) from our chemogenomic dataset described above. MDR genes are defined here as those that are required for growth in the presence of multiple stress conditions [at least 20% of tested conditions for homozygous deletion strains] [15]. This filtering removed apparent enrichment of genes involved in vesicle-mediated transport genes (*P*-value = 0.216), response to stress (*P*-value = 0.024), chromosome organization (*P*-value = 0.075) and protein modification process (*P*-value = 0.021). Following this, we found that cantharidin-specific *CRG1* interactors are significantly enriched for genes required for lipid metabolic process (multiple testing corrected *P*-value, 0.0003) (Figure S6A). In particular, lipid methyltransferases (*CHO2*, *ERG6*), glycosylphosphatidylinositol (GPI) lipid biosynthesis genes (*ARV1*, *GUP1*, *PDR1*) and lipid-related genes (*SAC1*, *MOT3*, *DEP1*, *RVS167*, *TTA7*) are essential in the double deletion strains in the presence but not absence of cantharidin treatment (Figure 5A). Furthermore, we demonstrated that an increase in cantharidin concentration (10 μM) did not result in cantharidin...
sensitivity for these genes compared to wild type (Figure 5A), confirming the dependence of the detected interactions on the presence of CRG1.

To explore further the role of CRG1 in lipid metabolism and related processes, we compared the lipid content (or “lipidome”) of wild type, crg1Δ/Δ homozygous deletion and CRG1-overexpressing crg1Δ/Δ strains in the presence and absence of cantharidin (250 μM) using electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis [43]. We observed significant changes in the abundance of most glycerophospholipids and sphingolipids in both the wild type and crg1Δ/Δ strains after growth in cantharidin-containing medium (P-value, 0.05, Kruskal-Wallis test). The strains with a CRG1-overexpressing construct did not exhibit significant cantharidin-induced lipid alterations (P-value, 0.08, Kruskal-Wallis test; Figure 5B, Table S4, Dataset S3). Specifically, in both the wild type and crg1Δ/Δ strains, cantharidin measurably increased the levels of short chain phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) species, while the levels of long-chain PCs and PIs were reduced (Figure 5B). In the crg1Δ/Δ strain we also noted a substantial decrease in the levels of mixed size phosphatidylethanolamine (PS) species after cantharidin stress, while the wild type and crg1Δ/Δ strain had increased levels of saturated short chain (C16 and C18) PI species compared to mono-unsaturated short chain PIs in cantharidin (Table S4). Such abundance changes with respect to acyl chain length and saturation were not observed in the CRG1-overexpressing mutant, suggesting that extra copies of CRG1 complemented the cantharidin-induced defects.

It has been previously reported that phospholipid and sphingolipid biosynthetic pathways are interconnected [44–46]. One way in which this interconnection is seen is when, a single gene deletion or chemical perturbation of cells results in the so-called “ripple effect” [45] characterized by lipidome-wide perturbations. We see evidence of this effect: the amounts of the most abundant sphingolipid inositolphosphoceramide (IPC) and mannosyl-inositolphosphoceramide (MIPC) were also affected by cantharidin in a crg1Δ/Δ mutant (Figure 5D). To investigate if other lipid intermediates are affected by the drug in a similar manner in crg1 mutants, we analyzed both sterol content and the formation of lipid droplets, which serve as storage pools of triacylglycerols and steryl esters [47]. We found no obvious changes in these lipid species in the presence of drug (Figure S6B and S6C). Taken together, these results demonstrated that cantharidin’s effect is specific towards phospholipids and sphingolipids in crg1 mutants.
To test if cantharidin-induced alterations in yeast lipidomes are evolutionally conserved, we examined the lipidome of the human fungal pathogen *Candida albicans* in response to cantharidin. A *C. albicans* homozygous *crg1* deletion (*orf19.633Δ/Δ*) displayed similar growth defects to those observed in *S. cerevisiae* when challenged with cantharidin (Figure S7A). Lipidomic analysis demonstrated that cantharidin treatment (2 mM, IC20 for *C. albicans* wild type) resulted in the significant changes in most phospholipid species in both wild type and *orf19.633Δ/Δ* homozygous mutant (*P*-value, 0.05). Furthermore, although to a more modest degree than seen in *S. cerevisiae*, we found that *C. albicans CRG1* may account for some difference between wild type and a mutant strain (*P*-value, 0.05; Figure S7B; Dataset S4). In addition, we have shown previously that the overexpression of *C. albicans* ORF *orf19.633* restored cantharidin resistance in *S. cerevisiae crg1Δ/Δ* mutant [14], further suggesting that the lipid homeostasis functions of this *C. albicans* putative SAM-dependent methyltransferase are conserved.

**Crg1-Dependent Effects of Cantharidin on Cytoskeleton Organization**

One of the phospholipids manifesting substantial changes in our lipidome analysis was phosphatidylinositol (PI) (Figure S7C). PI is an essential phospholipid with multiple roles in the biosynthesis and metabolism of phosphoinositides (PIP), inositol polyphosphates (IPs), complex sphingolipids, GPI anchors, and PIPs. (D) Comparison of sphingolipid profiles of *wt*, *crg1Δ/Δ* and *CRG1*-overexpressing *crg1Δ/Δ* mutants in the presence of cantharidin (250 μM). The suffixes -B, -C, and -D on IPC and MIPC denote hydroxylation states, having two, three, or four hydroxyl groups, respectively. Statistical significance in the abundance of lipid species in the presence of cantharidin between wild type and mutants was determined using Kruskal-Wallis test, *P*-value <0.05 (Table S4).

