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Developmentally regulated MAPK pathways modulate heterochromatin in *Saccharomyces cerevisiae*

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ABSTRACT

Variegated expression of genes contributes to phenotypic variation within populations of genetically identical cells. Such variation plays a role in development and host pathogen interaction and can be important in adaptation to harsh environments. The expression state of genes placed near telomeres shows a variegated pattern of inheritance due to heterochromatin formation, a phenomenon that is called telomere position effect (TPE). We show that in budding yeast, TPE is controlled by the *a1*/*α2* developmental repressor, which dictates developmental decisions in response to environmental changes. Two *a1*/*α2* repressed genes, *STE5*, a MAPK scaffold and *HOG1*, a stress-activated MAPK, are the targets of this heterochromatin regulation pathway. We provide new evidence that link MAPK signaling and heterochromatin formation in yeast. Our results show that the same components that regulate gene expression states in euchromatic regions regulate heterochromatic expression states and that stress can play a part in turning on or off genes placed in heterochromatic regions.

INTRODUCTION

In the nucleus of living cells, DNA is wrapped around histone octamers and forms nucleosomes. Nucleosomes in turn are folded to higher order structures, which make up chromatin. A large portion of the genome in higher eukaryotes is found in a repressed state, refractory to gene expression, called heterochromatin. Heterochromatin is marked in higher eukaryotes by the presence of deacetylated and methylated histones and repressive protein complexes that bind these histones such as HP1, Polycomb group proteins and the SIR complex (1). Changes in chromatin configuration are inherited epigenetically over the cell cycle. These changes in gene expression pattern in turn play a role in dictating the identity of each cell lineage.

During the life cycle of the budding yeast, haploid yeast cells mate to form a diploid cell, which can undergo meiosis but is unable to mate (2). The mechanisms that underlie these cell type differences between haploid or diploid yeast constitute a relatively simple example of developmental states and decisions. Haploid yeasts can have one of two mating types: *a* and α; only *a* cells can mate with α cells and vice versa. The fusion of *a* and α cells creates a diploid cell with an *a*/α genotype. The different cell types define different patterns of gene expression. *a* specific genes (*asg*) are expressed only in *a* cells, α specific genes (α*sg*) are expressed only in α cells. Genes that are expressed in both states but not in diploid cells are termed haploid-specific genes (*hsg*); finally, some genes, such as *IME4*, are expressed only in diploids (*dsg*). However their expression pattern is governed indirectly by the repressor of *hsg*, in the case of *IME4* through antisense transcription and in some cases through repression of a repressor (3).

The identity of cells as *a* or α is determined by the *MAT* locus, which houses two genes, *a1* and *a2* in the case of *MATa* cells and α1 and α2 in the case of *MAT*α cells. These genes regulate the expression of the haploid specific genes. In *a*/α cells, which are typically diploid, the homeobox-containing products of the *a1* and *α2* genes form a repressor complex that represses the *hsg*. The different states of gene expression dictate the various responses to environmental stimuli and developmental decisions made by haploid or diploid cells. For example, only *a*/α diploids can enter meiosis and they do so only in response to low glucose and nitrogen levels (2). Diploid cells enter a pseudohyphal state of growth in response to low nitrogen levels; haploid cells can enter a similar growth state known as invasive growth but do so in response to low glucose levels (4).

Mating and the developmental decisions made by yeasts are intimately linked to heterochromatin. This link originates from the silent copies of the mating-type genes placed at *HML* and *HMR*. *HML* and *HMR* contain

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silent copies of *α1* and *α2* or *a1* and *a2* respectively. Gene silencing in *HML* and *HMR* is maintained due to the activity of the SIR complex, which is responsible for the formation of heterochromatin in yeast. Mutation in any one of the *SIR2/3/4* genes leads to derepression of the silent-mating-type regions; consequently the *a1/α2* repressor is expressed turning off the *hsg* and rendering cells sterile (5).

In the silent-mating-type regions DNA-binding proteins such as Rap1, Abf1 and Orc1 bind DNA sequences termed silencers (6). These proteins interact with Sir1 and Sir4 to initiate the spreading of the repressive SIR complex (7). *SIR2*, a NADH histone deacetylase, is recruited to these nucleation sites through its interactions with Sir4p. Sir3p and Sir4p, in turn, show an affinity for the deacetylated tails of histone H3 and H4 and thus enable the SIR complex to spread along deacetylated nucleosomes (8). Heterochromatin is not limited to the silent-mating-type regions and can be found at the ends of eukaryotic chromosomes (9). Near telomeres, Rap1p, the major telomere-binding protein in budding yeast, again recruits Sir4p through an interaction between the C-terminal domain of Rap1p and Sir4p (7). As in mating-type silencing, several redundant mechanisms exist to recruit the SIR-silencing complex. The Ku70/Ku80, a complex that binds DNA ends, is also a component of telomeric chromatin and interacts with Sir4p, thus contributing to SIR complex nucleation near telomeres (10). Many yeast telomeres also contain binding sites for Abf1p and Orc1p, which may enhance the formation of the SIR complex at these telomeres (11). The SIR complex can spread to distances of 3–5 kb from the chromosome termini and affect the transcription of genes adjacent to telomeres (12).

In addition to telomeres and to the silent-mating-type regions, Sir2 also plays an important role regulating silencing and recombination in the ribosomal gene cluster. At the rDNA Sir2 interacts with Net1 and Cdc14 to form the RENT complex. Loss of Sir2 function leads to loss of rDNA silencing and increase recombination between rDNA repeats (13). Recombination between rDNA repeats results in the excision of extra chromosomal circles, which were shown to be one of the causes of yeast replicative ageing. Thus, Sir2, by virtue or its NAD^+-dependent histone deacetylase activity provides a link between cellular metabolism and ageing (14).

