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Cohesins coordinate gene transcriptions of related function within *Saccharomyces cerevisiae*

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Cohesion factors pair together sister chromatids from early S-phase until anaphase onset. Numerous findings also establish an additional role in transcription. In humans, mutations in cohesion factors result in developmental abnormalities such as Cornelia de Lange, Roberts Syndrome/SC-Phocomelia, Rothman-Thompson Syndrome and others. While clinically relevant, a detailed study that links experimentally-defined cohesin defects to transcriptional changes remains lacking. Here, we report on the effects of cohesin inactivation during an early and discrete portion of the cell cycle. Even transient cohesin inactivation in α-factor arrested cells to target the G_1 portion of the cell cycle results in significant and reproducible changes in transcription. Surprisingly, over a third of the affected genes exhibit inter-related functions, suggesting that cohesin positioning along chromosomes evolved to coordinate gene expression. Prior studies indicate that defects in rRNA maturation/ribosome biogenesis produce developmental maladies in humans. Thus, the identification of genes critical for rRNA maturation in this study is of particular interest.

Introduction

Images of dividing cells have driven researchers to focus on the mechanisms of chromosome segregation for well over a century. One current endeavor revolves around a fundamental facet of segregation termed sister chromatid cohesion. In budding yeast, cohesion requires the temporal activities of deposition factors (Scc2 and Scc4) that load cohesin complexes (Mcd1/Scc1, Smc1, Smc3 and Scc3/Irr1) onto chromatin. Subsequent conversion of cohesin-decorated sister chromatids to a paired state, termed establishment, requires the acetyltransferase Ctf7/Eco1.[1] During S-phase, Ctf7/Eco1 acetylates Smc3, which in turn counteracts the anti-establishment activity of Pds5 and Rad61/WAPL.[2-6] Ctf7/Eco1 can also become activated during G_2/M in response to DNA damage. In this case, Ctf7/Eco1 acetylates Mcd1/Scc1 to counteract anti-establishment factors.[7-10] Regardless of the cell cycle stage, cohesin modification is essential for conversion to a pairing-competent state. Currently, the structure that tethers together sister chromatids remains unresolved, but popular models include a double ring handcuff, a single ring lasso and others.[11-13]

While the first decade of cohesion research focused almost exclusively on chromosome segregation, early characterization of Drosophila Nipped B (homolog of yeast Scc2) presaged the now burgeoning field of cohesin-dependent gene expression regulation.[14,15] Recent findings obtained in fruit flies, fission yeast and vertebrate cell lines confirm the role of cohesins in long-range transcription regulation. In Drosophila, cohesion pathways are critical for eye and wing development, segment identity and axonal pruning of mushroom body neurons.[15] In budding yeast, cohesion factors appear to delineate both active euchromatin and silenced heterochromatin.[16] The interplay between cohesion and silencing is reciprocated in that cells mutated in particular silencing factors exhibit cohesion defects and that transcription can drive cohesin displacement/relocation.[17-19] In fission yeast, cohesins determine transcript termination, possibly by blocking Pol II migration. Thus, cell cycle differences in cohesin residence are posited to generate RNA run-ons that alter gene expression.[20] In vertebrate cells, cohesins target heterochromatic regions first occupied by CTCF—a transcription insulator and regulator of numerous loci that promotes immunofunction and hematopoiesis.[21-27]

Understanding cohesin function in transcription regulation is likely to be of clinical importance given that mutations in cohesion pathways directly result in developmental abnormalities that include Cornelia de Lange Syndrome, Roberts Syndrome/SC-Phocomelia, Rothman-Thompson Syndrome and others.[1,16,28,29] Importantly, cells from afflicted patients can contain normally paired sister chromatids—functionally separating cohesin roles in pairing and transcription. At present, little is known regarding how cohesins regulate transcription. Questions at the forefront of this issue include (1) is transient or extended cohesin inactivation required to alter gene transcription, (2) does cohesin inactivation randomly target genes or are specific gene subsets effected, (3) does cohesin inactivation affect a unique locus or spread to adjacent loci and (4) does cohesin inactivation during a discrete and early portion of the cell cycle affect genes normally transcribed later in the cell cycle? Here, we report for

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Table 1. Tabulation of all 29 loci (YGR272 combined in with YGR271-C) whose expression is altered in response to transient cohesin inactivation