To test if cantharidin-induced alterations in yeast lipidomes are evolutionally conserved, we examined the lipidome of the human fungal pathogen *Candida albicans* in response to cantharidin. A *C. albicans* homozygous *crg1* deletion (*orf19.633Δ/Δ*) displayed similar growth defects to those observed in *S. cerevisiae* when challenged with cantharidin (Figure S7A). Lipidomic analysis demonstrated that cantharidin treatment (2 mM, IC20 for *C. albicans* wild type) resulted in the significant changes in most phospholipid species in both wild type and *orf19.633Δ/Δ* homozygous mutant (*P*-value, 0.05). Furthermore, although to a more modest degree than seen in *S. cerevisiae*, we found that *C. albicans CRG1* may account for some difference between wild type and a mutant strain (*P*-value, 0.05; Figure S7B; Dataset S4). In addition, we have shown previously that the overexpression of *C. albicans* ORF *orf19.633* restored cantharidin resistance in *S. cerevisiae crg1Δ/Δ* mutant [14], further suggesting that the lipid homeostasis functions of this *C. albicans* putative SAM-dependent methyltransferase are conserved.

---

**Figure 5. Crg1 is important for lipid homeostasis during cantharidin stress.** (A) *CRG1* is synthetically lethal with lipid-related genes in the presence of cantharidin. Cells were normalized to an equivalent OD600,10-fold diluted, spotted onto synthetic complete defined medium containing cantharidin and incubated at 30°C. (B) Comparative phospholipid profiles of wild type and *crg1* mutants in response to cantharidin. Cells grown to mid-exponential phase in YPD were treated with cantharidin (250 μM) for 2 hours. Lipid standards were added to the cells, and extracted lipids were measured using ESI-MS/MS. The quantities of lipid species are expressed as ion intensities relative to the levels in DMSO, and converted to a log2 scale. Data are the average of three samples. Statistical significance in the abundance of lipid species in the presence of cantharidin between wild type and mutants was determined using Kruskal-Wallis test, *P*-value, 0.05 (Table S4). (C) A simplified diagram of phospholipid biosynthesis linked to sphingolipid biosynthesis. PI species contribute to biosynthesis of complex sphingolipids, GPI anchors, and PIPs. (D) Comparison of sphingolipid profiles of *wt*, *crg1Δ/Δ* and *CRG1*-overexpressing *crg1Δ/Δ* mutants in the presence of cantharidin (250 μM). The suffixes -B, -C, and -D on IPC and MIPC denote hydroxylation states, having two, three, or four hydroxyl groups, respectively. Statistical significance in the abundance of lipid species in the presence of cantharidin between wild type and mutants was determined using Kruskal-Wallis test, *P*-value, <0.05 (Table S4). doi:10.1371/journal.pgen.1002332.g005
Cells grown to mid-exponential phase were treated with cantharidin (250 μM) or DMSO for 1 hour. Cells fixed with formaldehyde were stained for actin with FITC-phalloidin and visualized by fluorescence microscopy. Bar, 5 μm. The number of actin patches per cell in each sample was quantified. Values are the mean of three independent replicates (n = 270–1000), error bars are the standard deviation; * P-value <0.025, ** P-value <0.0002 (Student’s t-test). (C) Cantharidin induces CRG1 transcription via the Cell Wall Integrity (CWI) pathway. Cells grown to mid-exponential phase were treated with cantharidin (250 μM) in YPD for indicated time. Total RNA was extracted, cDNA was prepared and analyzed by qRT-PCR. CRG1 transcript levels were normalized to ACT1. The simplified diagram of CWI pathway is shown. Slt2 is a kinase, Rlm1 is a transcriptional activator governed by the CWI pathway. (D) A preliminary model describing Crg1-cantharidin interaction. See text for details.

**Figure 6. Crg1 is important for actin patch formation during cantharidin treatment.** (A) Diagram showing how PIP species are involved in Pkc1-dependent changes in actin cytoskeleton. (B) Crg1 is important for actin patch integrity during cantharidin stress. Wt, crg1Δ/Δ mutant and crg1Δ/Δ cells were grown to mid-exponential phase at 30 °C in YPD in the presence of cantharidin (250 μM) or DMSO for 1 hour. Cells fixed with formaldehyde were stained for actin with FITC-phalloidin and visualized by fluorescence microscopy. Bar, 5 μm. The number of actin patches per cell in each sample was quantified. Values are the mean of three independent replicates (n = 270–1000), error bars are the standard deviation; * P-value <0.025, ** P-value <0.0002 (Student’s t-test). (C) Cantharidin induces CRG1 transcription via the Cell Wall Integrity (CWI) pathway. Cells grown to mid-exponential phase were treated with cantharidin (250 μM) in YPD for indicated time. Total RNA was extracted, cDNA was prepared and analyzed by qRT-PCR. CRG1 transcript levels were normalized to ACT1. The simplified diagram of CWI pathway is shown. Slt2 is a kinase, Rlm1 is a transcriptional activator governed by the CWI pathway. (D) A preliminary model describing Crg1-cantharidin interaction. See text for details.

doi:10.1371/journal.pgen.1002332.g006

---

CRG1 Transcription Is Regulated via the Cell Wall Integrity (CWI) Pathway

Finally, to determine how Crg1 is regulated at the transcriptional level in response to cantharidin, we explored which pathways, if any, are required for cantharidin resistance. Based on our observation that the homozygous deletion strains slt2Δ/Δ and bck1Δ/Δ (both CWI kinases) are hypersensitive to cantharidin [14,15], combined with the fact that the promoter region of CRG1 contains a binding site for Rlm1 (a transcriptional regulator of CWI pathway) [52], we asked if CRG1 expression is activated by cantharidin via the CWI pathway. We found that deletion of these genes blunted the increase of CRG1 transcript in response to cantharidin (250 μM) compared to the wild type (Figure 6C), indicating that CWI pathway components are required for CRG1 expression in the presence of cantharidin. The CRG1 promoter also contains a binding site for Yap1, a transcription factor required for cadmium tolerance and the oxidative stress response. In contrast to Rlm1 and Slit2, the relative amount of CRG1 transcript in the yap1Δ/Δ mutant was unchanged in the presence of cantharidin (Figure S9A). While these data suggest that Crg1 may be regulated via the CWI pathway and is transcriptionally responsive to numerous cell wall stressing agents (Figure S9B), we did not detect any drastic fitness defects when crg1 mutants were grown in the presence of cell wall perturbing agents (Figure S9B). However, overexpression of CRG1 in the crg1Δ/Δ mutant did confer resistance to lithium chloride and fenpropimorph, both of which are known perturbants of the cell membrane and other lipid processes (Figure S9C) [53–58]. Together these results support a model in which Crg1 is involved in lipid-related processes that indirectly impinge on cell wall integrity.