In order to respond to extracellular signals and environmental changes yeast cells employ a number of MAPK signal transduction cascades. Two of the most studied examples are the cellular response to mating pheromone and to osmotic stress. Response to pheromone is initiated by binding of pheromones to their respective receptors Ste2 or Ste3 and activation of a trimetric G-protein composed of Gpa1, Ste4, Ste18 which in turn trigger the activation through phosphorylation of Ste11 (MAPKKK), Ste7 (MAPKK) and the two functionally overlapping downstream MAPKs Fus3 and Kss1 (15). All the components of this cascade bind the Ste5 scaffold, which facilitates signal propagation. Phosphorylated Fus3 and Kss1 induce Ste12-dependent activation of mating and pseudohyphal genes (16). The high osmolarity glycerol pathway is triggered by at least two different membrane receptors and culminates in activation of a different MAPK, Hog1 (17). Many signaling components such as the pheromone receptors, the *STE5* scaffold and two MAPKs, *FUS3* and *HOG1*, are direct targets of the *a1/α2* repressor and their repression renders diploid cells unresponsive to pheromone and sterile (18). The very same components serve a signaling role in other processes such as pseudohyphal growth and virulence in pathogenic fungi. *HOG1* stands out among this group of developmentally repressed genes in several ways. First, in *Saccharomyces cerevisiae* (in contrast to *Schizosaccharomyces pombe*) *hog1* mutants are not defective for mating (19). Hog1 is a member of a conserved MAPK activated by various stresses. In *S. cerevisiae*, *hog1* cells are exquisitely sensitive to osmotic stress. It is worth mentioning that p38, the mammalian homolog of Hog1, while sharing its stress activation role, has a well-documented role in the development of myogenic cells (20). Another unique feature of *HOG1* is its physical interaction with transcriptional activation complexes. Like other MAPKs, Hog1p regulates the activity of several transcription factors via phosphorylation. However, in addition, it was found to bind induced genes directly (21) and was shown to recruit the Rpd3 histone deacetylase complex as part of its mechanisms for transcriptional induction (22).

We have isolated the *MAT* locus as a suppressor of telomeric silencing. Further examination showed that both Ste5 and Hog1 mediate this effect. These findings point to an intimate relationship between MAPK signaling and heterochromatin formation and suggest that such a relationship may play a role in developmental processes in higher eukaryotes.

MATERIALS AND METHODS

Plasmids

pCB115 is a *CEN LEU2* plasmid carrying the *a1* and *α2* genes expressed from their native promoters. p204-a/α was created by cloning the *Spe*I *Hind*III fragment containing *a1* and *α2* from pCB115 into a *Xba*I and *Hind*III sites of YIplac204. p112-*a*/α was created by *in vivo* recombination in yeast of YEplac112 digested with *Hind*III and the *Pvu*II fragment from p204-*a*/α. pRS303-STE5 was created from pSKM92 (23) using Eco47III digestion and self ligation that resulted in the removal of the 2 μm origin of replication.

Screening for telomeric-silencing suppressors

Screening for suppressors of the *elg1*-silencing defect was carried out in strain *Yuv112* (*leu2Δ0 trp1 ade2::KanMX elg1::HygB ura3 adh4::ura3-ADE2-TELVII*), derived from AJL442-4b (24). A YEp24-based genomic library was transformed into *Yuv112*. Cells were plated on Synthetic Dextrose lacking uracil and with 6 μg/ml of adenine. White colonies were picked and reexamined for their color phenotype and for its dependency on the library plasmids. Plasmids were rescued from the positive clones, reintroduced into *Yuv112* and the color phenotype of the transformants was examined again.

Silencing of HMR and rDNA-embedded genes

Strains carrying an *ADE2* marked HMR were grown over night in SD–TRP for plasmid maintenance, resuspended in H_2O, counted and diluted in 5-fold serial dilutions. *ADE2* derepression was only apparent on drop in media. SD–ADE plates contained 2% glucose, 0.67% yeast nitrogen base and 1.5 g/l from an equal mixture of leucine, histidine and uracil. Strains carrying the *ADE2-CAN1* cassette in the rDNA were streaked on minimal plates lacking arginine and adenine and grown over night in minimal media lacking adenine and arginine before being plated on 50 μg/ml canavanine plates.

Strain construction

Mating, sporulation and dissection were done according to standard protocols. Most strains were constructed using one-step gene replacement using PCR generated fragments from the deletion library strains with 300 bp of flanking sequences. Correct replacements were verified using PCR. When deletion strains were not available, long primers were used together with the KanMX cassette from pFA6-KamMX or the HygB cassette from pAG32 for the creation of full replacements of all coding sequences with the resistance cassette. Three independent colonies were analyzed for each strain constructed. A complete list of strains used in this study is found in Supplementary Table 3.

ChIP

Typically 50 ml of a log culture (5×10^7 cells/ml) was cross-linked for 30 min in 1% formaldehyde. The cross-linker was quenched by the addition of Glycine to 125 mM and the cells were incubated for 5 min at room temperature. Cells were washed twice with TBS + 10% glycerol. Cells were vortexed for 45 min in 600 μl of lysis buffer supplemented with protease inhibitors (Roche) and glass beads. The crude lysate was sonicated to an average fragment size of 300 bp after which the supernatant was clarified (14 000 rpm, 20 min). An amount of 450 μl of the clarified lysates was used for immunoprecipitations. Sir2 antibodies were purchased from Santa Cruz. SC-6666 was used for IP and SC-25753 to confirm the ChIP results and for western analysis. SC-789 (anti myc A14) was used for Sir3-9myc IP and detection. The immune complexes were retrieved using protein G beads (Adar Biotech) and washed using lysis buffer, wash buffer (250 mM LiCl, 0.5% NP40, 0.5% deoxycholate, 5 mM EDTA) and TE. DNA was eluted, cross linking was reversed and the DNA was ethanol precipitated and resuspended in 50 μl of TE. One microliter was used for PCR reaction; primer concentration and cycles' numbers were calibrated individually for each reaction. Every PCR reaction was carried out simultaneously on input DNA and on the relevant IP to control for changes in PCR conditions. Reaction products were resolved on 2.3% agarose gels. Each experiment was carried out at least three independent times. Enrichment ratios were calculated by dividing the IP ratio between the indicated experimental band and the Cup1 control with the same ratio obtained from the input sample. All ratios were compared to the ratio seen in the wild-type. All the PCR primers used are listed in the Supplementary Table 4.

RESULTS

MAT heterozygosity suppresses telomeric silencing in yeast

We have previously shown that Elg1, a protein that forms an alternative clamp loader complex (25), plays a part in regulating telomere length. In addition, *elg1* cells show enhanced levels of telomeric silencing and this is independent of its involvement in telomere length regulation (26). We screened a yeast high copy library for plasmids that suppress telomere position effect (TPE) in *elg1* strains. Our screen was carried out in a strain containing a telomere proximal *ADE2* gene. In the wild-type strain telomeric silencing confers a pink/white variegation, whereas *elg1* strains are red (hyper-silencing phenotype) (26). The yeast high copy number library was screened for plasmids that produced white colonies. After screening ~200 000 colonies, we identified several high copy suppressors of TPE (Supplementary Table 1).

