Genome-wide analysis of transcription regulation during the cell quiescence cycle

Genoom-brede analyse van transcriptie regulatie tijdens de quiescence cyclus van de cel

(met een samenvatting in het Nederlands)

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Eritis sicut Deus, scientes bonum et malum

(Faust, J. W. Goethe 1790)

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Chapter 1

General introduction

Eukaryotic quiescence and proliferation

All organisms have cells that are capable of exiting the normal cell cycle and entering a non-proliferative state termed quiescence or G0. Most eukaryotic cells, whether they exist as single-celled or multicelular organisms, spend the majority of their life-cycle in a quiescent state (Lewis, 1991). An important property of quiescent cells is that they can remain in G0 for long periods, retaining the capability to re-enter the proliferative cycle if necessary.

Mammalian cells enter quiescence in response to extracellular signals, including various growth inhibitors, high cellular density (contact inhibition) and deprivation of serum. Cells monitor their external environment during the G1 phase of the cell cycle and, on the basis of sensed signals, decide whether to proliferate, to enter quiescence (from which they may reemerge on some future occasion when extracellular signals permit), or to permanently renounce their proliferative potential upon terminal differentiation and become senescent. Within a normal tissue, antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. However, if the mechanisms controlling the decision between cell cycle progression and cell cycle exit are not functional, cells bypass quiescence and undergo uncontrolled proliferation. This results in development of cancer and other diseases (Hanahan and Weinberg, 2000; Malumbres and Barbacid, 2001; Yusuf and Fruman, 2003). For example, in acute leukemia, the normal balance between quiescence and cell cycle entry of hematopoietic stem cells is perturbed (Lacorazza et al., 2006). Inappropriate exit from quiescence is also proposed to be an important early pathway associated with neuronal death and the entire spectrum of pathological events associated with Alzheimer's disease (Raina et al., 1999). In addition, the development of multiple sclerosis is related to the inability of the oligodendrocyte population to exit quiescence and regenerate remyelination of cells (Wolswijk, 1998). Therefore, a better understanding of quiescence would be beneficial for the treatment of various diseases. Unraveling the molecular mechanisms that underlie regulation of quiescence should provide important insights into how normal cells become tumorigenic and how new therapeutic strategies could be devised.

Quiescence of yeast *S. cerevisiae* has been likened to mammalian quiescence (Gray et al., 2004). The fundamental condition that signals yeast and other microorganisms to enter quiescence is nutrient starvation, which is commonly encountered in nature. Entry of cells into quiescence upon depletion of essential nutrients represents a survival strategy; quiescent microbial cells acquire a series of properties that protect them against harsh environmental conditions, enabling long-term viability. When nutrients become available, cells exit quiescence and resume growth. In the wild, yeast has to cope with long periods of nutritional shortage, alternating with short periods of food abundance. Therefore, these microorganisms enter quiescence as a strategy to survive on very low nutrient levels while retaining the ability to rapidly respond to sudden abundance of nutrients and efficiently reset their metabolism and growth rate to a rich environment. The optimal regulation of entry and exit from quiescence is an essential property for yeast in nature, as the ability to promptly react to external changes provides evolutionary advantage in a competitive environment.

Full understanding of microbial quiescence has important medical and industrial implications. The remarkable resistance of quiescent microbes to environmental stress causes difficulties in treatments of human microbial diseases with antibiotics. More insight into the quiescence state would therefore benefit strategies for combating pathogenic microorganisms. In addition, *S. cerevisiae* is widely used in the food and biotech industry and better understanding of yeast quiescence would improve such applications (Schuller and Casal, 2005). Finally, since quiescent microbes represent most of the biomass on Earth, quiescence studies also have significant agricultural and environmental implications.

S. cerevisiae as a model for quiescence

It is not yet known if *S. cerevisiae* and mammalian quiescence are equivalent. They certainly do share a number of important properties. This includes unreplicated genomes (Hartwell et al., 1974), characteristically condensed "G0" chromosomes (Pinon, 1978), increased rate of autophagy (Noda and Ohsumi, 1998) and reduced rate of protein synthesis (Fuge et al., 1994). Both mammalian and yeast cells enter a quiescence-like-state in response to the immunosuppressant rapamycin (Schmelzle and Hall, 2000). In addition, mammalian cells share with yeast the ability to respond to starvation by entering quiescence, as it is has been shown for serum deprived fibroblasts (Winkles, 1998).

We chose to study eukaryotic quiescence in *S.cerevisisae* for several reasons. First, *S.cerevisisa* is one of the best-studied eukaryotes and it is an excellent system for a variety of experimental analyses. The availability of the genome sequence and the ability to easily test gene function by genetic analysis makes it a tractable system for genome-wide studies. Second, the successful study of the active phases of the yeast life cycle has resulted in many insights into the understanding of eukaryotic cell cycle in general (Amon, 1998; Nasmyth, 1996; Nurse, 2000). Because of conservation of the basic cellular processes among eukaryotes, the study of quiescence in yeast is likely to illuminate the equivalent mechanisms and states in other eukaryotes. This is supported by the fact that pathways implicated in control of yeast quiescence, such as PKA, PKC and TOR have orthologs in mammals with similar regulatory roles.

S. cerevisiae stationary phase culture is comprised of quiescent cells

In *S. cerevisiae*, quiescence is most commonly induced by allowing a liquid culture of haploid cells to deplete an essential nutrient. As a consequence of nutrient depletion, the culture enters stationary phase (SP) and cells within the SP culture enter quiescence. The term "stationary phase " is therefore used to describe a nutrient starved, saturated culture of quiescent cells, where no further increase or decrease of cell number is observed. The term "stationary phase" thus refers to the state of the entire population and the term "quiescence" describes the state of cells in the stationary phase population.

In laboratories, stationary phase cultures are normally obtained by growing yeast strains in rich, glucose-based medium (yeast extract-peptone-glucose, YPD). Strains are grown to saturation, usually for five to ten days at 30°C. During this period, cultures exhibit several distinct phases of growth (Figure 1). After a short period of adjustment to the medium (lag phase, LP), cells divide exponentially (exponential phase, EP) using energy derived primarily from glucose fermentation. Following exhaustion of glucose, cells experience a transient cell cycle arrest while they adjust to respiratory metabolism (diauxic shift, DS). After the diauxic shift, cell division resumes, although at a reduced rate, during the post-diauxic growth phase (PD). This slow-growth phase, also called respiratory growth phase, is driven by respiration of the end-products of fermentation and other available carbon sources. The post-diauxic phase may last more then one week during which the culture density usually triples. When all available carbon sources are exhausted, cells cease to divide and become quiescent, and the culture enters stationary phase (SP). Thus, entry of *S. cereviesiae* into quiescence in liquid culture is a stepwise process, with various characteristics of the quiescent state acquired either at the diauxic shift or on final cessation of proliferation (Gray et al., 2004).

Limitation of nutrients other then glucose, including nitrogen, phosphate and sulfur can also signal cells to arrest growth and develop some stationary phase characteristics. However, the morphology of the cells arrested in these alternative ways is different from cells arrested by glucose depletion (Fuge and Werner-Washburne, 1997). Another important difference is that only cells limited for glucose are able to survive long-term starvation (Granot and Snyder, 1991; Granot and Snyder, 1993). Therefore, it has been

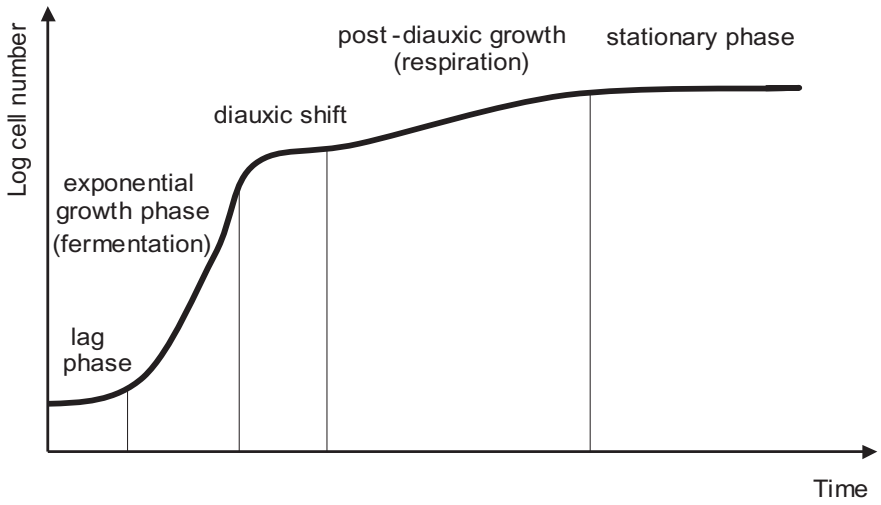

Figure 1. Growth phases of *S. cerevisiae*

When yeast cells are inoculated into a rich, liquid, glucose-based medium, the culture first passes through a short period of adjustment to the medium (lag phase), followed by the rapid cell proliferation fueled by glucose fermentation (exponential growth phase). Upon exhaustion of the fermentable sugar in the medium, the cells cease rapid proliferation and readjust their metabolism for utilization of non-fermentable carbon sources (diauxic shift). The consequent post-diauxic growth phase is driven by respiration and characterized by very slow cell proliferation. Finally, when all external carbon sources are exhausted, the culture reaches saturation and enters stationary phase, whereby the constituent cells enter quiescence.