**Discussion**

In this study we demonstrated that yeast genetic and chemical genome-wide approaches, when combined with rigorous biological follow-up, can effectively characterize a novel gene that, despite being subject to numerous large-scale phenotypic studies, had little functional annotation. Our previous work demonstrated that Crg1, a putative SAM-dependent methyltransferase, was a novel mediator of resistance to the protein phosphatase inhibitor, cantharidin [14]. Here we show that the mechanism of Crg1-cantharidin interaction is through direct methylation of the compound, and that, furthermore, Crg1 plays an essential role in the cellular response to cantharidin-induced lipid alterations.

Our initial observation that cantharidin cytotoxicity is suppressed by overexpression of CRG1 suggested a specific, although not necessarily direct, cantharidin-Crg1 interaction in vivo [14]. Here, we demonstrate that Crg1 is able to interact with cantharidin in vitro, resulting in the formation of a methylated
cantharidin species. Modification by methylation is known to remove negative charges on diverse molecules which can alter hydrophobicity and modulate cellular pathways and processes. Given the clear phenotype of Crg1-deficient cells and the results from our in vitro biochemical characterization of Crg1, we hypothesize that methylation of cantharidin alters its physical properties such that it is no longer harmful to cells. In a manner similar to other methyltransferases that are known to detoxify small molecules [17,59-62], chemical modification of cantharidin provides some insight regarding how its methylation may modify its activity. For example, endothall, an unmethylated, ring-opened form of cantharidin, has been assayed for protein phosphatase inhibition [26] and the methyl, ethyl, and propyl esters of endothall are still potent inhibitors of PP1 and PP2A. Several lactol derivatives of norcantharidin (the anhydride form of cantharidin, has been assayed for protein phosphatase inhibition. For example, endothall, an unmethylated, ring-opened form of cantharidin, has been assayed for protein phosphatase inhibition [26] and the methyl, ethyl, and propyl esters of endothall are still potent inhibitors of PP1 and PP2A. Several lactol derivatives of norcantharidin (the anhydride form of cantharidin, has been assayed for protein phosphatase inhibition [26] and the methyl, ethyl, and propyl esters of endothall are still potent inhibitors of PP1 and PP2A.

Another possible explanation for our observation that cantharidin-induced lipidome alterations can be suppressed by increasing the gene dose of CRG1 can be found in the transcriptional changes that occur in these strains. Genes involved in methionine biosynthesis are differentially expressed in CRG1-overexpressing strains in the presence of the drug compared to wild type and the crg1A/A mutant. This is of particular interest because changes in methionine metabolism can regulate methylation reactions by altering levels of SAM, a methyl donor [65,66]. For example, Tehlivets et al. showed that defects in methionine cycling enzymes result in an imbalance of phospholipid and triacylglycerol synthesis [67,68]. The mechanisms underlying these relationships are not yet clear, but it is possible that cells sense that the level of SAM is depleted via Crg1 activity, which results in transcriptional changes in methionine biosynthesis genes, in particular, the cystathionine beta-lyase Str3. These findings suggest that overexpression of Crg1 may buffer cantharidin-treated lipidome alterations in part through changes in the methionine cycle.

To define the ‘core’ buffering network to CRG1 in the presence of cantharidin, we compared the transcriptome and cantharidin SGA profiles. Although we did not find any obvious overlap in GO term biological processes between these datasets, in our cantharidin SGA one of the most sensitive mutants was MET22 (Figure 4C), a gene with a role in sulfur assimilation and methionine biosynthesis. This gene was also differentially expressed in CRG1-overexpressing mutant vs. wild-type strain (P-value <0.02; Table S2).

Our chemical genomics results were corroborated by traditional SGA analysis. This analysis demonstrated that CRG1 has an alleviating (suppressing) genetic interaction with RVS167. It is well established that a similar phenotype is observed when RVS167 is deleted in combination with genes involved sphingolipid biosynthesis (e.g. SUR1, SUR2, FEN1, ELO3 and IPT1), implicating sphingolipid biosynthesis in the regulation of the actin cytoskeleton [49,50,69–71]. Similarly to S. cerevisiae and C. albicans, studies in the ciliate Tetrahymena showed that cantharidin treatment also influences PI metabolism and the actin cytoskeleton [72], demonstrating the conservation of cantharidin-lipid-actin interactions.

Understanding the transcriptional regulation of CRG1 during cantharidin stress adds many layers to the picture of the complex physiological roles of this methyltransferase. CRG1 transcription is activated by cantharidin via the conserved MAPK family components of the CWI signaling pathway [52,73]. Hoon et al. previously demonstrated that deletion of slt2 and bck1 results in cantharidin sensitivity, suggesting that this pathway is critical for cantharidin resistance [14]. In mammalian cells, several studies have reported that the MAP kinases ERK and JNK are also activated by cantharidin [27,29], likely as a consequence of the inhibition of protein phosphatases. Moreover, other studies reported that an intact CWI cascade is essential for maintaining lipid homeostasis [74]. It remains to be determined what specific steps are involved in the activation of CRG1 by cantharidin. One possible scenario is that the CWI pathway is activated by the accumulation of aberrant lipid species in a manner analogous to previous reports that suggest that long chain bases induce the Pkc1-MAPK CWI pathway in yeast [75,76].

Based on our observations, we propose the following mechanism for Crg1-cantharidin interaction (Figure 6D). Cantharidin treatment inhibits PP2A and PP1, resulting in the perturbation of both lipid homeostasis and actin cytoskeleton organization. This perturbation activates the CWI pathway, which in turn induces CRG1 transcription. The resulting Crg1 protein directly methylates cantharidin, alleviating its cytotoxicity and restoring lipid homeostasis.
homeostasis, actin cytoskeletal architecture, as well as other cantharidin-associated effects.