We isolated two well-known effectors of TPE: *ASF1* and a truncated *SIR3* allele. Asf1 is a histone chaperone that participates in chromatin assembly and affects many aspects of chromosome metabolism in yeast and in other eukaryotes (27,28). $Sir3^{138–929}$ is a deletion of the first 137 amino acids of the Sir3 protein, and it is known to reduce silencing at telomeres and at the mating-type region. Both of these genes were previously isolated as suppressors of *HML* silencing and were shown subsequently to affect telomeric silencing in wild-type strains (29). In addition, we isolated several plasmids that carry either the *MATα* locus or the *HML* locus (without one of the silencer elements). Since we carried out our screen in *MATa* cells, the plasmids turned the **a** cells into a/**α**. While silencing of the mating-type regions plays a vital role in determining the *MAT* dependent gene expression pattern in the cell, the fact that expression of opposite *MAT* information affects silencing was very surprising. We therefore focused on determining the exact relationship between these factors.

First, we tested whether the effect observed was specific for *MATα*. Supplementary Figure 1B shows that only *MATα*-containing plasmids, but not those carrying *MATa*, were able to suppress telomere silencing in *MATa* haploids. Similarly, only *MATa*-carrying plasmids suppressed TPE in *MATα* strains (data not shown). We thus conclude that in *elg1* strains, mating-type heterozygosity can suppress TPE. The *MAT* locus may contain two sets of genes, *a1* and *a2* or *α1* and *α2*. In *MATa*/*α* cells the gene products of the *a1* and *α2* genes form a complex that represses the expression of the haploid-specific genes. In order to show that this repressor is responsible for the observed reduction in TPE, we transformed our strain with a plasmid that expresses a1 and α2 from their endogenous promoters. We found that introducing the *a1*/*α2* repressor alone is sufficient to reduce TPE in both *MATa* (Figures 1 and 2) and *MATα* (data not shown).

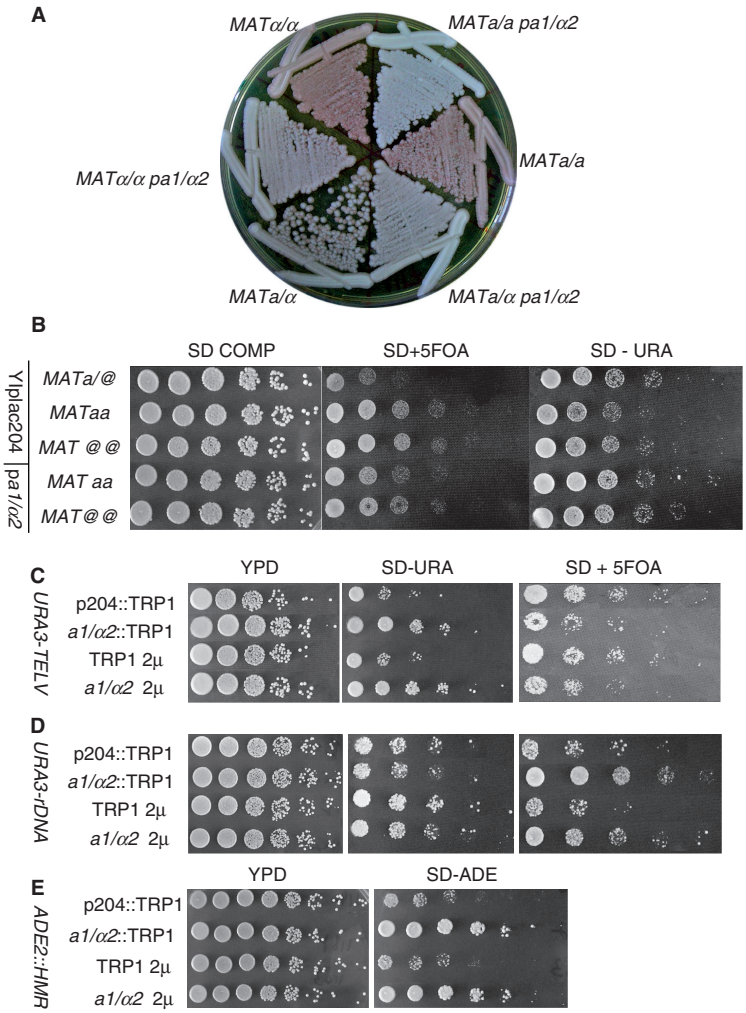

Figure 1. The effect of the *a1*/*α2* repressor on heterochromatin in yeast. (**A**) Diploid strains with the indicated mating-type configuration containing either empty plasmids or the *a1*/*α2* repressor integrated at the *TRP1* locus were streaked on YPD plates and incubated until color development. (**B**) Cultures were grown in YPD medium, 5-fold serial dilution were made and the strains were spotted on the indicated media. *MATa*/*α* reduces silencing as indicated by the reduced growth on 5FOA medium and increased growth on SD–URA plates. Growth assays were used to determine the effect of repressor expression on heterochromatin, wild-type strains with *URA3* marked telomeres (**C**), *URA3*-marked rDNA repeats (**D**) or *ADE2*-marked *HMR* (**E**), carrying either an integrated copy of the *a1*/*α2* repressor, an empty plasmid integrated at the *TRP1*, an empty 2 μm plasmid or a 2 μm plasmid carrying the same repressor construct were spotted on appropriate plates to determine the extent of silencing in the respective regions.

We next sought to determine whether this effect is dependent on the Δ*elg1* mutation. For that purpose, we introduced high copy number plasmids that carried either *MATα* or *MATa* into wild-type yeast strains carrying *ADE2* and *URA3* marked telomeres. As seen in Supplementary Figure 1C we found that independently of the *ELG1* gene, expressing the opposite-mating-type information reduces telomeric silencing. This effect is therefore general and not dependent on the *elg1* mutation.