Common		Gene	
YNL141W	AAH1	YBR294W	SUL1
YJL200C	ACO2	YCR061W	ORF:YCR061W
YDR448W	ADA2	YDL037C	BSC1
YJR047C	ANB1	YDL182W	LYS20
YOL058W	ARG1	YDR021W	FAL1
YJL088W	ARG3	**YDR445C**	ORF:YDR445C
YDL037C	BSC1	**YDR446W**	ECM11
YJR150C	DAN1	**YDR448W**	ADA2
YHR144C	DCD1	YEL065W	SIT1
YLR348C	DIC1	YER124C	DSE1
YER124C	DSE1	YGR271C-A	EFG1
YHR143W	DSE2	YHL023C	RMD11
YDR446W	ECM11	**YHR143W**	DSE2
YGR271C-A	EFG1	**YHR144C**	DCD1
YDR021W	FAL1	YIR020W-B	ORF:YIR020W-B
YLR068W	FYV7	YJL088W	ARG3
YPL017C	IRC15	YJL200C	ACO2
YOR084W	LPX1	YJR047C	ANB1
YDL182W	LYS20	YJR150C	DAN1
YNR050C	LYS9	YLR068W	FYV7
YPR167C	MET16	YLR348C	DIC1
YHL023C	RMD11	YNL141W	AAH1
YEL065W	SIT1	YNR050C	LYS9
YBR294W	SUL1	YOL058W	ARG1
YOR081C	TGL5/STC2	**YOR081C**	TGL5/STC2
YOR083W	WHI5	**YOR083W**	WHI5
YCR061W	YCR061W	**YOR084W**	LPX1
YDR445C	YDR445C	YPL017C	IRC15
YIR020W-B	YIR020W-B	YPR167C	MET16

Left: Loci listed in alphabetical order based on common name. Right: Loci listed in positional context based on gene name.

the first time the identity of those genes whose regulations are effected by transient and cell cycle specific cohesin inactivation.

Results

Transient cohesin inactivation during G_1 alters gene expression. Mcd1/Scc1 proteolysis is promoted by Polo/Cdc5-dependent phosphorylation and requires Mcd1/Scc1 chromatin-association. However, a significant fraction of mitotic cohesin is soluble. This pool is largely refractile to both phosphorylation and proteolysis and thus is thought to persist into G_1, albeit at low levels.[30-35] Currently, little is known regarding the cohesin subsets (G_1, S and G_2/M) that participate in transcription regulation.[1,20-27] Given the likely transcriptional-based role of cohesins in numerous human developmental abnormalities,[1,28] it becomes clinically relevant to address this issue. Cohesin inactivation during a discrete portion of the G_1 portion of the cell cycle presented many advantages. First, G_1-deposited cohesin occurs prior to sister chromatid pairing, providing a mechanism to inactivate cohesins without affecting either sister chromatid pairing or cell viability. Second, since only a relatively minor cohesin pool may persist into G_1, inactivation of this limited cohesin subset might identify an equally discrete subset of genes whose expression is cohesin-dependent.

To accomplish this, we exploited conditional *mcd1-1* mutant yeast strains to inactivate cohesins specifically during late G_1. Mcd1/Scc1 is a logical choice because it is the most thoroughly characterized cohesin integral to sister chromatid pairing. Briefly, log growth wildtype *MCD1* or temperature-sensitive *mcd1-1* mutant yeast cells were grown at the permissive temperature of 23°C and then synchronized in G_1 using alpha factor. The resulting G_1-synchronized cultures were then shifted to 37°C for 1 hour to inactivate mutant mcd1-1 protein, a regimen well documented as effecting neither cell viability nor sister chromatid pairing.[36-38] To assess the transcriptional profile of each gene affected by this regimen, RNA was extracted from G_1-synchronized and temperature shifted wildtype and *mcd1* mutant cultures, hybridized and analyzed by microarray. We performed this experiment three separate times, in each case identifying genome-wide changes by microarray hybridization for each set of wildtype and mutant cultures. We limited our analyses to those genes whose expression was either increased or decreased 1.5 fold or greater, relative to wildtype cells (**Suppl. Table 1**). Out of this data set, Venn diagram analyses identified with high confidence 30 loci that exhibit highly reproducible and significant changes common to all data sets (**Table 1, Fig. 1**). One open reading frame (YGR272C) was later determined to be a part of YGR271C-A (*EFG1*), reducing to 29 the number of genes common to all data sets. This observation attests to the validity of the approach (**Table 1**). In combination, the results show that even transient cohesin inactivation, and one targeting a portion of the cell cycle that is prior to sister chromatid pairing, is sufficient to significantly alter transcriptional profiles of many genes.