In summary, our study demonstrates the value of combining classic biology approaches and chemical genomics with other "omic"-based methods for de-orphaning proteins and elucidating previously unknown mechanisms of therapeutics action.

Materials and Methods

Strains, Plasmids, and Growth Conditions

Yeast strains and plasmids used in this study are described in Table S5 and Table S6. Unless otherwise stated, wild-type (wt) strain is BY4743; crg1Δ/Δ was derived from BY4743. Yeast cells were grown in YPD (2% yeast extract, 1% peptone, 2% glucose) or in synthetically defined medium, SD (0.67% yeast nitrogen base, 2% glucose, and amino acids). Cantharidin, norcantharidin, cantharidic acid, and fenpropimorph were purchased from Sigma Aldrich (Toronto, Canada). Cantharidin, cantharidic acid, norcantharidin, and fenpropimorph were dissolved in DMSO and stored at −20°C. The IC\textsubscript{50} of cantharidin in YPD for wild-type is 250 μM, in SD it is 5 μM, both determined in liquid culture as described [77].

Site-Directed Mutagenesis of CRG1

CRG1 was amplified from wild-type strain using primers (Table S7) with homology to the vector p426-GAL1-TAP at the 5’ end. The amplified CRG1 and HindIII linearized vector were directly co-transformed into a crg1Δ/Δ mutant and transformant colonies were selected in synthetic defined media lacking uracil (SD-URA). CRG1 was cloned downstream of a GAL1 inducible promoter and in frame with the TAP coding sequence. Transformants were screened by PCR and for cantharidin resistance. CRG1 missense mutants were prepared using the QuickChange Lightning Site-directed mutagenesis kit (Stratagene - Agilent Technologies Company, La Jolla, CA, USA). Clones were sequenced to verify the mutations. To express Crg1, transformants were grown to mid-exponential phase in SD-URA and raffinose (2%), then induced by the addition of galactose to a final concentration of 2%. 30 μM of cantharidin was used to test sensitivity of mutants. Cells were harvested after 3 hours of induction, and Crg1 expression was verified by Western blots of 12% SDS-PAGE gels using anti-TAP antibodies (OpenBiosystems – Thermo Fisher Scientific, Huntsville, AL, USA).

RNA Isolation, cDNA Preparation, and Quantitative Real-Time PCR Analysis

Cells grown to mid-exponential phase in YPD medium were incubated with or without cantharidin (250 μM) for various amounts of time, harvested by centrifugation, frozen in liquid N\textsubscript{2} and stored at −80°C. RNA was extracted with hot acidic phenol [78] and treated with the Turbo DNase (Ambion – Applied Biosystems, Austin, TX, USA). RNA purity was tested using a spectrophotometer and integrity was evaluated by denaturing gel electrophoresis. First-strand cDNA was synthesized from 1 μg DNase-treated RNA with 0.5 μg of oligo(dT\textsubscript{12-18}) primers (Invitrogen, Burlington, ON, Canada) using 200 units of Superscript II Reverse transcriptase (Invitrogen, Burlington, ON, Canada). Real-time PCR analysis was conducted with Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and gene-specific primers (Table S7) at a final concentration of 250 nM. qRT-PCR was carried out on a 7900HT Fast system (Applied Biosystems) using Sequence Detection System software version 2.3. Fold change in CRG1 transcript level normalized to ACT1 was calculated using the 2\textsuperscript{−ΔΔCT} method. At least three independent replicates of each reaction were performed. Student’s t-test was applied for statistical analysis (paired for drug vs. DMSO treatments, and unpaired for mutants vs. wild type).

Microarray Analysis

Cells grown to mid-exponential phase in YPD medium were incubated with or without cantharidin (250 μM) for 1 hour, harvested by centrifugation. Isolation of RNA and hybridization to the tiling arrays was performed as described [79], except that actinomycin D was added in a final concentration of 6 μg/mL during cDNA synthesis to prevent antisense artifacts [80]. Two independent replicates were used for the analysis. Hybridization to Affymetrix Tiling Arrays using the Gene-Chip Fluidics Station 450 (Affymetrix) was followed by the extraction of intensity values for the probes using the GeneChip Operating Software (Affymetrix). Acquisition and quantification of array images were performed using the Affymetrix tiling analysis software (http://www.affymetrix.com/support/developer/downloads/TilingArrayTools/index.affx). The resulting BAR files containing probe position and intensities were further analyzed by aligning the probes that match the position of the S. cerevisiae Genome Database list of defined ORFs (http://downloads.yeastgenome.org/chromosomal_feature/saccharomyces_cerevisiae.gff). The log2 of signal intensity of each ORF was defined as the average across the probes associated with the ORF. Quantile normalized datasets were clustered with a correlation similarity metric and the average linkage method using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The cluster was visualized using TreeView software (http://jtreeview.sourceforge.net/). The significance for differential expression was set as log2 (drug/DMSO) >1 and <−1, P-value <0.05 as determined by Student’s t test. Significantly up- and downregulated transcripts were further tested for Gene Ontology (GO) biological process enrichment using FunSpec (http://lunspec.med.utoronto.ca/) with P-value cutoff of 0.01 and multiple testing correction (Bonferroni) (Table S1; Dataset S6). The probability was calculated using a test employing hypergeometric distribution (see below). To detect cantharidin-specific genes the genes involved in ESR [36] were eliminated from the gene-set.