So far, we have shown the effect of the *a1*/*α2* repressor in haploid strains. However, one possible explanation for our results is that telomeric silencing operates in a different and mutually exclusive manner in haploid and diploid yeasts. To separate the effect of ploidy from that of the different transcriptional programs of *MATa*, *MATα* and *MATa*/*α* cells we constructed isogenic diploid strains that are either *MATa*/*α, MATa*/*a or MATα*/*α* and compared telomeric silencing between these strains. We found that *a*/*a* and *α*/*α* diploids silence telomere-proximal genes better than a/α diploids (Figure 1). This effect is completely dependent on the presence of the *a1*/*α2* repressor since expressing the a1/α2 repressor ectopically from the *TRP1* locus in *MATa*/*a* or *MATα*/*α* strains restores gene silencing to levels equivalent to that of *MATa*/*α* cells (Figure 1). Conversely, deleting either *MATa* allele in a *MATα*/*α* diploid increased TPE (data not shown). We therefore conclude that the sole reason for the observed suppression of TPE is the transcriptional difference brought about by the *a1*/*α2* repressor and not changes in ploidy.

The *a1*/*α2* repressor modulates heterochromatin globally

Transcriptional silencing occurs at several loci in the yeast genome: the silent-mating-type regions, telomeres and the

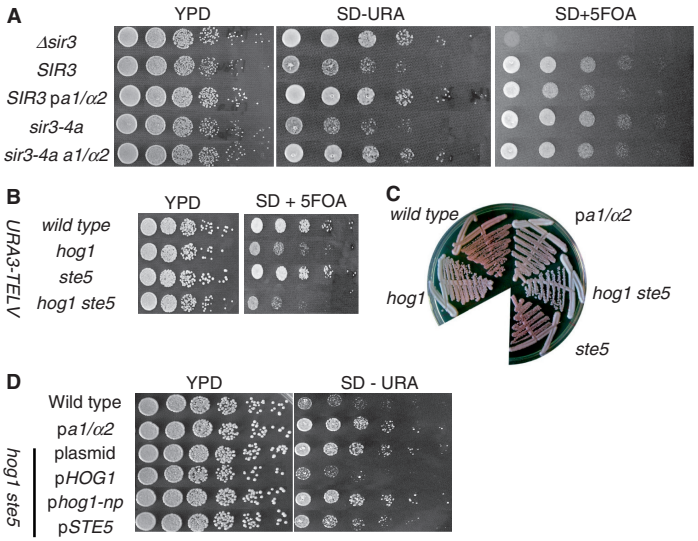

Figure 2. (**A**) Sir3 phosphorylation is not necessary for changes in telomeric silencing. Δ*sir3* cells were transformed with either a *SIR3* or *sir3-4a* integrating plasmids and an *a1*/*α2* expressing plasmid. Cells were grown in YPD and 5-fold serial dilutions were plated on indicated plates. Expression of the *a1*/*α2* repressor reduces telomeric silencing irrespective to the *sir3* allele present. (**B–D**) *HOG1* and *STE5* positively regulate TPE. *ste5*, *hog1* and *ste5 hog1* cells were plated on YPD to assess growth and on 5FOA-containing medium to assess *URA3* expression (A), or streaked on YPD plates to monitor *ADE2* expression from a telomeric marker (B). A *ste5 hog1* strain was transformed with either *HOG1*, *hog1-np* or a plasmid carrying *STE5* to determine the extent of TPE (**C**).

rDNA locus (6). While Sir2p is required for silencing in all these loci, the mechanisms responsible for its recruitment differ among them (6). Therefore, the silencing phenotypes observed in the three loci can provide mechanistic insights on the effect of the *a1*/*α2* repressor on telomere silencing. For example, *sir3* and *sir4* mutations abrogate telomeric and *HML*/*HMR* silencing while *sir4* mutations strengthen rDNA silencing (13). We used ectopically expressed *a1*/*α2* repressor either integrated at the *TRP1* locus or from a high copy plasmid to examine the effects of the repressor on heterochromatin. Figure 1C shows that a *URA3* gene integrated near a telomere exhibits TPE, allowing only partial growth on plates without uracil. Expression of the *a1*/*α2* repressor allows better growth in this medium. A reciprocal growth pattern can be seen in plates containing 5-fluoroorotic acid (5-FOA), which selects against cells expressing the *URA3* gene (Figure 1C). We found that the effect of the *a1*/*α2* repressor is not limited to telomeric chromatin. As seen in Figure 1E an *ADE2* gene placed at *HMR* was expressed at higher levels in strains expressing the *a1*/*α2* repressor than in control trains as evidenced from the increased growth of this strain on media lacking adenine. When we examined a *mURA3* construct placed at the *rDNA* locus, we observed the opposite effect: *a1*/*α2*-expressing cells grew better than their controls on 5FOA medium (Figure 1D) indicating that silencing in the rDNA is strengthened in these cells. The dosage of the repressor does not seem to play an important part in silencing as we found that identical constructs expressed form a high copy plasmid or from a chromosomal location have similar effects (Figure 1).

HOG1 and *STE5* regulate TPE

We first investigated whether expression of the *a1*/*α2* repressor alters the expression of members of the SIR complex. We did not detect differences in the expression levels of either Sir2, Sir3 (Supplementary Figure 2) or Sir4 (data not shown). Previously, TPE was shown to be regulated by a MAPK, *MPK1*. *SIR3* is a direct target of *MPK1* and *MPK1* phosphorylation decreases *SIR3* association with telomeres and therefore reduces silencing (30). Although the *a1*/*α2* repressor does not directly regulate *MPK1*, we explored the significance of *SIR3* phosphorylation. If changes in *SIR3* phosphorylation are the underlying cause of the changes observed in silencing we would expect that these changes should disappear in a *SIR3* allele unable to undergo phosphorylation. We examined the effect of a *SIR3* allele in which all the four known phosphorylation sites were mutated to prevent *MPK1*-dependent phosphorylation [*sir3-4a*, (30)]. As seen in Figure 2A, introducing the repressor into both *SIR3* and *sir3-4a* expressing cells caused a similar decrease in telomeric silencing, showing that *SIR3* phosphorylation is not needed for the effect of *a1*/*α2* on silencing.