Cohesins coordinate the expression of genes of inter-related functions. A robust body of literature reveals that cohesins provide important regulatory functions for a wide array of genes and pathways.[22-24,26,39] Based on this, we expected that cohesin inactivation would deregulate a highly diverse set of genes and biochemical pathways. Surprisingly, the gene sets obtained from Venn analyses reveal instead that cohesins target genes of highly related function. For instance, genes within biosynthetic pathways for lysine (*LYS9* and *LYS20*) or arginine (*ARG1* and *ARG3*), cell wall metabolism (*DAN1, DSE1* and *DSE2*) or 18s rRNA maturation (*FAL1, FYV7* and *EFG1*) are coordinately effected by transient inactivation of cohesin in α-factor arrested cultures. In fact, more than 30% of the genes identified in this screen participate within related pathways. We decided to test for alternate mechanisms through which these loci might be targeted. In each case, we were able to refute these models. For instance, cohesin targeting was not limited to genes that are expressed similarly through the cell cycle (**Table 2** and see below). Nor is cohesin targeting proximity-dependent. In fact, genes of inter-related functions

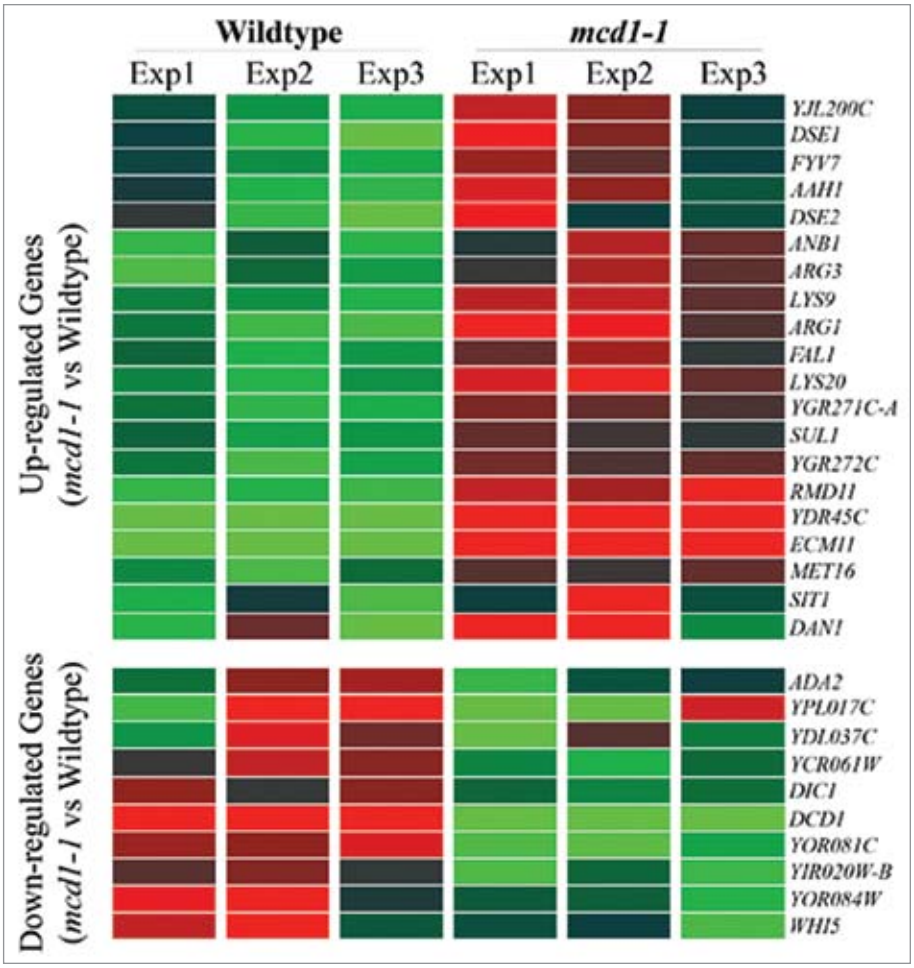

Figure 1. Heat map of all 30 loci identified by microarray as either upregulated (top) or downregulated (bottom) in response to transient cohesin inactivation. Pooled and common results for each of the three independent experiments are shown.

identified in this screen (*ARG1* and *ARG3; LYS9* and *LYS20; DNA1, DSE1* and *DSE2*) are located on separate chromosomes and on different DNA strands (**Table 1**). In combination, these findings suggest that cohesin positioning along the chromosome length evolved to regulate multiple genes within a given cellular pathway to provide concerted and coordinated regulation.