Expression and Purification of Crg1 Fusion Protein

Purification of 6xHis-Crg1 was performed as previously described [81]. Wild-type yeast strain Y258 carrying a vector pBG1805-GAL1-CRG1 with a triple affinity tag at C-terminal (His\textsubscript{6}-HA\textsubscript{1}-pIle\textsubscript{1}protease site-ZZprotein A) was grown in 660 mL of synthetically defined medium (SD-URA and 2% raffinose) to mid-exponential phase at 30°C. To induce expression of CRG1 340 mL of 3x YP (yeast extract and peptone) and 6% galactose was added to the final concentration of 2%. Cells were harvested by centrifugation at 3,000 rpm for 5 min. All steps following harvest were performed at 4°C. Cells were washed with PBS buffer, resuspended in 7 mL of resuspension buffer (20 mM HEPES pH 7.5, 1 M NaCl, 5% glycerol), and lysed using an acid washed Zirconia beads in the presence of protease inhibitors (1 mM Pefabloc, 2.5 μg/mL pepstatin A, 2.5 μg/mL leupeptin, 1 mM PMSF). The cell lysate was centrifuged at 20,000 rpm for 45 min, and diluted two fold with binding buffer (20 mM HEPES pH 7.5, 40 mM imidazole, 5% glycerol), 300 μL of Ni Sepharose 6 Fast Flow beads (50% slurry in 20% ethanol) was added to the sample and rotated for 1.5 hours, followed by three washes with 40 mL of wash buffer (20 mM HEPES pH 7.5, 40 mM imidazole, 5% glycerol, 0.5 M NaCl). To elute Crg1 the Ni beads were
resuspended in 1 mL of elution buffer (20 mM HEPES pH 7.5, 250 mM imidazole pH 7.7, 5% glycerol, 0.5 M NaCl), and Crg1p was released by rotating the mixture for 15 min at 4°C. The protein was further concentrated with Amicon Ultra tubes (10 K) (Millipore, Etobicoke, ON, Canada) to 100 µL.

Purification of TAP-tagged wild-type and mutant forms of Crg1 was performed in BY4743 carrying p426-GAL1-CRG1-TAP as described in Rigaut et al. [82]. Cell growth, induction of Crg1 with galactose (2%), and preparation of cell lysates were performed as described for the Crg1-6xHis fusion. 300 µL IgG-agarose was added to the extract and incubated for 2 hours followed by a triple wash with 25 mL of Low Salt and High Salt Wash Buffer (50 mM HEPES pH 7.5, 10% glycerol, 150 mM/750 mM NaCl, 0.1% Tween20). The final wash was performed with 15 mL TEV Cleavage Buffer (10 mM, Tris pH 8.0, 150 mM NaCl, 0.05% Tween20, 10% glycerol, 0.5 mM EDTA, 1 mM DTT). The extract was incubated overnight with 100 U TEV protease (Invitrogen, #12575-015). 1.2 mL CaM Binding Buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween20, 10% glycerol, 1 mM MgOAc, 1 mM imidazole pH 8.0, 2 mM CaCl2, 1 mM DTT) and 2.4 µL 1 M CaCl2 were added to the protein eluates. The eluates were then incubated with 400 µL (50% slurry) Calmodulin Sepharose in 5 mL CaM Binding Buffer for 2 hours, followed by a wash with 25 mL CaM Binding Buffer and elution with 5×200 µL Elution Buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween20, 10% glycerol, 1 mM MgOAc, 1 mM imidazole pH 8.0, 2 mM EGTA, 1 mM DTT). Protein eluates were stored at 0°C in the presence of 50% glycerol. Recovery of Crg1 was determined using Bradford reagent (BioRad Laboratories, Mississauga, ON, Canada) and its integrity and purity was assessed with 12% silver-stained SDS-PAGE gel.

**Preparation and Analysis of Radiolabeled *In Vitro* Crg1 Reactions**

*In vitro* enzymatic reactions were prepared with 0.09 µg 6xHis-tagged Crg1, 0.2 mM cantharidin dissolved in DMSO (Sigma Aldrich, St. Louis, MO, USA), and 20 µM S-adenosyl-[methyl-14]Cmethionine (55.8 mCi/mmole) (GE Healthcare, Piscataway, NJ, USA) in 0.1 M sodium phosphate, pH 7.4 with a final volume of 50 µL and 2% DMSO. The complete reactions and relevant controls were incubated at 30°C for 2 hours. Following incubation, the enzymatic reactions were separated by reverse-phase high-performance liquid chromatography (Series II 1090 Liquid Chromatograph, Hewlett Packard, Palo Alto, CA, USA). The chromatography gradient was adapted from [83], except that the flow rate was 1 mL/min. Mobile phase A contained 0.1% trifluoroacetic acid in water and mobile phase B was 0.1% trifluoroacetic acid in acetonitrile. A BetaBasic-18 column (250 mm×4.6 mm; 5-µm particle size) (Thermo, Waltham, MA) was used. 40 µL was injected, 2-minute fractions were collected, and 350 µL of each fraction was mixed with 5 mL of Safety-Solve (Research Products International, Mt. Prospect, IL, USA) before quantification of radioactivity with a LS6500 liquid scintillation counter (Beckman Coulter, La Brea, CA, USA). Each fraction was counted three times for 3 minutes.

Other *in vitro* reactions were prepared in an identical manner with 0.09 µg of either mutant or wild-type TAP-tagged Crg1 and varying concentrations of cantharidin (USB, Cleveland, OH, USA). After incubation at 30°C for 2 hours, 40 µL 2 N HCl was added to each 50 µL reaction. Immediately, 80 µL of this mixture was transferred to a 1.9-cm×9-cm folded piece of filter paper in the neck of a scintillation vial containing 5 mL of Safety-Solve and the vials were capped. After 4 hour incubation at room temperature, the pieces of filter paper were removed from the neck of each vial and the acid-labile volatile radioactivity was quantified with a liquid scintillation counter as described above [84].