The role of mating-type information goes beyond simply controlling mating behavior in yeast and affects many aspects of cell behavior such as response to starvation, DNA damage and ageing. The fact that the repressor dosage does not play a part in telomere silencing supports an indirect model to explain its involvement in silencing. In this model, one or more of the genes regulated by the repressor affects telomeric silencing. All of the targets for the *a1*/*α2* repressor have been identified using complementary genome-wide techniques (18). We systematically deleted each of the known repressor targets and examined telomeric silencing in these mutants (see Supplementary Figure 3 and Supplementary Table 2 for a complete list). No single mutant affected telomeric silencing to the same extent as the repressor, supporting a hypothesis of multiple contributions. From the genes examined,

HOG1, a stress activated MAPK, showed relatively low levels of TPE suggesting that it is one of the relevant targets of the repressor. Among the other genes only *STE5*, a MAPK scaffold, showed a slight phenotype. In spite of the marginal phenotype that we observed in *ste5* strains, the fact that, like *HOG1*, *STE5* is involved in MAPK signaling drove us to test TPE in the double mutant. In this double mutant, we observed that telomere silencing further decreases (Figure 2B and C). From the phenotype of the double mutant, we conclude that both *HOG1* and *STE5* positively regulate TPE in yeast. In order to confirm that indeed the effect observed stems from the *hog1* and *ste5* mutations we carried out complementation analysis on the double mutant strain. As shown in Figure 2D, a plasmid expressing Hog1 restores silencing almost to wild-type levels as expected from the weak phenotype of *ste5* strains. When the *hog1-np* allele that carries two mutations in its MAPK-activation loop (31) was transformed into the same strain, the levels of silencing remained unchanged, showing that Hog1 activation is necessary for the reduction in TPE and not simply the presence of the Hog1 protein. A plasmid carrying Ste5 also restores silencing, although, as expected, its effect is lower than the one of Hog1 (Figure 2D).

Hog1 and Ste5 regulate heterochromatin globally

Since we observed that the repressor mediates a global effect on silencing, we examined the effect of *hog1* and *ste5* on silencing at the rDNA and the *HMR* loci, expecting to phenocopy the effect of the *a1*/*α2* repressor. Indeed, as shown in Figure 3A, deleting *hog1* and *ste5* reduced *HMR* silencing to levels comparable to those observed in *a1*/*α2*-expressing cells. Interestingly, the *ste5* deletion had a more pronounced effect than the *hog1* deletion, suggesting a 'division of labor' between these separated MAPK pathways with respect to distinct regions of heterochromatin. We measured rDNA silencing using the *CAN1-ADE2* cassette inserted in the rDNA locus. The *CAN1* gene encodes a pump that imports the toxic arginine analogue canavanine into the cells. As seen in Figure 3B, similarly to the effect of the *a1*/*α2* repressor, silencing in the rDNA locus *increases* in *hog1 ste5* double mutants, allowing better growth in the presence of canavanine. The extent of canavanine resistance was slightly smaller than the one caused by expression of the repressor suggesting that an additional gene regulated by the repressor affects rDNA silencing. Taken together our results show that MAPK signaling plays a global role in heterochromatin regulation.

Hog1 and Ste5 negatively regulate Sir2 localization to heterochromatin

Having shown that the mating and stress-signaling pathways regulate silencing, we wanted to determine their effect on the distribution of the SIR complex, the major silencing complex in yeast. We followed Sir2 and Sir3 localization at telomeres, silent-mating-type regions and in the rDNA regions, using a ChIP assay. We could detect both Sir2 and Sir3 binding at the right telomere

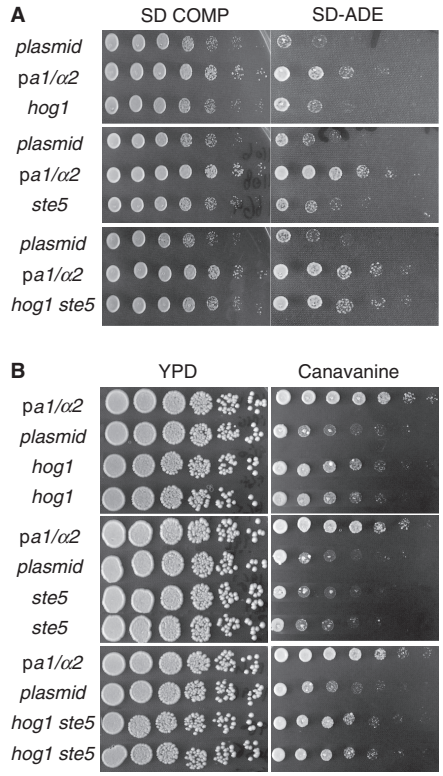

Figure 3. *HOG1* and *STE5* regulate heterochromatin globally. *ste5*, *hog1* and *ste5 hog1* double mutants cells were 5-fold serially diluted and plated on YPD plates to asses growth and on SD–ADE plates to assess the extent of HMR silencing (**A**) or Canavanine-containing plates (50 μg/ml) to assess the repression of the *CAN1* gene at the rDNA locus (**B**).

of chromosome *VI*. As expected, Sir2 binding was Sir4-dependent, as we saw no Sir2 enrichment in *sir4* strains at both telomeres and *HML* (Figure 4B). We found reduced binding of both Sir2 and Sir3 at telomeres in *a1*/*α2* expressing cells and in *hog1* cells in comparison to their wild-type controls (Figure 4B and C). This effect on SIR complex occupancy is relatively mild, as we still observed relatively efficient binding at a distance of 1 kb from the telomere (Figure 4B and C). At a distance of 5 kb from the telomere we did not observe any enrichment for either Sir2 or Sir3, consistent with previously published results. We observed that rDNA-silencing levels increase in our mutants and consistently with this we observed increased binding of Sir2 at the rDNA locus in *hog1*, *ste5* and *hog1 ste5* double mutants. Thus, the reduced levels of silencing we observe are correlated with, and can be explained by, the lower occupancy of the SIR complex at the examined loci, showing that MAPK signaling can affect the distribution of silencing complexes.

MAPK pathways regulate telomeric heterochromatin

We have already established that Hog1 activation is necessary in order to reduce TPE (Figure 2D). This suggests that the entire Hog1 and pheromone response pathways participate in heterochromatin regulation. To examine this hypothesis we created single and double mutants of various pathway components. As seen in Figure 5A,

deleting *pbs2*, the HOG pathway MAPKK, recapitulates both the phenotype of *hog1* and its relationship with *ste5* with regard to telomeric silencing, showing that indeed we are looking at pathway interactions. We further created combinations of both *ste7* (the pheromone response MAPKK) and *ste18* (the G protein gamma subunit of the pheromone response pathway) with either *hog1* or *pbs2* and found similar patterns of interactions (Figure 5B and C). In summary, our results show that both MAPK-signaling pathways contribute to heterochromatin regulation via separate mechanisms.