implied that the "real stationary phase" can be established exclusively upon glucose starvation. In addition, cells seem to require complete (YPD) medium to properly enter stationary phase. When strains are grown to SP in minimal media, the kinetics of entry into stationary phase and viability over time is significantly affected (Fuge and Werner-Washburne, 1997). Cells in minimal medium grow more slowly during the exponential phase, exhibit a prolonged diauxic shift and enter SP earlier and at lower cell density then cells cultured in YPD. The importance of appropriate kinetics of SP entry is also demonstrated by experiments comparing abrupt versus gradual glucose limitation. Differences in gene expression under the two conditions suggest that cells employ distinct pathways in response to slow and rapid glucose depletion (Destruelle et al., 1994).

Characteristics of quiescent cells

The morphology of cells in stationary phase cultures is distinct from those of exponentially growing cells. Visualised by phase-contrast microscopy, quiescent cells grown in YPD appear as round, unbudded and bright cells, with large and prominent vacuole. The number of mitochondria and lipid vesicles is increased in these cells compared to their abundance in exponential phase (Fuge and Werner-Washburne, 1997). In quiescent cells, the cell wall becomes thicker, increasing resistance of cell to digestion by zymolase and to treatment with certain toxic drugs (de Nobel et al., 2000). Other developed means of protection from harsh environmental conditions are increased thermotolerance (Plesset et al., 1987) and resistance of quiescent cells to osmotic and oxidative stress (Blomberg et al., 1988; Izawa et al., 1996; Sousa-Lopes et al., 2004). Resistance

to environmental stress is in part mediated by trehalose that accumulates in quiescent cells (Francois and Parrou, 2001). Trehalose and glycogen are reserve carbohydrates with the obvious function in providing carbon and energy reserves for cells deprived from nutrients. However, trehalose has been shown to additionally function as an important protectant against a variety of stresses (Wiemken, 1990). A high content of trehalose increases thermotolerance (De Virgilio et al., 1994; Elliott et al., 1996) and protects cells from oxidative (Benaroudj et al., 2001), osmotic (Hounsa et al., 1998) and hydrostatic pressure stress (Iwahashi et al., 2000). In addition, it can protect native proteins from denaturation and also suppresses the aggregation of denatured proteins (Singer and Lindquist, 1998). One proposed mechanism for trehalose contribution to stress resistance is the ability of this carbohydrate to stabilize the structure of membranes (Francois and Parrou, 2001).

One of the most notable characteristics of quiescent cells is their decreased metabolic rate and biochemical activity. Despite this overall reduction, specific aspects of cellular metabolism are induced. This includes metabolic pathways involved in energy derivation, mainly from respiration and beta-oxidation of fatty acids. Also, glyoxalate pathway genes are found to be upregulated in cell cultures on entry into stationary phase (Gasch et al., 2000).

Similar to metabolism, overall transcription and translation rates of quiescent cells are severely reduced (Boucherie, 1985; Choder, 1991; Fuge et al., 1994). Reduction in transcription rate is largely caused by inactivation of ribosome biosynthesis, since in a rapidly growing yeast cell, 60% of total transcription is devoted to ribosomal RNA and 50% of RNA polymerase II transcription is devoted to ribosomal proteins (RPs) (Warner, 1999). However, a small subset of genes required for specific aspects of stationary phase is strongly induced (Werner-Washburne et al., 1996), and these mRNAs are efficiently translated (Dickson and Brown, 1998). In addition to the global reduction in biosynthesis, mRNA degradation and proteasome-dependent proteolysis also decrease in quiescent cells (Bajorek et al., 2003; Jona et al., 2000).

One process that is enhanced in stationary phase cells is autophagy (Nair and Klionsky, 2005). Autophagy is a degradation system, responsible for turnover of cellular components. It includes a membrane transport pathway leading from the cytoplasm to the vacuole in yeast (or to lysosomes in mammalian cells) for degradation and recycling. Under most conditions, autophagy is suppressed to a very low basal level, probably contributing to the turnover of cellular components at steady state. Some conditions, including starvation in yeast and hormonal stimulation in mammalian cells, can trigger dramatic induction of autophagy. Starvation activated autophagy is thought to contribute to maintenance of an amino acid pool for gluconeogenesis and for the synthesis of proteins that are necessary for survival (Gray et al., 2004). Efficient autophagy is essential for survival of quiescent cells, as autophagy-deficient mutants die rapidly under starvation conditions (Tsukada and Ohsumi, 1993).

Lastly, an important property of quiescent cells is their ability to respond to environmental signals other than carbon source availability. A variety of treatments, including heat shock, irradiation, oxidative and toxic stress, can induce the expression of similar genes in both quiescent and proliferating cells (Cyrne et al., 2003; Jelinsky et al., 2000). This shows that quiescence is a sensitive and actively maintained state, responsive to external stimuli.

Quiescence cycle

Our knowledge of quiescence in any organism including yeast is fragmented and the mechanisms that regulate entry into, maintenance of and exit from quiescence are poorly understood. Historically, one major factor limiting the study of quiescent cells has been their modest life style: standard biological, physiological and biochemical assays detect little or no activity in these cells. The thick cell wall, developed to protect yeast quiescent cells from environmental stress, obstructs lysis and makes preparation

of cellular extracts more challenging. As a result of these difficulties, quiescence has been long viewed as an uninteresting state, often considered as non-distinct from G1 arrest; or, if it was recognized as a distinct phase, expression of the quiescence program was simply interpreted as a downstream consequence of exit from the cell cycle. A number of studies have disproved this traditional view. In both mammals and yeast, quiescence has been confirmed to be an actively maintained, unique developmental state with its own program of control, rather than a default pathway in the absence of signal (Coller et al., 2006; Werner-Washburne et al., 1996; Yusuf and Fruman, 2003).

The decision whether to enter the cell division cycle or quiescence is made during the G1 phase of the cell cycle (Hartwell, 1974; Pardee, 1989) (Figure2). In the presence of sufficient nutrient supplies, a G1 cell passes START and enters the proliferative cell cycle (Hartwell, 1974). The subsequent removal of nutrients will not affect the progression of the cell cycle, which is driven by internal signaling of cyclin-dependent protein kinases (Nurse et al., 1998). In the absence of sufficient carbon source, a G1 cell is unable to pass START and enters quiescence (G0). Quiescence is separate, out-of-cell cycle state, as it is shown by identification of mutants that specifically lose viability in stationary phase or are unable to reproliferate from quiescence (Drebot et al., 1987). When nutrients are resupplied, cells are triggered to exit from quiescence and the proliferative cell cycle resumes (Gray et al., 2004) (Figure 2).

Entry into quiescence is triggered when a proliferating cell senses carbon limitation. In contrast, exit from quiescence is induced in an entirely different cellular state (quiescence), upon sensing the presence of a carbon source. By analogy to the proliferative cell cycle, entry into, maintanance of, and exit from quiescence are

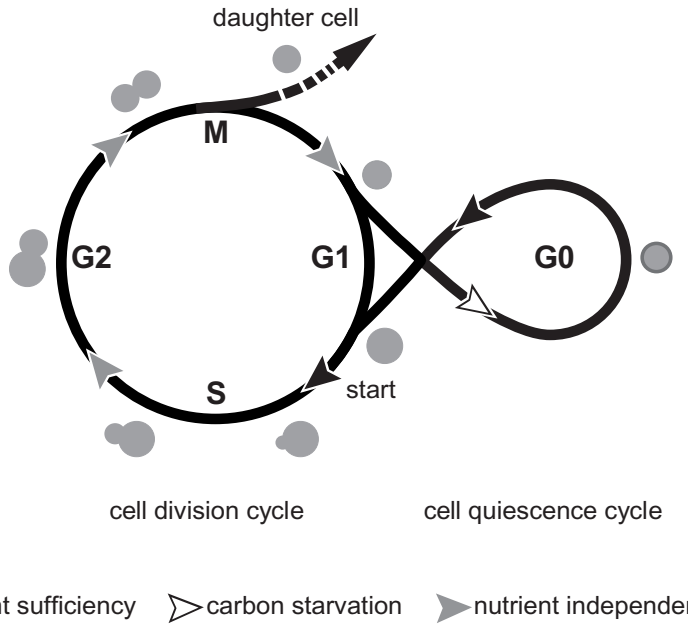

Figure 2. *S. cerevisiae* cell quiescence cycle and its relationship to the cell division cycle