**Preparation of *In Vitro* Enzymatic Reactions for Analysis by Mass Spectrometry**

*In vitro* enzymatic reactions using unlabeled SAM (Sigma Aldrich) were prepared in a similar manner with 0.09 µg of 6xHis-tagged Crg1, 200 µM cantharidin (Sigma Aldrich), and a SAM concentration of 1.6 mM. These reactions were quenched with addition of 200 µL acetonitrile, and 12.5 µL of 15% ammonium bicarbonate was added to reduce product degradation. After concentration with a vacuum centrifuge and resuspension in 50 µL of water, these reaction mixtures were analyzed by liquid chromatography-tandem mass spectrometry (1100 Series Liquid Chromatograph, Agilent, Santa Clara, CA; QSTAR Elite Mass Spectrometer, Applied Biosystems, Foster City, CA) in positive ionization mode with a Turbo Spray source. 8 µL of sample was injected onto a reverse-phase column (Luna C18 (2), 150 mm×1 mm, 5-µm particle size, Phenomenex, Aschaffenburg, Germany) with a flow rate of 50 µL/min and the following gradient: t = 0–1 min, 2% mobile phase B; t = 10 min, 35% B; t = 14–16 min, 90% B; t = 16.5–35 min, 2% B. Mobile phase A was 0.1% formic acid in 98% water and 2% acetonitrile, while mobile phase B was 0.1% formic acid in 98% acetonitrile and 2% water. Spectra were collected with the instrument information-dependent acquisition mode (full scan: m/z = 70–600, 1,000,007 s; 5 product experiments: m/z = 65–500, <2 s per experiment). The following mass spectrometer parameters were used: declustering potential, 85 V; focusing potential, 300 V; declustering potential II, 15 V; ionspray voltage, 5500 V; ion source gas II, 5 units; curtain gas, 45 units; collision gas, 7 units.

**Fitness Profiling of Double Deletion Mutants with Cantharidin**

A pool of double deletion mutants (crg1ΔΔxxΔ) was prepared by generating viable double deletion mutants using Synthetic Genetic Array (SGA) technology with crg1 as a query strain [40] (Text S1). ~4800 viable double deletion mutant colonies were collected, normalized to 50 OD's/mL and stored at ~80°C in media containing 7% DMSO. Two independent pools were generated for the analysis. Each was tested in triplicate. The pooling of the strains was possible due to the presence of strain-specific sequence tags flanking each gene deletion region [3]. The double deletion pool was treated with 30 µM of cantharidin, a dose which inhibits growth of the crg1 double deletion pool by ~20%. Fitness analysis using a tag-specific algorithm that takes into account the intensities of each tag in cantharidin-treated cells compared to non-treated cells was performed as described [41]. Hybridization to Affymetrix Gene Chips using GeneChip Fluidics Station 450 (Affymetrix) was followed by the extraction of intensity values for the probes using the GeneChip Operating Software (Affymetrix). The data was quantile normalized, outliers (one standard deviation off) were omitted, and fitness defect scores as the log2 ratio between the mean signal intensities of the control (DMSO) and the drug were calculated for each deletion strain in the pool as previously described [41]. As a control, the relative fitness of the double-gene deletion mutants exhibiting high sensitivity to cantharidin (log2 (drug/DMSO) < -1; P-value <0.05) was compared to the relative fitness of the corresponding single gene deletion mutants. For a given double deletion mutants, the resulting interaction was evaluated using comparison of the observed double mutant growth rate to the expected assuming that there is no interaction exists.
Log2 ratio $< -1$ represents those strains with a measurable growth defect (or lethality) and log2 ratio $> 1$ demonstrates resistance to the drug. Cantharidin-specific CRG1 negative genetic interactors were further verified as individual clones in liquid growth assays and/or by spot dilutions. To evaluate the chemogenomic dataset for statistical significance enrichment for general biological processes (Gene Ontology Slim mapper), we used a standard hypergeometric test, that assesses the probability that the intersection of given list with any given functional category occurs by chance. Obtained $P$-values were corrected for multiple testing correction (Bonferroni) by multiplying $P$-values with the number of genes in the test. The probability was calculated as follows: the $P$-value of observing $x$ genes, belonging to the same functional category, is:

$$P = \frac{\max(M, n)}{\sum_{i=x}^{\min(M, n)} \frac{M!}{i!} \frac{(N - M)!}{(n - i)!} \frac{N!}{n!}}$$

where $M$ is the total number of genes involved in a functional category, $n$ is the total number of genes in the cluster, and $N$ is the total number of yeast ORFs.

**Lipidome Analysis by Liquid Chromatography-Tandem Mass Spectrometry**

Cells were grown to mid-exponential phase and treated with cantharidin (250 μM) for 2 hours. Lipids were extracted from 25 OD600-equivalent of cells and analyzed as described [43]. All lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA), with the exception of dioctanoyl glycerophosphoethanolamine, which was obtained from Echelon Biosciences (Salt Lake City, UT, USA). Quantification of individual molecular species was carried out using multiple reaction monitoring (MRM) with an Applied Biosystems 4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA, USA). 25 μl of samples were subjected to analysis as described previously [43,85]. Lipid levels in each sample were normalized to internal standards. For each lipid species, the mean normalized signal from the wild type and mutant strains grown in the presence or absence of cantharidin were calculated. Three independent experiments were used for analysis. Lipid levels were calculated relative to relevant internal standards. The quantities of lipids are expressed as ion intensities relative to the levels without cantharidin, and then converted to a log2 (drug/DMSO) scale. The difference in levels of individual lipid species in DMSO vs. cantharidin was determined with Kruskal-Wallis test (Table S4). Similarly, the difference between wild type and mutants was assessed statistically using the Kruskal-Wallis test, with a $P$-value cutoff of 0.05.

**Actin Staining**

Cells were grown to mid-exponential phase in YPD media, and treated with and without cantharidin (250 μM) for 1 hour. Cells were then fixed by addition methanol-free formaldehyde (Polysciences, Warrington, PA) to 4% for 1 hour, centrifuged at 3,000 rpm 5 min and washed with PBS buffer three times. Cells were permeabilized with 0.2% Triton X-100 in PBS at 25°C for 15 min, washed with PBS three times and a normalized number of cells were stained with Alexa Fluor 488 phalloidin (Invitrogen, Burlington, ON) in the dark at 25°C for 1 hour. Cells were observed with 100× objective, and fluorescence images were acquired using AxioVision software on an Axiovert 200 M fluorescence microscope (Carl Zeiss) using a 1.5 s exposure for all images. The average number of actin patches per cell was determined by dividing the total number of actin patches per total number of cells ($N = 3, n \geq 300$ cells).