The SIR complex disassociates from DNA in response to osmotic shock

So far we have examined loss of function mutations in the relevant signaling pathways. The phenotypes of these mutants are expected to be the opposite of the ones expected for pathway activation. Thus, our results predict that pathway activation may positively regulate telomeric and mating-type silencing. To directly examine the consequences, if any, of *HOG1* and pheromone pathway-signaling stimulation on heterochromatin, we exposed cells to osmotic stress and to pheromone, and monitored the association of the SIR complex components with chromatin under these conditions. First, we exposed wild-type and *hog1* cells to severe osmotic shock (using 1M NaCl). We observed that Sir3 binding is rapidly lost at both telomeres and *HML* upon exposure to osmotic stress (Figure 6A). We examined the association of Sir2 with the rDNA locus and found that Sir2 is lost from rDNA with similar kinetics. We also followed the association of Sir2 with telomeres and HML and obtained identical results to those of Sir3 (data not shown). While the kinetics of dissociation appeared similar in all tested loci, the re-association of Sir3 to telomeres and those of Sir2 to rDNA occurred rapidly and could be detected as early as 30 min after osmotic shock while the assembly process in the *HML* region seemed to take a longer time. After 4 h of incubation in 1M NaCl we observed that binding of both Sir3 and Sir2 to all loci returned to its pre-stressed levels. This assembly is *hog1*-dependent as we observed no reassembly of the SIR complex in *hog1* cells (Figure 6A).

Next, we examined whether exposure to pheromone can lead to changes in the binding of SIR complex to telomeres and to the silent mating-type loci. We exposed cells to alpha factor for 120 min, at which time point virtually all the cells had arrested in G1 (as determined by visual inspection). We then released the cells into fresh medium and monitored their progression into the cell cycle. We assayed Sir3 binding at various time points during this experiment but did not detect any change throughout the experiment (Figure 6C). We therefore conclude that osmotic shock, but not pheromone exposure, regulates the location of the SIR complex to telomeres and silent-mating-type loci.

DISCUSSION

In this study, we uncovered the surprising fact that yeast cells regulate heterochromatin formation in a global fashion, via the master regulator encoded by the *MAT* locus. We first observed these changes during a screen designed to uncover suppressors of TPE in cells in which the large subunit of the alternative clamp loader, Elg1, is deleted. We believe that screening for suppressors of TPE in this background with higher levels of telomeric silencing facilitated the identification of this relatively mild effect. The effect, however, can be clearly seen in Elg1$^+$ cells too (Supplementary Figure 1 and Figure 2).

Since silenced domains are known to be connected in *trans* through Sir2 availability (32), we asked whether other heterochromatin domains are affected by *a1*/*α2*. We found that silencing in the silent-mating-type regions was affected in a similar manner to telomeres while silencing in the rDNA locus was enhanced rather than reduced. These changes in silencing are similar in tendency (although smaller in magnitude) to those seen when *SIR4* is deleted or in yeast cells undergoing replicative ageing (13,33). Yeast cells expressing the *a1*/*α2* repressor have been shown to exhibit increased replicative lifespan (33). These cells also exhibit higher levels of homologous recombination, in comparison to haploids expressing either only *MATa* or only *MATa* information (34). Such a high level of recombination may produce extrachromosomal rDNA circles (ERCs), which induce ageing and senescence (35). Increased heterochromatin formation at the rDNA may therefore be advantageous in these circumstances, as it may prevent the creation of ERCs. Our results show that the MAPK pathways participate in this process, as in *hog1* or *ste5* cells silencing is increased (Figure 3) and higher levels of Sir2 can be found at the rDNA locus (Figure 4).

A connection between stress and developmental pathways to heterochromatin formation

Another aspect of the regulation of heterochromatin formation concerns the expression state of genes natively placed at the subtelomeric region. There are several examples in which the expression state of subtelomeric genes plays a role in resistance to stress, pathogenicity and adaptation (30,36). Epigenetic control of master genes has been shown to affect pathogenicity in *Candida glabrata* (EPA genes) (37), in *Pneumocystis carinii* (MSG genes) (38) and in *S. cerevisiae* (FLO genes) (39). In most of these cases, each member of the protein family encode a slightly different protein with unique cell-surface properties, and the variability in expression of the various genes allows the formation of a population exhibiting heterogeneous antigenic properties. A similar strategy is used by the VSG genes in *Trypanosome bruceii* (40). These genes are usually located at subtelomeric regions, and chromatin variegation may contribute to the heterogeneity described.

Previously, Mpk1-dependent *SIR3* phosphorylation was shown to be induced under stress and subsequently to play a part in stress resistance, through its regulation of subtelomeric gene expression (30). We examined a *sir3* allele in which all known phosphorylation sites were mutated and found that the *a1*/*α2* repressor can still reduce TPE in this strain, demonstrating that the effect of *a1*/*α2* on TPE is not dependent on phosphorylation