The decision of cells whether to enter the division cycle or quiescence is made during the G1 phase of the cell cycle. In the presence of sufficient nutrient supplies, G1 cells pass START and enter the proliferative cell cycle. The further progression of the division cycle is driven by internal signals and it is nutrient- independent. If the medium is deficient in carbon source, G1 cells are unable to pass START, and instead enter quiescence (G0) cycle. Cells remain quiescent until nutrients again become available in the medium. Re-addition of nutrients triggers the exit from G0, leading to completion of the cell quiescence cycle and consequently, resumption of the cell division cycle.

regarded as the cell quiescence cycle (Gray et al., 2004; Pardee, 1989) (Figure 2). The obvious difference between these two processes is that cell division cycle results in a doubling of cell number, whereas the quiescence cycle does not. However, in both proliferative and quiescence cycles, each turn of the cycle changes the state of the starting cell. As a result of either cycle, the cell becomes older: it undergoes replicative ageing (i.e. the mother cell becomes one generation older) as the result of proliferative cycle, or experiences chronological ageing (i.e. shortening the period of viability in stationary phase) as the consequence of quiescence cycle (Ashrafi et al., 1999; Bitterman et al., 2003). Studies of *S. cerevisiae* quiescence are therefore also regarded as a highly informative model for the study of life span regulation.

Full understanding of the cell quiescence cycle would require identification of specific factors that regulate each distinct phase of this cycle. Analysis of mutants defective in key transitions of the cycle is a powerful method for identification of such regulators. Three subclasses of "quiescence" mutants are proposed to exist, all of which may be defective in one or more transition of the cell quiescence cycle (Gray et al., 2004). The first subclass of quiescence mutants includes those defective in entry into stationary phase. Failure to properly enter quiescence diminishes the ability of these mutants to remain viable when starved, and consequently, they will die upon nutrient depletion. The second proposed subclass includes maintenance mutants, which successfully enter quiescence but are unable to maintain long-term viability in that state. Both entry and maintenance mutants are characterized by decreased viability in stationary phase, however, this lethality has distinct causes: entry mutants fail to establish quiescence and to acquire protective properties that are essential for survival, and maintenance mutants acquire the key characteristics of quiescent cells, but can not survive long starvation periods. The third subclass of proposed quiescence mutants includes those that are defective in the exit from quiescence. These mutants are viable during stationary phase but unable to generate colony forming units (CFU) upon refeeding. The distinction of mutants defective in individual phases of the quiescence cycle has been rarely addressed (Drebot et al., 1990; Drebot et al., 1987), and in systematic screens quiescence mutants have usually simplistically been defined as those that lose viability when cultured to stationary phase.

Signaling pathways regulating entry into stationary phase

The precise signaling pathways that sense nutrient starvation and subsequently regulate the entire cascade of events that lead to quiescence are still unknown. However, several pathways that control cellular changes during diauxic shift and environmental stresses are shown to contribute to acquisition of numerous characteristics of quiescent cells. Metabolic changes that occur during diauxic shift are considered to be a necessary precursor for the establishment of nonproliferative quiescent state, thus it is likely that regulation of the signaling network that acts at the diauxic shift also promotes the final entry into quiescence. Four signaling pathways have been implicated to be involved in regulation of entry into quiescence (or similar state). This includes the protein kinase A (PKA) and TOR (Target Of Rapamycin) pathways, which act as inhibitors of quiescence, and Protein Kinase C (PKC) and Snf1p pathways that act as positive regulators of quiescence (Gray et al., 2004) (Figure 3).

The Ras/PKA (cAMP - dependent protein kinase) pathway coordinates cell cycle progression and metabolic activities with respect to nutritional status, and it is conserved in all eukaryotes (Edelman et al., 1987; Walsh and Van Patten, 1994). The PKA pathway is regulated by cellular cAMP concentration, which is controlled by Ras1 and Ras2 GTPases. In response to the presence of glucose, the Ras pathway promotes cAMP synthesis and elevated cAMP levels act as the signal for induction of the PKA pathway, which in turn stimulates growth via its downstream effectors. Upon glucose depletion, cAMP levels drop and the PKA pathway and consequently,

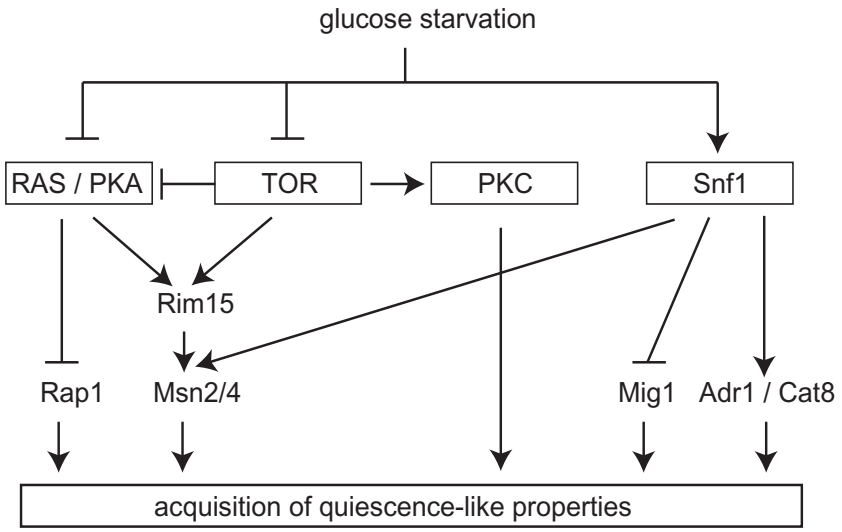

Figure 3. Overview of known signaling pathways controlling aspects of entry into quiescence

Nutrient starvation inactivates the TOR and RAS/PKA pathways, which act as the central controllers of growth in *S. cerevisiae*. This inactivation leads to acquisition of many properties of quiescent cells, achieved in part via subsequent induction of the PKC pathway. In addition, glucose starvation activates the Snf1 pathway, which facilitates the switch from fermentative to respiratory metabolism, necessary for entry into quiescence. Some of the known downstream effectors of RAS/PKA, TOR and Snf1 pathways are also shown (see the section: "Transcription regulation of quiescence").

growth, are inhibited (Thevelein and de Winde, 1999). Mutants lacking PKA activity are unable to proliferate and they arrest in a quiescence-like state (Boutelet et al., 1985; Broek et al., 1987; Tripp and Pinon, 1986; van Aelst et al., 1991). They acquire some of the properties of quiescent cells, including accumulation of storage carbohydrates and increased resistance towards heat and oxidative stress (Broach and Deschenes, 1990; Tatchell, 1986). In contrast, constitutive activation of adenylate cyclase activity, e.g. by inactivation of suppressive subunit of the PKA complex (Bcy1), causes cell death at the diauxic shift (Cameron et al., 1988; Cannon and Tatchell, 1987; Werner-Washburne et al., 1989). Therefore, proper downregulation of the PKA pathway is necessary for a successful transit to the diauxic phase (and subsequently quiescence) (Matsumoto et al., 1985; Shin et al., 1987; Uno et al., 1982). On the other hand, activation of PKA is required for efficient exit from quiescence, as mutants with low PKA activity exhibit a long delay in reentering the cell cycle when nutrients are replenished (Jiang et al., 1998). Ras/PKA is suggested to control entry into and exit from quiescence via regulation of the global transcription shutdown that occurs in this state. This regulation may be achieved through multiple mechanisms, including control of chromatin structure, the general transcription factor TFIID, carboxy-terminal domain (CTD) of RNA Polymerase II (Pol II) and components of the Mediator complex of Pol II holoenzyme (Howard et al., 2002; Howard et al., 2001).

A second signaling network implicated in regulation of quiescence is the TOR pathway. TOR components are phosphatidylinositol kinase-related proteins and are structurally and functionally conserved in all eukaryotes (Cutler et al., 1999; Schmelzle and Hall, 2000). In yeast, two TORs, Tor1 and Tor2 control many growth-related processes in response to environmental conditions (Cardenas et al., 1999; Crespo et al., 2002; Hardwick et al., 1999; Komeili et al., 2000; Schmelzle and Hall, 2000). Both rapamycin treated and TOR-inactivated yeast cells display several characteristics similar to those in quiescent cells. They arrest as unbudded cells

with unreplicated DNA, transcription of rRNA and tRNA is repressed by inhibition of RNA polymerases I and III, and protein synthesis is reduced to 50% of exponentially growing cultures. Downregulation of TOR also induces expression of many quiescence-characteristic stress response genes, and stimulates autophagy and accumulation of storage carbohydrates (glycogen and trehalose) (Schmelzle and Hall, 2000). TOR is therefore proposed to repress the quiescence program when nutrients are abundant, while upon starvation, inactivation of TOR would lead to derepression of the quiescence program and consequently entry into quiescence (Crespo and Hall, 2002; Duvel and Broach, 2004; Lorberg and Hall, 2004; Powers et al., 2004; Rohde and Cardenas, 2004; Schmelzle and Hall, 2000).