**Supporting Information**

**Dataset S1** Chemical SGA. Fitness profiling of wild-type double deletion mutants (uraAΔhexxΔ) with cantharidin. (XLS)

**Dataset S2** Chemical SGA. Fitness profiling of crg1 double deletion mutants (crg1ΔhexxΔ) with cantharidin. (XLS)

**Dataset S3** Glycerophospholipid and sphingolipid analysis by liquid chromatography-tandem mass spectrometry in S. cerevisiae. (XLS)

**Dataset S4** Glycerophospholipid and sphingolipid analysis by liquid chromatography-tandem mass spectrometry in C. albicans. (XLS)

**Dataset S5** SGA scores for crg1 double deletion mutants in the absence of cantharidin. (XLS)

**Dataset S6** Transcriptome profiling of crg1 mutants upon exposure to cantharidin treatment (250 μM for 1 hour). (XLS)

**Figure S1** (Related to Figure 1.) (A) CRG1 gene dose is important for cantharidin tolerance. Wt, crg1A/crg1A heterozygous, crg1A/ΔA homozygous and CRG1-overexpressing crg1A/ΔA mutants were assessed in the presence of cantharidin in YPD. Growth curves were obtained by plotting OD600 vs. time at the tested concentrations of cantharidin. At least three independent replicates were analyzed and the representative growth curves are shown. (B) Cantharidin is more potent in SD media than in YPD. Wt, crg1A/ΔA and CRG1-overexpressing crg1A/ΔA mutants were assessed in the presence of cantharidin in SD medium. Dose-response curves were obtained by plotting OD600 at saturation point vs. tested drug concentrations. Values are means of three independent replicates, and error bars represent standard deviation. (C) Crg1 is important for resistance to the cantharidin analogues cantharidic acid and norcantharidin. Growth of wt, crg1A/ΔA and crg1A/ΔA cells overexpressing CRG1 was assessed in the presence of cantharidin analogues in YPD. Dose-response curves were obtained by plotting OD600 at saturation vs. drug concentration. Values are the mean of three independent replicates, and error bars represent the standard deviation. (PDF)

**Figure S2** (Related to Figure 1.) (A) Cantharidin induces CRG1 transcription in a gene-dose dependent manner. Wt, crg1A/crg1A heterozygous, crg1A/ΔA homozygous deletion mutants and CRG1-overexpressing crg1A/ΔA mutant grown to mid-exponential phase were incubated with or without cantharidin (250 μM) for 1 hour. Total RNA was extracted, cDNA synthesized and the relative abundance of CRG1 transcript was analyzed by qRT-PCR. Data are the mean of at least three independent experimental replicates, and error bars are the standard deviation. (B) GFP-tagged Crg1 is localized to the cytosol after 1 hour treatment with cantharidin (low fluorescence medium at 4 μM). Bar, 2.5 μm (see Text S1). (C) Chemical inhibition of protein phosphatases with calyculin A results in the transcriptional induction of CRG1. Wt cells grown to mid-exponential phase in YPD were treated with calyculin A (2 μM) for 30, 60 and 120 min. Total RNA was extracted, cDNA synthesized, and the relative abundance of CRG1 transcript was analyzed by qRT-PCR. Data represent the mean of at least three
independent experimental replicates, and error bars are the standard deviation. (D) CRG1 is a stress-responsive methyltransferase. Expression profile of CRG1 was compared with other yeast genes during 174 diverse environmental stresses [36]. Expression profiles of CRG1, ACT1 and SSE2 are shown. (E) Crg1 protein level was not altered by mutations in methyltransferase domain. Cells were collected after induction with galactose (2%) for 3 hours. The cell lysates were analyzed by western blotting with anti-TAP antibody. Tubulin was used as an internal loading control and detected by an anti-tubulin antibody.

Figure S3 (Related to Figure 1.) (A) Viability of crg1 mutants treated with cantharidin for 1 hour. Wt, crg1A/CRG1 heterozygous, orf19.633 heterozygous deletion mutants and CRG1-overexpressing orf19.633 mutants grown to mid-exponential phase were incubated with or without cantharidin (30 μM and 250 μM), the IC20 for crg1A/ΔA and the IC20 for wt, respectively for 1 hour. Cells were normalized to an equivalent OD600, 10-fold diluted, spotted onto YPD solid medium and incubated at 30°C. (B) The comparison of GO term Biological processes for the genes that are upregulated and downregulated (log2 ratio >1 and <−1) in a crg1A/ΔA mutant treated with cantharidin (30 μM and 250 μM) for 1 hour. (C) Heat map of the transcriptional profiles of wt, crg1A/ΔA and CRG1-overexpressing orf19.633 mutants in response to cantharidin were compared. (D) Hierarchical clustering was used to group all significantly expressed genes (at least two fold) in at least one of the strains in the presence of cantharidin (250 μM). Clusters of genes exhibiting highly similar profiles across the strains are boxed. The overrepresentation of GO Biological processes in these gene clusters are indicated on the right. (E) Cantharidin significantly induces STR3 transcript levels in CRG1-overexpressing orf19.633A mutant. Wt, orf19.633 and CRG1 heterozygous, orf19.633A homozygous deletion mutants and CRG1-overexpressing orf19.633A mutants grown to mid-exponential phase were incubated with or without cantharidin (250 μM) for 1 hour. Total RNA was extracted, cDNA synthesized and the relative abundance of STR3 transcript was analyzed by qRT-PCR. Data are the mean of at least three independent experimental replicates, and error bars are the standard deviation. (F) Treatment of cells with the non-specific methyltransferase inhibitor SAH increases sensitivity to cantharidin. Wt cells were grown in SC media with or without cantharidin (4 μM) and SAH (50 μM).

Figure S4 (Related to Figure 3.) (A) Silver stained 12% SDS-PAGE of purified TAP-tagged wild-type and mutated Crg1 (see Materials and Methods for details). (B,E) Single ion chromatograms of the major species identified in the spectra shown in Figure 3.