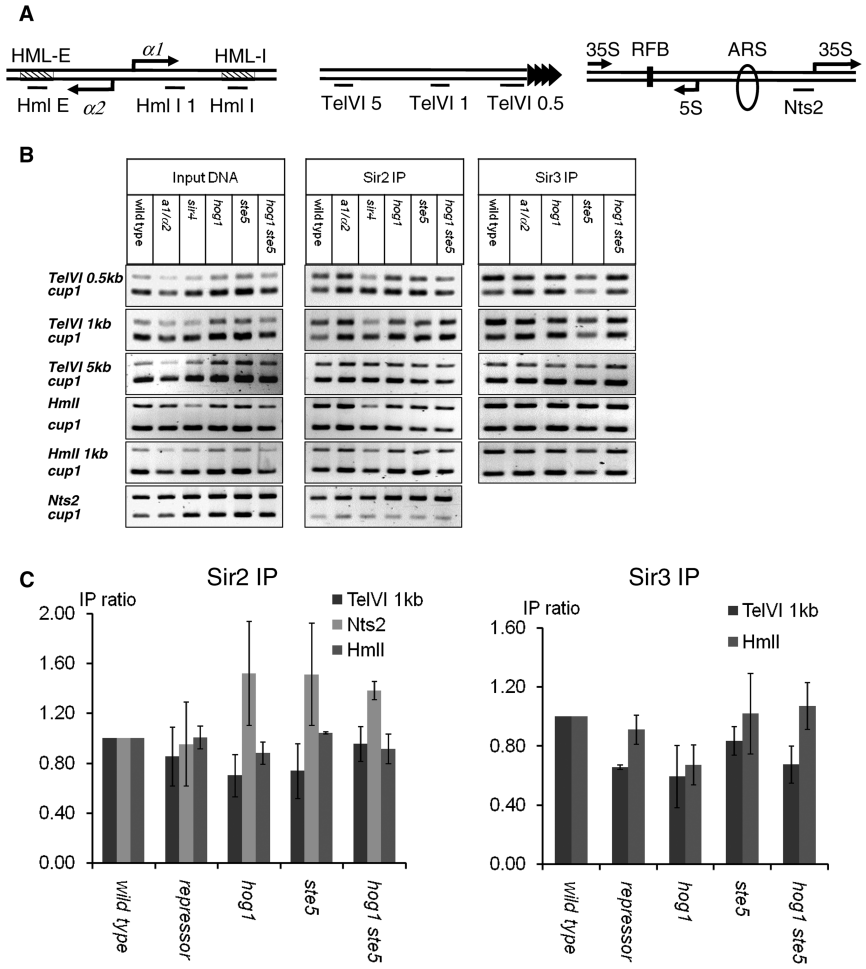

Figure 4. Expression of *a1*/*α2* or deletion of *HOG1* and *STE5* affects the association of the SIR complex with silenced regions. (**A**) The location of the various PCR fragments used in a ChIP assay to follow SIR complex association with DNA. (**B**) Representative ChIP experiments performed using anti-Sir2 or anti-myc (for Sir3) antibodies. (**C**) Enrichment ratios obtained for wild-type strain, an isogenic strain expressing the a1/α2 repressor, and various mutants, at telomeric, rDNA or silent mating-type regions.

of Sir3 by Mpk1. *MPK1* is not the only MAPK induced under stress. Yeast cells adapt to osmotic stress by raising intracellular glycerol levels to reduce osmotic pressure. This stress adaptation pathway converges on Hog1 activation. *HOG1* is known to be under the control of *a1*/*α2* and accordingly diploid cells are more sensitive to osmotic stress than haploid cells; however, the cellular function of this repression is unknown (18). We have found that *hog1* also reduces telomeric silencing, linking this stress-responsive MAPK to subtelomeric gene expression. We also observed this reduction in TPE in strains deleted for the *HOG1* MPKK, *PBS2* (Figure 5), showing that the whole MAPK pathway plays a role in TPE. While the single mutant *ste5* exhibited only a mild effect on TPE, when we deleted *ste5* in *hog1* cells we were able to further reduce telomeric silencing, supporting a model in which both of these pathways contribute independently to silencing.

Taken together, our findings and those of others point to an intimate relationship between developmental decisions, MAPK signaling and heterochromatin. Different MAPKs have different effects and act in opposite directions with respect to silencing. This stems from the distinct mechanisms at work; Sir3 phosphorylation does not seem to be involved in Hog1- and Ste5-dependent regulation and indeed while activating the *MPK1* pathway reduces telomeric silencing, inactivating the pheromone response pathway or the osmosensing pathways leads to a similar effect. In agreement with this, *ste11-4*, a mutation that leads to constitutive activation of pheromone signaling, is able to increase telomeric silencing (41). Studies in fission yeast have established a similar connection between stress and heterochromatin formation. In *S. pombe* two stress-activated transcription factors, Atf1 and Pcr1 are important for heterochromatin formation at the silent mat2/3 locus (42). Atf1 and Pcr1 interact with the histone deacetylase Clr6 (43) and are activated by Sty1, the fission yeast homolog of Hog1 (44). Although the effect of these mutations on telomeric and rDNA silencing was not examined in these studies, they did show that the involvement of stress-activated MAPK in heterochromatin assembly is conserved in evolution and therefore is functionally significant. In Drosophila, the JNK pathway (a stress-activated MAPK pathway, similar to the HOG

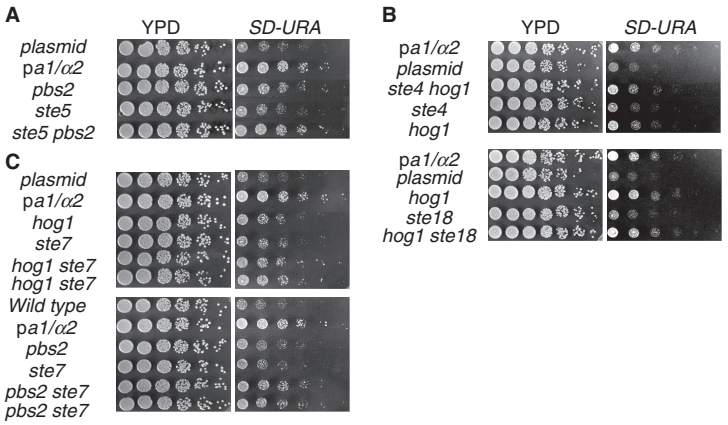

Figure 5. MAPK pathways regulate heterochromatin: All strains contain a telomere-proximal *URA3* gene. Strains were 5-fold serially diluted and plated on YPD plates to assess growth and on SD-URA plates to assess the extent of telomeric silencing. (**A**) Effect of mutations in two scaffold proteins, Ste5 and Pbs2. (**B**) Effect of mutations in Ste4 and Ste18, components of the trimeric G protein, in combination with *hog1*. (**C**) Effect of mutation in Ste7, the MAPK kinase, combined with either *hog1* or *pbs2*.

pathway) is involved in developmental processes such as dorsal thorax closure (45).

The link between signal transduction pathways able to respond to external stimuli and heterochromatin formation suggests that cells have evolved to dynamically regulate heterochromatin formation thus increasing phenotypic variation in genetically identical populations in response to changing environments.