In addition to its redundant function with Tor1 in a rapamycin-sensitive signaling pathway, Tor2, a homolog of mammalian phosphatidylinositol kinases, also has a specific rapamycin-insensitive function in the cell cycle dependent polarization of the actin cytoskeleton (Schmidt et al., 1996). This unique regulatory function of Tor2 is executed by activation of PKC1, which signals to the cytoskeleton via a MAP kinase cascade (Helliwell et al., 1998). The yeast protein kinase C (PKC) pathway is a third pathway in regulation of quiescence and it is essential for the maintenance of cell integrity in response to environmental stresses. The PKC pathway is negatively regulated by TOR, thus upon TOR inactivation by starvation, the Pkc1 pathway is activated and promotes remodeling of the cell wall, providing resistance to cell wall digesting enzymes. Mutants defective in Pkc1-MAP kinase pathway die during carbon or nitrogen starvation and during stationary phase, underscoring the necessity of formation of the proper cell wall structure for establishment of quiescence (Krause and Gray, 2002).

Another positive regulator of entry into quiescence is the Snf1 signaling pathway. Snf1 is a yeast homologue of AMP activated protein kinase (AMPK) (Hardie et al., 1998). Both mammalian AMPK and yeast Snf1 are activated when ATP/AMP ratio drops, as occurs during glucose starvation. Activation of the Snf1 pathway derepresses genes required for the ATP-generating metabolic pathways and for use of alternative carbon sources (Wilson et al., 1996). In addition, Snf1 also has a role in glycogen metabolism (Cannon et al., 1994) and it is positive regulator of autophagy (Wang et al., 2001). Mutants lacking *SNF1* are unable to utilize alternative carbon sources such as ethanol and glycerol (Hardie et al., 1998) and die soon after the diauxic shift, suggesting that adaptation to poor carbon through the Snf1 pathway is indispensable for proper entry into quiescence.

Interactions among signaling pathways involved in quiescence regulation appear to form a complex network that acts at the diauxic shift in response to changes in carbon quality. Several common effectors of the PKA and TOR pathway have been described, including the Msn2 and Msn4 transcription factors that regulate stress-responsive genes (Beck and Hall, 1999; Cameroni et al., 2004; Monteiro and Netto, 2004; Thevelein and de Winde, 1999) and the Rim15 protein kinase that activates the diauxic-shift response (Cameroni et al., 2004; Pedruzzi et al., 2003) (Figure 3). Recent studies propose that the Ras/PKA pathway might be acting downstream of TOR (Schmelzle et al., 2004). Also, Snf1 function converges with both PKA and TOR pathways in modulating various outputs, including activity of transcription factors Gln3, Msn2p/Msn4 and Adr1 (Bertram et al., 2000; Denis and Audino, 1991; Mayordomo et al., 2002; Taylor and Young, 1990), autophagy and peroxisome function (Budovskaya et al., 2004; Igual and Navarro, 1996; Navarro and Igual, 1994; Wang et al., 2001). It is likely that each of the described signaling pathways contributes to establishment of quiescence by regulating specific aspects of the starvation response and providing stepwise adaptation of cells to nutrient deficiency.

Transcription regulation of stationary phase

Entry into quiescence is accompanied by global changes in gene expression that facilitate adjustment of cells to starvation and promote a long-term survival program. Lack of nutrients

acts as a signal that activates receptors on the cell surface, consequently triggering specialized signaling cascade that ultimately relays the information to the nucleus, where the regulation of gene expression can be executed.

Transcription of protein-coding genes is a complex process that can be regulated through various mechanisms, including sequence-specific DNA binding factors, the RNA polymerase II transcriptional machinery, chromatin-remodeling factors, and a variety of enzymes that catalyse the covalent modification of histones and other proteins (Lee and Young, 2000). Rapid and accurate transcription response to environmental challenges is an essential feature of eukaryotic cells and the program of transcription regulation is of particular interest for understanding the processes underlying entry into, maintenance of and exit from quiescence. However, regulatory mechanisms that control transcription reprogramming in quiescence are largely uncharacterised. A general shut-down of transcription by RNA polymerase II has been described, but the mechanism underlying the inhibition of gene expression in quiescence is poorly understood.

One proposed mechanism attributes the global transcriptional shut-down to changes in DNA topology, which are governed by topoisomerase I (Choder, 1991). Cells lacking the gene that encodes topoisomerase I (*TOP1*), grow and transcribe their genomes normally during exponential growth, but are defective in the transcription repression that normally takes place in stationary phase. Following entry into quiescence, these mutants continue to transcribe most of genes relatively efficiently. The manner in which Top1 promotes transcription inhibition in quiescence is not known; it is possible that this is achieved through changes in DNA topology, which modifies chromatin structure that in turn results in the transcriptional repression. This possibility is supported by the fact that chromatin structure of stationary phase differs from the one of exponentially growing cells (Lohr and Ide, 1979). However, *top1* mutants do not lose viability when cultured to late stationary phase, showing that Top1 activity is not essential for successful quiescence. This suggests that topoisomerase I mediated repression is not the only mechanism that contributes to global transcriptional shut-down necessary for long-term survival (Choder, 1991). Changes in DNA topology observed during quiescence may in fact be the consequence of global transcription shut-down, rather then the cause.

Other proposed mechanisms of transcription inhibition in quiescence suggest that the composition and/or state of the Pol II holoenzyme might be the key determinant in regulation of gene expression. In eukaryotes, there are three RNA polymerases of which RNA polymerase II (Pol II) transcribes mRNAs for protein coding genes and a number of small nuclear RNAs. Pol II is conserved from yeast to humans and it is composed of 12 subunits (Rpb1 to Rpb12), which are all required for normal growth (Lee and Young, 2000). However, the Rpb4 subunit has some unique features: as for its interacting partner Rpb7, but in contrast to other subunits, the stoichiometry of Rpb4 in the Pol II complex is subjected to changes in response to environmental stimuli. As the cells encounter heat shock or enter the slow growth phase prior to quiescence, the proportion of Pol II molecules that contain the Rpb4 subunit increases (Choder and Young, 1993). Rpb4 is required for transcription activity and viability exclusively during stress, and it is proposed to stabilize the structure of the entire Pol II enzyme under unfavorable conditions (Choder and Young, 1993). Therefore, it is possible that Rpb4 also has a role in regulation of quiescence. This regulation could be achieved in cooperation with Rpb7, since Rpb4 functions as the enhancer of the interaction of Rpb7 with Pol II and overexpression of Rpb7 supresses the phenotype of *rpb4* deletion mutant (Sheffer et al., 1999).

Another essential and highly conserved feature of Pol II is a unique carboxy-terminal domain (CTD) of the largest subunit Rpb1, which consists of tandem repeats of a consensus heptapeptide sequence. The CTD is subject to regulation by phosphorylation, and many functions of Pol II are dependent on the phosphorylation pattern of the CTD. Generally, Pol II molecules engaged

in transcription have a multiple phosphorylated CTD, and this pattern changes in response to environmental stress and entry into stationary phase (Patturajan et al., 1998). Thus, a global shut-down of transcription in quiescence may be reflected in the overall extent of CTD phosphorylation. The Ras/PKA pathway has been implicated in regulation of Pol II activity via the CTD during stationary phase, but it is not clear if its control mechanism involves phosphorylation (Howard et al., 2002).

Pol II promotes transcription by acting in concert with general transcription factors (GTFs) and the multisubunit Mediator complex. Loss of general transcription factors (such as TFIID) during stationary phase has been proposed to influence the global transcriptional shut-down that occurs in quiescence (Walker et al., 1997). Also, there is evidence that several subunits of the Mediator complex (Med12, Med13, Cdk8 and CycC) are required for the normal transcriptional response to nutrient limitation and proper entry into stationary phase (Chang et al., 2001). The Mediator complex has a function in integrating signals from gene specific transcription factors at promoters, and its composition may change as cells encounter new environments to allow coordinate control of specific sets of genes (Lee and Young, 2000). Some Mediator subunits are shown to be repressors of a number of genes that are normally induced during stationary phase entry. Mutants of *MED12*, *MED13, CDK8* and *CYCC* exhibit constitutive expression of quiescence-specific genes in exponential phase, are unable to undergo normal growth arrest and assume many of the characteristics normally associated with stationary phase cells (Chang et al., 2001).