To regulate expression of genes of related function, cohesins could either bind directly to specific DNA sequences or become recruited by either early boundary element binding subunits or transcription co-factors. To pursue this possibility, we tested whether loci regulated by cohesins might contain a common motif within or distal from the open reading frame. Pair-wise alignment of DNA sequences, including 1,000 nucleotides of flanking sequence both up- and down-stream of the open reading frame, were performed for genes of related functions (*LYS9* and *LYS20*) using the multiple sequence alignment program ClustalW2 provided by EMBL (www.ebi.ac.uk/Tools). A multitude of short regions of nucleotide identity were obtained, suggesting that this search offered insufficient specificity to uncover a common binding motif (data not shown). We note that none of these short consensus sequences contain a canonical CTCF-like binding motif.[23] We expanded our search to include the remaining gene sets of inter-related functions (*LYS9, LYS20; ARG1, ARG3; DAN1, DSE1, DSE2; FAL1, FYV7* and *EFG1*). However, computer-assisted search methods failed to identify any sequence motif. We note that prior efforts similarly suggest that cohesins do not bind specific DNA sequence motifs.[40-42] In combination, these results suggest that if cohesins indeed evolved to coordinate expressions of inter-related genes, then their recruitment requires early-binding boundary element subunits or transcription factors analogous but not identical to cohesin-recruitment factors human CTCF and zebrafish RUNX.[20-26]

Cohesin inactivation alters the expression of positionally clustered genes. To date, analyses of cohesin-dependent transcription identify limited spatial effects such that individual loci are regulated—possibly through enhancer access.[22-24,26] Our data set provides a new resource from which we could test whether cohesin inactivation targets individual loci or if deregulation would spread to neighboring genes. Given the extent of our data set (29 of ~6,300 yeast genes), the odds of finding immediately apposed cohesin-regulated genes are extremely low—much less than 1 in 200 or 0.5%. In contrast to this expected outcome, the results show that a full 20% of the identified genes (6 of 29) are immediately adjacent to one another. When we include in our

Table 2. Loci affected by cohesin inactivation clustered according to expression profile through cell cycle in unchallenged cells

Non-periodic	0–35%	36–66%	67–100%
YJL200C	YER124C (17%)	YNL141W (57%)	YNR050C (69%)
YLR068W	YHR143W (17%)	YPR167C (58%)	**YDL037C (88%)**
YJR047C	**YOR084W (33%)**	YEL065W (65%)	
YOL058W	**YOR083W (35%)**		
YJO088W			
YDL182W			
YDR021W			
YFR150C			
YDR446W			
YDR445C			
YBR294W			
YHL023C			
YDR448W			
YPL017C			
YCR061W			
YLR348C			
YHR144C			
YOR081C			

Red indicates downregulation; Green indicates upregulation.

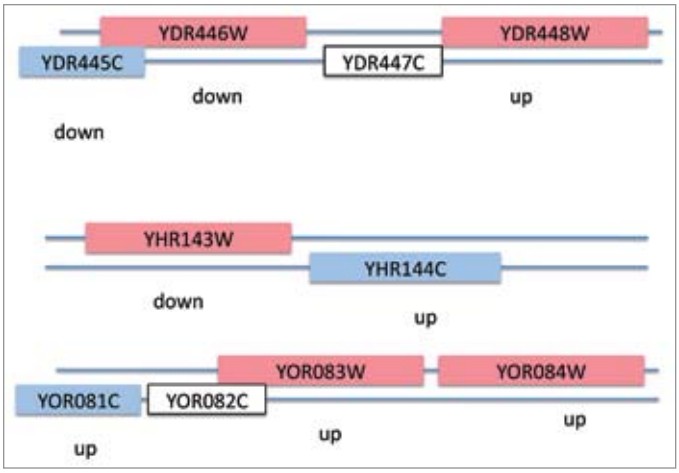

Figure 2. Schematic indicates affected loci, relative to DNA strand, and resulting change in transcription (up vs. downregulation) upon transient cohesin inactivation.