Figure S5 (Related to Figure 4.) (A) Two independent replicates for crg1A/xxxA pools are highly correlated. Only strains with significant log2 (drug/DMSO) (P-value <0.05) are included in the analysis. (B) The deletion of DBF2 suppresses CRG1 sensitivity to cantharidin. Cells were normalized to an equivalent OD600,10-fold diluted, spotted onto synthetic complete medium containing cantharidin (10 μM) and incubated at 30°C. (C) CRG1-dependent changes of DBF2 transcript levels in the presence of cantharidin. Wt, crg1A/CRG1 heterozygous, crg1A/ΔA homozygous deletion mutants and CRG1-overexpressing orf19.633A mutants grown to mid-exponential phase were incubated with or without cantharidin (250 μM) for 1 hour. Total RNA was extracted, cDNA synthesized and the relative abundance of DBF2 transcript was analyzed by qRT-PCR. Data are the mean of at least three independent experimental replicates, and error bars are the standard deviation.

Figure S6 (Related to Figure 5.) (A) GO term enrichment analysis of cantharidin-specific CRG1-interactors before and after MDR gene filtering. Only the terms with significant FDR-corrected P-values (<0.05) are shown. Bonferroni correction was applied to the MDR-filtered terms. (B) Representative images of cells stained with Nile Red for lipid droplets. Cells were grown at 30°C for 42 hours to reach stationary phase, and inoculated into fresh medium with cantharidin (250 μM) for 2 hours. Cells were fixed and stained with Nile Red. Bar, 2.5 μm. (C) Sterol species are not affected by the deletion of CRG1 and cantharidin treatment (see Materials and Methods for details).

Figure S7 (Related to Figure 5.) The C. albicans functional homolog of CRG1, orf19.633, has a role in lipid homeostasis. (A) orf19.633, a putative methyltransferase, is required for cantharidin resistance. Fitness of wt (SN87) and orf19.633A/ΔA mutant were measured in liquid YPD medium in the presence of cantharidin (2 mM, the IC20 for wt). Dose-response growth curves were obtained by plotting OD600 at saturation point in liquid versus tested drug concentrations. (B) Comparative lipidomics of cantharidin-treated C. albicans wild type and orf19.633A/ΔA mutant. The cells were treated with 2 mM cantharidin (2 hours) and further prepared as described in Figure 5. Only those lipid species with significant changes in their abundance between wt and orf19.633A/ΔA mutant are shown (P-value <0.05, Student’s t-test). (C) PI species are significantly affected by cantharidin in Crg1l-dependent manner. Cells were prepared as described in Figure 5. Only those PI species with significant changes in their abundance between wt and orf19.633A/ΔA mutant are shown (P-value <0.05, Student’s t-test). The PI content of CRG1-overexpressing mutant is not significantly different form the one in wild-type strain (P-value >0.05).

Figure S8 (Related to Figure 5.) The genetic interactome of CRG1 reveals functional networks required for buffering the absence of CRG1 in standard laboratory condition. (A) Genetic interactors of CRG1 identified through SGA analysis. The double deletion mutants orf19.633A were generated by SGA as previously described [86]. The double and single mutant fitness (based on colony sizes) from two independent SGA screens were used to quantify the strength of genetic interaction between CRG1 and other gene. Quantification was performed using the quantitative SGA scoring algorithm as described in [87]. The significant genetic interactors (P-value <0.05) common between two SGA screens were considered as hits. The mutants with SGA score >0.08 are significant positive or alleviating interactors, the ones with <0.08 are significant negative or aggravating interactors. (B) Deletion of CRG1 suppresses fitness defect of orf19.633A mutant. Double and single mutants were grown in synthetic complete (SC) medium to the saturation. ura3Δ his3Δ is used as the control strain.

Figure S9 (Related to Figure 6.) (A) Transcriptional activator Yap1 is not required to activate CRG1 transcription during cantharidin stress. Sample treatment and qRT-PCR analysis were performed as described in Figure 1B. (B) CRG1 is transcriptionally activated by cell wall perturbing agents, however, it is not required for the growth in their presence. qRT-PCR analysis was performed as described in Figure 1B. Cells normalized to equal OD600 were 10-fold diluted, spotted onto solid YPD medium containing various cell wall and membrane perturbing agents, and incubated at 30°C for 2–3 days. (C) Overexpression of CRG1 confers resistance to fenpropimorph. Mid-exponentially grown cells were normalized to equal OD600 were 10-fold diluted, spotted
onto solid YPD medium containing fenpropimorph (250 μM), and incubated at 30°C for 2–3 days.

**Table S1** Significantly enriched (P-value <1.0×10^{-5}) Gene Ontology Biological processes for significantly up- and downregulated genes (log2 >1 and <-1) in the presence of cantharidin.

**Table S2** Differentially expressed genes of methionine biosynthesis in crg1 mutants under cantharidin stress.

**Table S3** Cantharidin-specific genetic interactors of CRG1.

**Table S4** Statistical analysis (Kruskal-Wallis test) of lipid abundance in crg1 mutants exposed to cantharidin.

**Table S5** Yeast strains used in this study.

**Table S6** Plasmids used in this study.

**Table S7** Primers used in this study.

**Text S1** Supporting Materials and Methods.

**Acknowledgments**

We thank Kyle Tsui (University of Toronto) for assistance with microarray analysis, Cheuk Hei Ho (University of Toronto) for providing plasmid YEp351-CRG1, Mariemna Gobchia (University of Toronto) for experimental assistance, Larry Heisler and Anu Surendra (University of Toronto) for computational analysis, and Ilia Tikhomirov for critical reading and discussion of the manuscript. We are also grateful to Michael Jung and Kym Faulk (UCLA) for their advice on cantharidin chemistry. Finally, we thank Gregg Czerwieniec at the Molecular Instrumentation Center at UCLA for his help with mass spectrometry. **Author Contributions**

Conceived and designed the experiments: EL BY JL MLU SGC GG CN. Performed the experiments: EL BY XLG IR. Analyzed the data: EL BY XLG HR GG CN. Contributed reagents/materials/analysis tools: MLU AB JL SH MM MRW HR LEC. Wrote the paper: EL GG CN.

**References**