In addition to regulation trough the general transcription machinery, gene expression is also controlled through gene-specific transcription factors (TFs). These sequence-specific DNA binding factors are positioned on transcription factor binding sites at upstream activating sequences (UAS), promoters or on distal transcriptional regulatory elements, such as enhancers of eukaryotic genes (Lee and Young, 2000). Gene specific DNA binding transcription factors largely affect the specificity of the transcriptional response. Genes with similar function often share common DNA motifs upstream of their transcription start sites, which can be recognized by specific DNA binding factors, so that entire sets of genes required for a response to a specific condition can be synchronously regulated. Thus, sequence-specific TFs interpret and transmit information that is encoded in the primary DNA sequence to the factors and cofactors that mediate the synthesis of RNA transcripts from the DNA template, acting as a key interface between genetic regulatory information and the transcription system (Kadonaga, 2004).

Multiple factors act coordinately in regulating transcriptional response to the environmental stimuli. The complete network of transcription factors that regulate quiescence has not been studied yet. Although “quiescence-specific” transcription factors remain elusive, TFs that promote the diauxic shift transition and stress response probably do have a role in regulating at least certain aspects of quiescence. Examples of these are Rap1 and Msn2 and Msn4 (Msn2/4) transcription factors, involved in the general environmental stress response (ESR) (Gasch et al., 2000). Rap1 is a regulator of ribosomal protein (RP) genes, and it is responsible for the high level of expression of the RP genes in actively growing cells (Morse, 2000). It is not clear how Rap1 activity changes in stationary phase, however, RP genes need to be repressed in quiescence and it is possible that this occurs via the regulatory function of Rap1. Msn2 and Msn4 are homologous transcription factors acting as positive regulators of stress and starvation induced genes. They belong to the zinc-finger family of TFs (Estruch and Carlson, 1993) and bind the STRE sequence (CCCCT) found in the UAS of stress-responsive genes. Msn2/4 have a broad effect on gene expression during stress: they regulate 90% of genes induced in ESR (Causton et al., 2001; Gasch et al., 2000). However, the extent of their involvement varies under each set of environmental conditions and it remains to be discovered what is the role of these factors in establishing quiescence.

Apart from the stress response, perturbation of metabolism is another important quiescence-related event that is regulated by transcription factors. Most of the metabolic changes occur in the diauxic shift, during the transition to a poor carbon source, and it is assumed that these changes are a necessary adaptation to entry into quiescence. A central glucose-responsive repressor is Mig1, which represses the transcription of gluconeogenic genes, respiratory genes and genes involved in utilization of carbon sources other then glucose (Carlson, 1999; Klein et al., 1998). All of these processes are required for quiescence; therefore, repression of Mig1 is probably needed for proper entry into quiescence and reactivation of Mig1 for the exit from quiescence. In addition, there are several positive regulators of the diauxic shift transition, which are probably also positively regulating entry into quiescence. Examples of these are Hap4, Adr1 and Cat8. Hap4 binds a sequence known as the CCCAAT box and it has been shown to be responsible for induction of genes involved in respiration and the tricarboxylic acid cycle (Forsburg and Guarente, 1989; Lascaris et al., 2003). Adr1 and Cat8 are both carbon source-responsive zinc-finger transcription factors required for derepression of the glucose-repressed genes. These two factors act as coregulators for some of their target genes (Tachibana et al., 2005), but also have separate functions: Adr1 is involved in induction of alcohol dehydrogenase, peroxisomal protein genes and genes required for ethanol, glycerol and fatty acid utilization (Simon et al., 1991; Tachibana et al., 2005), and Cat8 activates gluconeogenic and glyoxylate cycle genes (Hedges et al., 1995).

Overall knowledge about transcription under starvation and stress conditions is still obscure compared to the understanding of gene expression during optimal growth. Transition between active proliferation and quiescence is likely to involve the extensive reprogramming of regulatory networks and remodeling of most if not all intracellular structures and processes. The ultimate challenge in deciphering quiescence regulation would be to define a core quiescence program that prevents cell growth and proliferation, that provides cells with the ability to survive better under harsh conditions, and that allows a rapid transition back to the proliferating state when conditions again become favourable. The complexity of the transcription response to starvation is only beginning to unfold and this is to a great extent facilitated by the development of genome-wide techniques.

Genome-wide studies of transcription regulation

Advances in high-troughput DNA-sequencing technology during the late 1980s facilitated development of genomics approaches to studying genes and their function. In 1996, the complete 12-megabase (Mb) sequence of the *Saccharomyces cerevisiae* was released (Goffeau et al., 1996), followed by rapidly increasing number of genome sequences for other eukaryotic organisms. The availability of complete genome sequences has created a demand for novel techniques to emerge, which would enable analysis of gene functions that are as comprehensive as the genome sequences themselves. A major breakthrough in this field was the development of DNA microarrays (Chee et al., 1996; Schena et al., 1995). Consisting of thousands of individual gene sequences printed in a high-density array on a glass microscope slide (Schena et al., 1995; Shalon et al., 1996), DNA microarrays provide a practical and economical tool for analyzing thousands of DNA samples (or entire genomes) in a single, hybridization-based assay (DeRisi et al., 1996; Lockhart et al., 1996).

The classical application of DNA microarrays is in monitoring relative levels of mRNA transcripts on a genome-wide scale (transcriptome analysis). The whole-genome expression profiling involves differential labeling (usually by fluorescent dyes Cy3 and Cy5) of two population of nucleic acid, derived from two experimental conditions (Figure 4A). Both labeled sample populations are co-hybridized to the DNA array and the fluorescence intensity of each spot on the array is measured. The ratio of Cy5 to Cy3 signal per spot provides the quantitative indication of the

relative abundance of each target sequence in the two sample populations, identifying genes expressed differentially under two experimental conditions (Kumar and Snyder, 2001). In this way, transcriptional response of each gene to a change in cellular state can be measured, whether it is disease, a cellular state such as cell division cycle or a response to chemical or genetic perturbation.

In *S. cerevisiae*, genome-wide expression

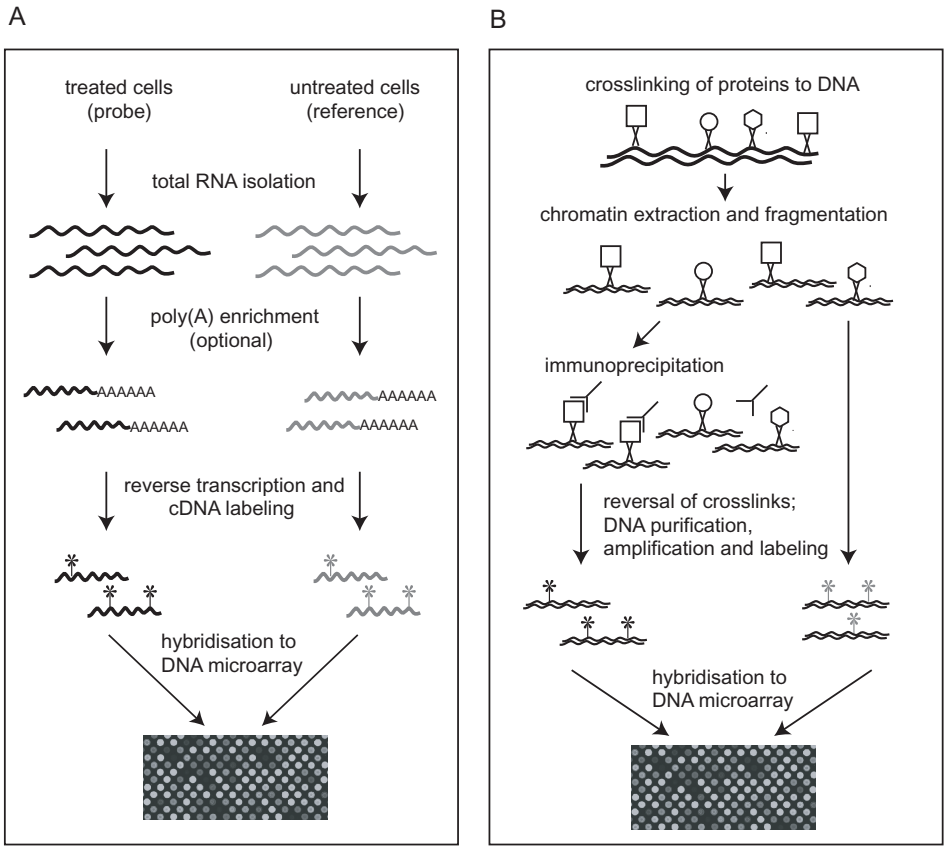

Figure 4. Utilization of DNA microarrays for genome-wide expression profiling and location analysis

(A) Genome-wide expression profiling typically includes the following steps: RNAs from treated and untreated cells are prepared in parallel and reverse transcribed into cDNAs. The obtained cDNAs are further differentially labeled with fluorescent dyes and co-hybridized on the DNA microarray containing DNA fragments that represent all or part of gene coding regions. The intensity of the signals measured on each spot of the microarray indicates the relative enrichment of the transcript abundance for the target sequence, identifying genes expressed differentially under the two experimental conditions.