analyses affected genes separated by a single loci via single gap analysis,[43] the incidence of co-regulated adjacent genes rises to 25%—with some instances in which three contiguous loci are affected by cohesin inactivation (**Table 2**). Strand bias does not account for this extension of deregulation. Nor do the results reveal that gene expressions within a domain are altered in a similar fashion: downregulation occurs immediately adjacent to upregulation (**Fig. 2**). In summary, these results provide new evidence that cohesins can elicit changes in expression and that this deregulation can spread to neighboring domains.

Cohesin inactivation in G_1 synchronized cultures deregulates genes specific to other cell cycle stages. How does cohesin inactivation targeting G_1 arrested cultures effect transcription of genes typically not expressed during G_1? Of the 29 genes deregulated by cohesin inactivation, expression profiles for 27 are readily available. We assayed the transcriptional profiles for each of these genes in unperturbed cells to determine in which portion of the cell cycle each is typically expressed.[44-46] Of these 27 genes deregulated by transient cohesin inactivation during G_1, 4 are typically expressed in G_1 synchronized cells the first third of the cell cycle (**Table 2**). Thus, loci expressed during G_1 are regulated by cohesins that remain after anaphase onset. Future studies are required to determine the extent that this G_1 cohesin pool directly regulates gene transcription or alters expression through an indirect mechanism.

Our results further reveal that cohesin inactivation in G_1 synchronized cells profoundly effect transcription of genes typically expressed in other phases of the cell cycle (**Table 2**). For instance, over a fifth (5 of 27 genes) of loci affected by transient and G_1-targeted cohesin inactivation normally exhibit transcriptional peaks in S-phase or as late as G_2/M. The data further suggests that G_1 cohesins are important regulators of different S-phase and G_2/M genes (**Table 2**). In combination, these results document that cohesins participate in transcription regulation of genes throughout the cell cycle. Genes within the largest class (18 of 27) of cohesin-dependent transcription typically exhibit constitutive (non-periodic) expression (**Table 2**).[44-46] Further research will be required to test whether cohesin-inactivation in other portions of the cell cycle would similarly effect expression of non-periodically expressed genes or if later expression can compensate for gene deregulation during G_1.

Discussion

One caveat of this study is that we do not directly measure cohesin levels in G_1 synchronized cells. Instead, we rely on numerous findings that a significant cohesin fraction is soluble and resistant to proteolysis at anaphase. Thus, and despite the efficiency of our synchronization regimen, cohesin inactivation might have occurred in a small fraction of non-arrested cells. With this caveat in mind, our findings reveal for the first time that even transient alterations in cohesin function are sufficient to elicit significant and reproducible changes in transcriptional control. Of particular interest is the cohesin-dependent deregulation of multiple factors required for rRNA maturation and ribosome biogenesis. In humans, the importance of rRNA processing and ribosome biogenesis in embryonic development is readily evident. Treacher Collins syndrome phenotypes range from mild (external ear and auditory canal deformities) to severe (lethality ensuring from airway failure) and arise from mutations in Treacle (TCOF1). Treacle is a nucleolar phosphoprotein required by pre-rRNA processing proteins and for transcription of rDNA genes.[47] Compounding this effect is that depletion of ribosomal proteins is "contagious"—resulting in decreased levels of associated ribosomal subunits and loss of ribosome maturation.[48] Diamond Blackfan anemia phenotypes include a bone marrow failure that results in reduced red blood cell production and a

predispostion to cancer.[49,50] Diamond blackfan anemia also arises from mutations in ribosomal proteins. Our finding that transient inactivation of the G_1 cohesin pool deregulates numerous rRNA maturation factors likely provides invaluable new insight into the molecular basis of human developmental disorders such as Cornelia de Lange syndrome, Roberts syndrome/SC-phocomelia and α-Thalassemia/mental retardation X linked. All of these maladies directly result from mutations within selected cohesion genes (NIPLB/Scc2, SMC1A, SMC3, PDS5, EFO2/ESCO2/Ctf7).[28,51-54] In combination, these findings suggest the importance of testing such afflicted individuals for defects in either rRNA maturation or ribosome biogenesis—efforts that may provide a unifying molecular basis for the diverse array of developmental abnormalities. More recent findings provide a conceptual link between cohesion and BRCA1 function: BRCA1 expression is conditionally lethal in cohesion genes and other factors central to chromosome segregation.[43,55,56]