Interplay between MAPK signaling and Sir2 distribution

Sir2 localization to telomeres/silent-mating types and to the rDNA depends on at least two distinct mechanisms. Recruitment to telomeres and to silent-mating-type regions is mediated by Sir4-Sir2 interaction, while rDNA localization of Sir2 depends on its interaction with Net1. Recently, Sir2 homotrimerization was shown to be important specifically for rDNA silencing and recruitment, adding an additional mechanism that regulates Sir2 distribution (46). We show that MAPK signaling drives the cellular Sir2 pool towards the rDNA. This does not stem from changes in the levels of SIR complex components and may reflect some sort of change in one or both of the mechanisms that are known to regulate Sir2 localization. One possibility (which we are currently exploring), is that the *HOG1* pathway regulates the assembly of one of the Sir2 containing complexes, by directly phosphorylating one of its subunits or accessory proteins. In addition, it is possible that inactivating *HOG1* signaling induces transcriptional changes in the cell and that these changes are responsible for the effect we observe. As far as we know *hog1* and wild-type cells do not exhibit large differences in gene expression under regular growth conditions, however some basal activity of this pathway may play a part during normal growth. This is supported by our finding that the *hog1-np* allele behaves indistinguishably from the Δ*hog1* allele.

It is also possible that Hog1 and Ste5 may affect heterochromatin in a direct manner through physical association. Both Ste5 and Hog1 were shown to localize to the promoters of activated genes following the activation of their respective signaling pathways (47,48). It is in principle possible that both are recruited to sites of heterochromatin, where they could collaborate with the SIR complex and additional silencing factors. Although in preliminary experiments we have failed to obtain evidence of such a physical interaction, a possible joint direct role in heterochromatin formation is a possibility that remains to be tested.

MAPK stimulation and its effect on SIR protein localization

We examined the effect of MAPK stimulation on Sir proteins localization and found that both Sir2 and Sir3 are removed from DNA immediately after exposure to osmotic stress. It has been previously shown that many transcription factors rapidly and transiently disassociate from DNA in response to osmotic stress (49). This effect likely stems from the increased ion concentration within the cell. The disassociation of the SIR complex that we observe probably results from this effect. Indeed, one of the transcription factors studied in (49) was Rap1. It was shown that Rap1 disassociate from promoters immediately after stress induction and rises back to its wild-type levels 10 min post-induction. We have shown a similar kinetics for the SIR complex at silenced regions. We attribute the delayed kinetics we observe to the more severe form of stress that we employed in our experiments. Cells probably recover more slowly from the initial shock at higher salt concentrations and therefore conditions that permit binding appear only after 10–30 min. Consistent with this, it was shown that more severe forms of stress leads to delayed Hog1 dependent transcriptional response (50). Alternatively, it takes longer for cells to assemble the SIR complex as it only forms after DNA-binding proteins can bind at the proper site. Clearly the role of Hog1 in this process is more related to stress adaptation but given the silencing phenotypes of *hog1* we cannot exclude the possibility that it also plays a direct role in the reassembly process.

The mating-type system in yeast plays a central role in determining cell type in this organism, and can be regarded as a master developmental switch mechanism. Unicellular organisms use environmental signals to direct

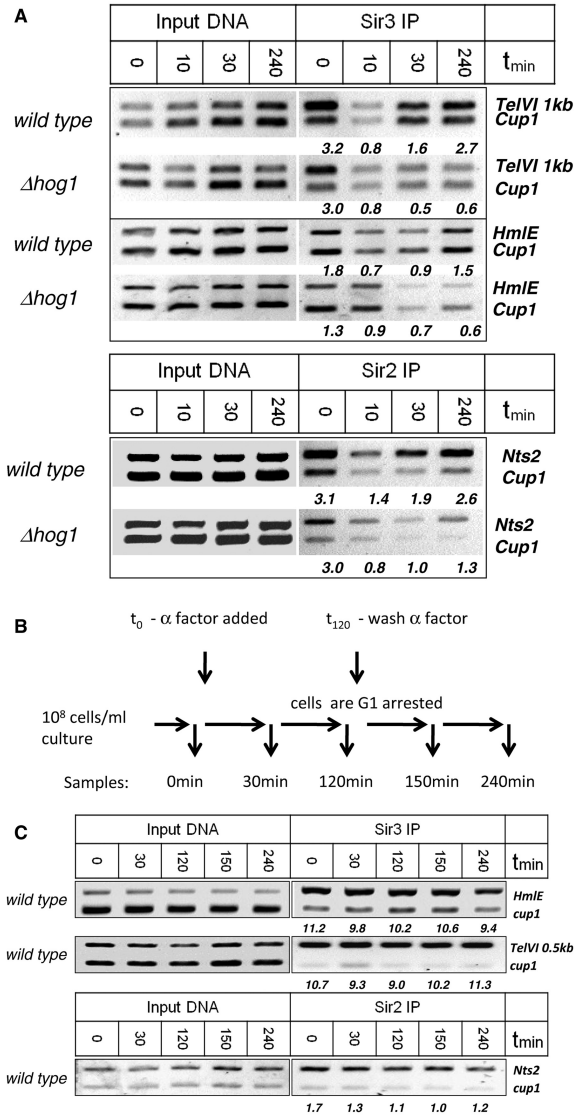

Figure 6. The SIR complex is removed from DNA after exposure to osmotic stress. (**A**) The SIR complex is displaced from telomeres, HML and from rDNA after 10 min of exposure to 1 M NaCl and re-associate with all regions in a Hog1-dependent manner after 4 h. Time 0 samples were taken prior to the addition of salt. ChIP and PCR reactions were carried out as in Figure 6. (**B**) A depiction of the pheromone exposure experiments. (**C**) Pheromone stimulation does not affect the association of the SIR complex with DNA.

developmental decisions. We have shown that the remodeling of cellular transcription that takes place in diploids can serve additional roles other then abrogating mating and enabling meiosis. By regulating the activity of MAPK pathways, diploid cells can achieve additional capabilities, such as the stabilization of rDNA locus in the face of age-associated recombination events (51). In addition, the change in telomeric heterochromatin that allows subtelomeric gene expression under stress may confer a selective advantage on the cells under these physiological conditions.

Collectively we observe that heterochromatin-affecting complexes that silence developmental regulators are in turn regulated by the action of the very same developmental regulators. In yeast the effect of the *a1*/*α2* repressor on the MAPK pathways affects silencing of the (usually silent) mating-type copies, possibly creating a fine-tuning mechanism on the abundance of the *a1*/*α2* repressor itself. As this regulation stems from the effect of the *a1*/*α2* repressor on the conserved stress-activated MAPK pathway, this mechanism should allow the cells to respond to external stimuli in a dynamic fashion. In higher eukaryotes a similar connection might involve the regulation of Polycomb complexes by the very homeotic genes that they silence during development. Indeed, in drosophila JNK signaling was shown to suppress Polycomb protein activity and induce transdetermination, a process by which cells can switch from one developmental faith to another (52).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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