(B) To investigate genome-wide location of DNA binding proteins, protein-DNA interactions are captured *in vivo* by crosslinking proteins to their genomic binding sites (usually by formaldehyde). After chromatin extraction, crosslinked samples are sonicated to generate DNA fragments of 200-600 base pairs (bp) in length. One part of the sample is further subjected to immunoprecipitation with the antibody directed against the protein of interest, while the remainder of the sample is not purified and can be used as the reference sample. After reversal of the crosslinks, DNAs are amplified, differentially labeled and co-hybridised to the microarray containing intergenic (or both genic and intergenic) regions. The ratio of target sample to reference sample at each array spot provides an indication of the binding frequency of the protein of interest to each corresponding genomic locus.

profiling has been used to describe transcriptome changes associated with many cellular processes. This has included pioneering work describing the transition from exponential growth to DS (DeRisi et al., 1997), cell cycle transitions (Cho et al., 1998; Spellman et al., 1998), sporulation (Chu et al., 1998), response to various environmental changes (Gasch et al., 2000), DNA damaging agents (Jelinsky and Samson, 1999), and starvation for diverse nutrients (Boer et al., 2003).

Genome-wide expression profiling can also be more directly used for elucidating mechanisms of transcription regulation. Transcriptome analyses identify global mRNA changes that indicate the level of transcriptional activity under particular condition. In addition, gene-specific changes can be monitored to determine which transcription factors are differentially expressed under given condition. These factors can be then further examined by expression profiling of their deletion mutants or by overexpression, identifying all genes that exhibit changes in mRNA levels upon inactivation or overexpression of this transcription factor. This approach has been used for identification of targets for components of general transcriptional machinery (Holstege et al., 1998; Kobor et al., 1999) and many gene-specific transcription factors (Hughes et al., 2000; Lyons et al., 2000; Natarajan et al., 2001; Pan and Heitman, 2000). The expression profiles can also be used to identify transcription factor binding sites, as these sequences will be statistically enriched upstream of genes co-regulated in response to the activation or inactivation of a given transcription factor (Wyrick and Young, 2002). However, detected expression changes in these assays are in part result of canonical regulation of multiple factors, and some of the targets can be regulated by factors acting downstream of the mutated transcription factor. Thus, only a portion of the identified target genes represents direct targets of the transcription factor. Microarray expression profiling on its own is insufficient to discriminate between direct and secondary effects of transcription regulators.

A more effective approach for identification of direct targets and regulatory motifs of transcription factors is to determine transcription factor location on DNA *in vivo* by chromatin immunoprecipitation (ChIP). The ChIP procedure is used to study protein-DNA interactions (Orlando, 2000) and it can be coupled to microarray tehnology (ChIP on chip) to obtain a panoramic view of protein binding throughout the genome (Ren et al., 2000) (Figure 4B). In this approach, cells are treated with formaldehyde to crosslink DNA-binding proteins to their target sites *in vivo.* Crosslinked DNA is extracted and sheared by sonication, and DNA bound to a transcription factor of interest is subsequently purified by immunoprecipitation using antibodies against the chosen transcription factor. After reversal of crosslinks, this purified DNA is amplified, fluorescently labeled by PCR, and co-hybridized to microarrays with differentially labeled reference DNA (obtained from non-immunoprecipitated whole cell extracts or a “mock” immunoprecipitation). The ratio of fluorescence intensities measured at each element in the array is indicative of the relative enrichment of each target sequence after immunoprecipitation, and, therefore, indicates the extent to which each site is bound *in vivo* (Ren et al., 2000). Ideally, hybridizations are performed on microarrays that contain the entire genome sequence, so that binding of the factor can be monitored on both intergenic and genic regions of the genome (Pokholok et al., 2005).

Genome-wide location analyses have been used for detection of targets of gene-specific transcription factors (Lieb et al., 2001; Ren et al., 2000) and general transcriptional machinery components (Harismendy et al., 2003; Kim and Iyer, 2004; Moqtaderi and Struhl, 2004; Zanton and Pugh, 2004). The most comprehensive assay to date has been performed by Harbison and colleagues (Harbison et al., 2004). These authors investigated genomic occupancy of 203 *S. cerevisiae* DNA-binding transcription regulators in rich media, and 84 of these were also assayed in an alternative environmental condition. This work highlighted the need for introducing environmental perturbations as a means to increase the range of transcription factors targets and to uncover new biological roles that cannot be detected under standard

conditions.

Genome-wide expression profiling, location analysis and computational identification of DNA biding motifs are the commonly used approaches for identification of transcription factor target genes and the circuitry that is responsible for transcription regulation (Wyrick and Young, 2002). When solely employed, each of these approaches has pitfalls that can be compensated by integration of methods. For example, expression profiling identifies both direct and indirect targets of the regulators, which can be further discriminated by location analysis. Conversely, knowledge of the location of a transcription factor on a target promoter provides no information regarding its function, and this can be deduced from up- or down-regulation of the target's mRNA. In addition, multiple transcription factors may recognize the same DNA sequence, causing failure of computational sequence analyses to distinguish targets of highly related factors. This can be compensated by high, antibody-based specificity of genome-wide location screens. Therefore, the most powerful approaches towards identification of complete sets of transcription factor target genes and unraveling underlying transcriptional regulatory networks are the ones that combine several strategies, merging information obtained under a variety of conditions through multiple genome-wide methods (Banerjee and Zhang, 2002; Blais and Dynlacht, 2005; Chua et al., 2004; Holstege and Clevers, 2006).

Outline of this thesis

The aim of the work described in this thesis is to investigate transcription regulation during the transition between proliferation and quiescence in *S. cerevisiae*, employing genome-wide approaches. The starting point, presented in **Chapter 2**, is a time-course DNA microarray study describing global and gene specific genome-wide mRNA expression changes that occur during entry, maintenance and exit from stationary phase. The observed global changes prompted a further investigation of the role of RNA polymerase II in the transcriptional control of these growth transitions. To correlate global changes with the activity of the general transcription machinery, we examine the composition and the genome-wide location of RNA polymerase II in exponential and stationary phase of growth, and subsequently, propose a novel mechanism of transcription initiation upon exit from quiescence. In **Chapter 3**, we shift the focus of the study from global to gene specific expression changes. We analyse functions and phenotypes of genes co-induced during entry into and exit from stationary phase to identify the most important biological processes and genes required for a successful quiescence cycle. Further, in **Chapter 4**, we use the collection of gene expression profiles to identify a novel gene-specific transcription factor, Yjl103c, and propose its involvement in regulation of entry into stationary phase. We employ combined genome-wide approaches, including DNA microarray expression profiling, genome-wide location analysis and DNA motif searches, to identify a complete set of targets and processes regulated by this transcription factor. The overall findings that emerge from these studies and their impact on the current understanding of the quiescent state are discussed in the final section, **Chapter 5**.

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Chapter 2

Genome-wide expression and location analyses reveal RNA polymerase II located upstream of genes poised for response upon *S. cerevisiae* stationary phase exit

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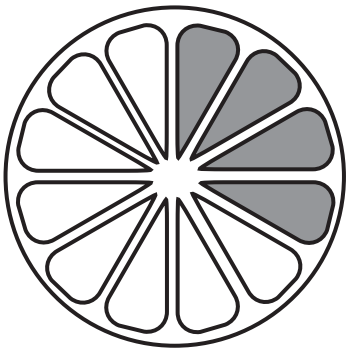

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Genome-wide expression and location analyses reveal RNA polymerase II located upstream of genes poised for response upon *S. cerevisiae* stationary phase exit

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Summary

The resting state of eukaryotic cells (G0) is relatively uncharacterized. We have applied DNA microarray expression-profiling of *S. cerevisiae* to reveal multiple transitions during a complete 9-day growth cycle between stationary phase (SP) exit and entry. The findings include distinct waves of transcription after the diauxic-shift, identification of genes active in SP and upregulation of over 2500 genes during the first minutes of lag-phase. This provides a framework for analyzing large-scale reprogramming of gene expression. Despite global repression, the general transcription machinery is found to be present in quiescent cells, but largely inactive. Genome-wide location analysis by ChIP on chip reveals that RNA polymerase II is more predominantly bound at intergenic regions in SP, upstream of hundreds of genes immediately induced upon exit. In contrast to current models of activation-coupled recruitment, the results show that RNA polymerase II is located and maintained upstream of many inactive genes in quiescence.