A priori, the cohesin structure that couples together sister chromatids is likely to be the same as the one that participates in transcription regulation. Increasingly, however, growing evidence indicates that these cohesin functions—and thus structures—are separable.[1] For instance, prolonged RNAi knockdown of cohesin in post-mitotic vertebrate cells is sufficient to alter gene transcription, even though post-mitotic cells are unlikely to require sister chromatid pairing activity.[20-26] Moreover, inactivation meant to target the soluble cohesin pool that persists into G_1 from anaphase onset is sufficient to alter gene transcription even though this cohesin subset is neither essential for cell viability nor functions in sister chromatid pairing reactions.[30-32,34,36-38,57] The notion that the cohesin structures required to regulate transcription and effect chromosome segregation are separable is immensely intriguing and amplifies the fact that the structures that tether together sister chromatids remain quite enigmatic with models that range from a two-ring handcuff, single ring hula-hoop and a host of other cohesin subunit configurations.[1,12]

In vertebrate cells, cohesins require transcriptional insulators human CTCF or zebrafish Runx1 for target-specificity and recruitment. CTCF binds 1,000s of sites within vertebrate genomes such that mutations in CTCF (or Runx1) generate a diverse array of developmental phenotypes.[23-27] Results from the current cohesin-inactivation study in budding yeast identify related gene sets that are regulated in a concerted fashion. This finding raises the intriguing possibility that cohesin positioning along the chromosome evolved to coordinate specific and related gene sets. The lack of a definable regulatory motif among the genes identified in this study suggests that cohesins, similar to those in vertebrate cells, must be recruited to coordinated loci by early boundary establishment subunits or transcription factors if the effect is directly mediated. Comparing these G_1-targeted loci to those identified in mitotic yeast cells (which include rDNA repeats) will provide an important platform from which to proceed.[35,40-42,58,59]

Materials and Methods

10 ml of log growth wildtype *MCD1* or temperature-sensitive *mcd1-1* mutant yeast cells were grown at the permissive temperature of 23°C and then synchronized in G_1 using alpha factor. G_1-synchronized cultures were then shifted to 37°C for 1 hour to inactivate mutant mcd1-1 protein, a regimen proven not to adversely effect yeast cell viability nor impact sister chromatid pairing. The resulting cultures were harvested by centrifugation and RNA extracted from the pellets using either hot phenol or RNeasy (Qiagen) procedures. In all cases, RNA quality was first assessed by 260/280 and 260/230 absorption ratios (Nanodrop) and further validated by Agilent 2100 Bioanalyzer. One-color Cy-3 labeled cRNA was generated using the Low RNA Input Linear Amplication Kit (Agilent). Samples were hybridized using Agilent Yeast Oligo Microarrays (V2) 4X44k format (G2519F), which includes >6,200 ORFs with a total of 45,018 features of 60-mer controls and gene probes. Each experiment was independently repeated three times with paired comparisons made between RNA obtained from wildtype and mcd1-1 mutant cell extracts.

One-color microarrays were scanned with an Agilent Microarray Scanner System (G25053), which generated the TIFF images of low and high intensity scans utilized by Agilent Feature Extraction Software (v9.5). Feature Extraction processing of fluorescent data corrected signals for background noise, foreground intensities, positive and negative spot controls, background subtraction and signal normalization. Tab delimited text files generated for each of the four experimental arrays were then analyzed using Agilent Technologies software GeneSpring GX (v10.0.2). Data were processed in GeneSpring GX (v10.0.2) by first filtering on expression intensities to retain features within the 20.0 to 100.0 percentile range followed by filtering on flags for features either present or marginal in the microarray. A fold change threshold of 1.5 was imposed for each of the three independent data sets (*mcd1* mutant vs. wildtype). Venn analyses of these data sets produced a list of 30 genes differentially regulated in mcd1-1 mutant cells, compared to wildtype cells, that were common to all data sets. Of these, 20 genes were reproducibly upregulated and 10 were reproducibly downregulated. Bi-color heat maps of the one-color arrays was generated by assigning average pixel intensity for each hybridization feature a color value of 0 (dark green) to 255 (bright red).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/SkibbensCC9-8-Sup.xls

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