Introduction

Eukaryotic cells reside for most of their life-span in a resting state, also known as quiescence or G0. An important property of quiescent cells is the maintained potential for proliferation. Studying quiescence control is important for understanding cancer, development and aging (Herman, 2002; Malumbres and Barbacid, 2001; Yusuf and Fruman, 2003). In unicellular organisms, quiescence confers increased protection against harsh treatment (Gray et al., 2004). More insight into the quiescent state would therefore also benefit strategies for combating pathogenic microorganisms.

The budding yeast *S.cerevisiae* has developed several ways of surviving harsh conditions. Nutrient depletion of haploid cells in liquid media results in stationary phase (SP) cultures, consisting of quiescent cells that have been likened to mammalian G0 (Gray et al., 2004; Herman, 2002). Our understanding of the cycle between proliferation and quiescence is obscure in comparison to knowledge of the active cell cycle of yeasts. Most progress has been made in identifying signal transduction pathways such as TOR, PKA and PKC, which are probably linked to quiescence through their regulation of nutrient limitation (Wilson and Roach, 2002). These are evolutionary conserved pathways and although the extent of similarity remains to be determined, it is likely that increased understanding of *S. cerevisiae* quiescence will also shed light on eukaryotic quiescence in general.

Effects downstream of signal transduction pathways during the transition to and from quiescence in *S. cerevisiae* are not well established. Of particular interest is the program of transcription regulation. Whereas studies

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of actively proliferating yeast cells and their responses to environmental changes have been beneficial towards developing models of eukaryotic transcription (Hampsey, 1998), gene expression during quiescence has been poorly characterized. In SP, a general shut-down of transcription by RNA polymerase II (Pol II) has been described which may be attributed to changes in DNA topology (Choder, 1991) or loss of general transcription factors such as TFIID (Walker et al., 1997). At what point does transcription shut-down occur? Are there genes that escape repression? How is the transcription apparatus reprogrammed for proliferation upon exit from quiescence? What is the sequential order of events during the return to proliferation?

Addressing these questions has in part been confounded by different methods of inducing quiescence, insufficient expression markers and lack of mutants with quiescence-associated phenotypes (Gray et al., 2004; Herman, 2002). In laboratories, quiescence can be induced by allowing haploid cells to deplete an essential nutrient. The ensuing stationary-phase (SP) cultures consist mostly of quiescent cells, similar to mammalian G0 in their potential to restart proliferating, smaller size, lower metabolic and transcriptional activity (Gray et al., 2004; Yusuf and Fruman, 2003). Allowing cells to deplete glucose is the most frequently used approach. When inoculated in rich media supplemented with glucose, cells first pass through a period of rapid fermentative growth on glucose, then adjust their metabolism to other carbon sources during the non-proliferative diauxic shift (DS). This is followed by slow respiratory growth, until complete carbon source depletion results in cells entering quiescence (Figure 1A).

DNA microarrays have been used to determine changes in gene expression associated with many cellular processes. This has included pioneering work describing the transition from exponential growth to DS (DeRisi et al., 1997), as well as to late post-diauxic growth/early quiescence (Gasch et al., 2000). Exit from quiescence has also recently been examined (Martinez et al., 2004). The full series of events including exit, proliferation and entry into quiescence have not been described in high temporal resolution in a single experiment. Furthermore, in most microarray studies, normalization of gene expression data is based on artificially balancing up-regulated genes with down-regulated genes, regardless of whether global shifts may be taking place (van de Peppel et al., 2003). This has confounded quantitative descriptions of events during major transitions, which may be important for understanding mechanisms of large-scale gene expression reprogramming.

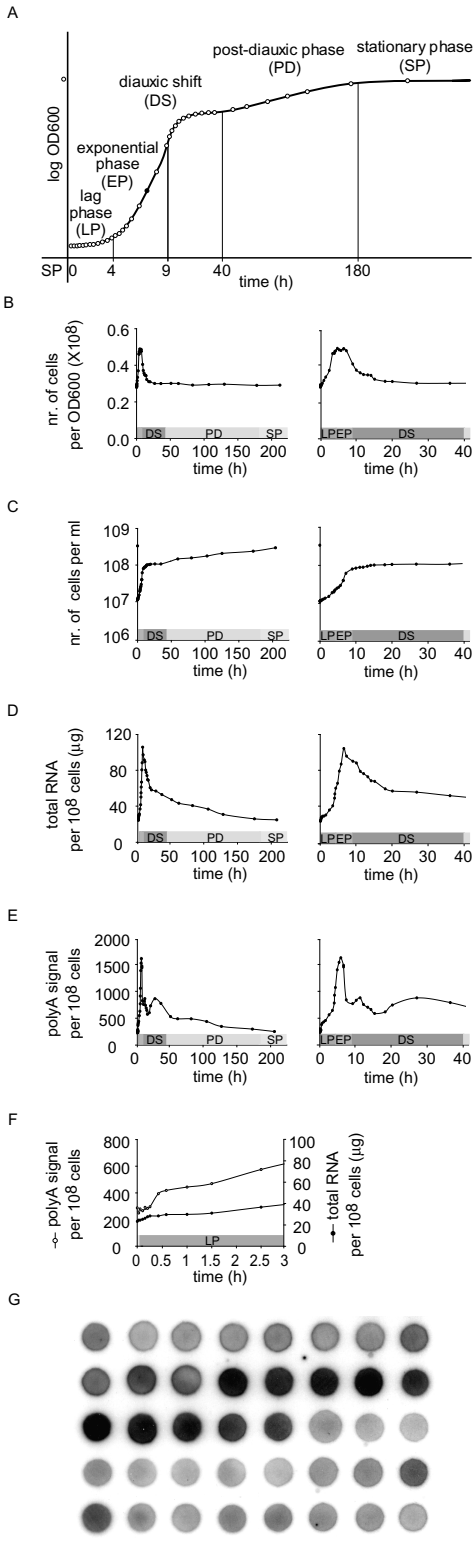
We describe an expression-profiling analysis of *S. cerevisiae* quiescence exit and entry, including the intervening events, in a single experiment that covers 9 days of culture. Several transitions are revealed in detail for the first time and the data provide a framework for further mechanistic analyses of large-scale gene expression reprogramming. Here, we focus on the extremely rapid response upon exit from SP involving immediate upregulation of at least 2500 genes within 6 min. Whereas current models of transcription regulation maintain that recruitment of Pol II is a rate-limiting step subject to several layers of regulation (Orphanides and Reinberg, 2002; Roeder, 2005), it is shown here that for a commonly occurring cellular state, Pol II is already located upstream of several hundred genes, prior to their activation.

Results

Poly(A) mRNA levels fluctuate several times during glucose starvation

To delineate changes in gene expression associated with *S. cerevisiae* quiescence entry and exit, a 9-day SP culture was refed by dilution in glucose-rich medium and allowed to grow again for 9 days. This period was chosen because true SP may not be reached before 7 days (Herman, 2002). Pilot experiments indicated which periods exhibit most change. The exit phase was therefore monitored every 3 min and the latter stages were monitored less frequently, resulting in a total of 39 samples (Figure 1A).

Cell count was monitored to relate changes in



gene expression to cell number. An almost two-fold difference in cells per OD_{600} is observed between SP and rapid exponential growth (Figure 1B), likely reflecting differences in size and cell surface morphology (Verstrepen et al., 2004). Immediately after refeeding, total RNA levels start to rise (Figure 1F), continue to do so during the period of rapid exponential growth, decline just before growth slows and tail off thereafter (Figure 1D).

Changes in global mRNA were monitored by poly(A) dot-blot hybridization (Figure 1E-G). Three peaks of poly(A) mRNA accumulation occur, indicating several major transitions (Figure 1E). Between 15 and 25 minutes after refeeding, poly(A) levels show a small sharp rise (Figure 1F), followed by a more gradual increase that continues throughout most of the rapid exponential growth phase (Figure 1E). Poly(A)

Figure 1. Global changes during quiescence exit and entry

(A) Schematic representation of a glucose starvation experiment, with circles representing the 39 samples analyzed. The single filled circle in the rapid exponential growth phase represents the common reference sample used in the microarray experiments. The open circle, left of the horizontal axis, represents the aliquot of the SP culture (day 9) that was used to start the experiment. See Experimental Procedures for a complete list of time points.

(B) Cell count per OD600 throughout the culture (left) and for the first 40 hours (right). The colored bar represents the various culture periods, with abbreviations according to A.

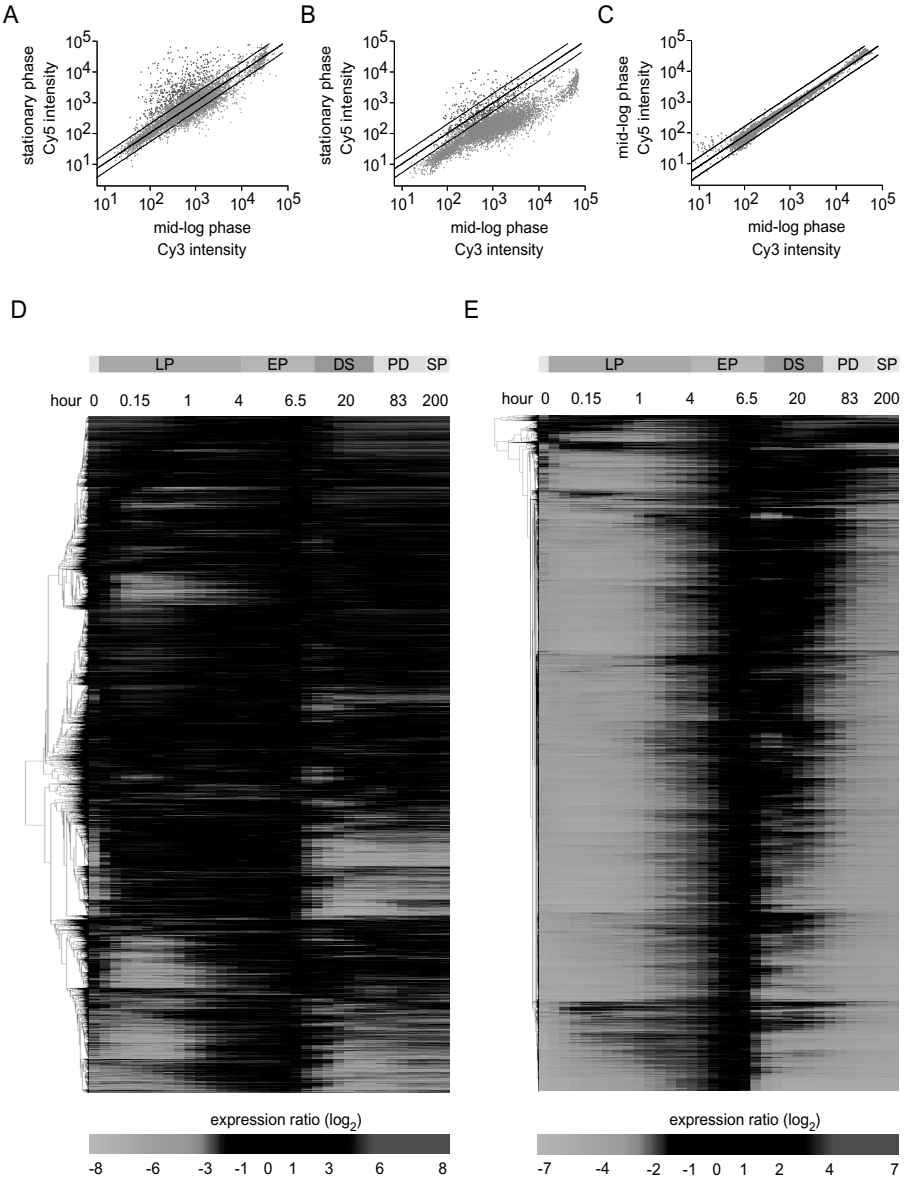
(C) Number of cells/ml throughout the culture (left) and for the first 40 hours (right).

(D) Total RNA per 10^8 cells.

(E) Average poly(A) dot blot ^{32}P radioactive signal per 108 cells as determined by analysis of two dot-blot experiments, one of which is depicted in G.

(F) poly(A) signal per 10^8 cells (open circles) and total RNA per 10^8 cells (filled circles) for the first 3 hours.

(G) poly(A) dot blot for determination of mRNA levels throughout the experiment. The first dot (left upper corner) represents the nine day SP culture from which the experiment was started. The 20^{th} and 21^{st} spot (counting from left to right) are duplicates of the 6.5 hour time point.


A
B
C
stationary phase Cy5 intensity
mid-log phase Cy5 intensity
mid-log phase Cy3 intensity
D
E
LP
EP
DS
PD
SP
hour 0 0.15 1 4 6.5 20 83 200
expression ratio (log2)
-8 -6 -3 -1 0 1 3 6 8
-7 -4 -2 -1 0 1 2 4 7
F
G
expression ratio
time (h)

mRNA levels first peak at mid-log (ML, 6 hrs) and start to decline rapidly, even before proliferation slows (7 hrs). Interestingly, the DS adaptation to non-fermentable carbon is characterized by two poly(A) peaks: first, a minor, early DS peak at 11 hrs and second, major DS poly(A) accumulation at 25 to 35 hrs (Figure 1E). These both occur prior to the resumption of slow proliferation at 40 hours. Hereafter poly(A) levels tail off during the transition from post-diauxic growth to SP. The almost six-fold difference in poly(A) mRNA levels between SP and rapid exponential growth agrees with a previous report of these end-points in colony growth experiments (Meunier and Choder, 1999). Previous analysis of poly(A) tails shows the presence of two distinct populations of mRNA, with either long (90 base) or short (20-30 base) poly(A) tails. In SP the relative abundance of the short poly(A) tail mRNA population increases (Sogin and Saunders, 1980). This means that the poly(A) levels monitored here are an underestimation of overall mRNA levels in SP.

The dynamic changes in poly(A) and total RNA reflect the combined results of changes in polyadenylation, *de novo* synthesis, constitutive and perhaps also active RNA degradation, all in response to changes occurring within the culture environment. Two peaks of poly(A) accumulation during DS have not been described before. These along with the SP exit and entry transitions were further examined at the gene specific level by expression-profiling analysis.

External control normalization more accurately determines mRNA level changes

mRNA samples were converted to labeled cDNA, mixed with an alternatively labeled common reference sample from the exponential growth phase and hybridized on 70-mer oligonucleotide microarrays. Aliquots from 11 to 15 hours systematically failed to yield sufficient and efficiently labeled cDNA in repeated experiments, also with larger amounts of mRNA. These samples resulted in poor microarray hybridizations and are excluded from further analysis. At these time-points, levels of poly(A) transcripts are similar to quiescent samples (Figure 1E,G), which do result in good quality

Figure 2. Genome-wide changes in transcript levels during quiescence exit and entry

(A) Standard, all genes normalized scatter-plot of average background subtracted microarray gene spot intensities, comparing ML samples with SP samples (9 days). The three lines represent two-fold up, no change and two-fold down boundaries, from top to bottom respectively. Colored in red and green are genes that were determined to show statistically significant changes in mRNA expression in the analysis of the eight SP versus ML replicate measurements.

(B) External control normalized result of the experiment shown in A, with the same genes colored red and green as in A.

(C) External control normalized result of a same versus same, ML replicate microarray hybridization.

(D) Standard, all genes normalized, hierarchical clustering diagram for the entire time-course (left to right), of all 6357 yeast genes represented on the microarrays. The colored bar on top represents the various culture periods, with abbreviations according to Figure 1A. Clustering was by average linkage analysis (UPGMA) with a standard (cosine) correlation. Supplemental table S1 contains a fully annotated version of the cluster diagram.

(E) Hierarchical clustering diagram of the external control normalized microarray data.

(F) Expression ratios of 123 ribosomal protein genes throughout the time-course when the data is normalized by the standard, all genes approach. The collective expression ratio of all genes is artificially set at one (no change) in each individual time-point by this approach. The RPGs appear only to fluctuate once during the time-course, at 20 hours. This actually represents the time-point when these transcripts deviate most from the behavior of all the genes, with which the data is normalized here.

(G) Expression ratios of 123 ribosomal protein genes when data is normalized with external controls. This results in ratios for each individual transcript, relative to the level of that transcript in the reference sample. The reference sample was ML, which is why all transcripts show a ratio of one at this time-point.

microarray hybridizations. We suggest that the inability to generate cDNA from the 11 to 15 hour samples reflects modification of poly(A) transcripts during the early part of the DS and may be related to a transient decrease in translation at this time (Fuge et al., 1994).

cDNA samples for the microarray time-course were generated using oligo-dT priming. To investigate whether poly(A) tail reduction confounds analysis of gene-specific changes, a control experiment was performed comparing random-priming of total RNA with oligo-dT priming of mRNA (Supplemental Figure S1). Transcripts determined down-regulated in SP when using oligo-dT priming are also determined down-regulated when random-priming is applied, indicating that poly(A) tail reduction in SP does not confound the analysis of gene-specific changes. This agrees with the poly(A) tail lengths previously reported for SP, which should still be amenable to oligo-dT priming (Sogin and Saunders, 1980). The advantages of oligo-dT priming are significantly higher microarray signal and sensitivity, with no cross-hybridization from the large amounts of labeled tRNA and ribosomal RNA present in the random-priming method (Supplemental Figure S1).

Two distinct procedures were applied for normalization of microarray data, which is required to counteract technical variation in preparing RNA, labeling samples and array hybridizations (Quackenbush, 2002). Standard normalization equilibrates the signals from all genes, based on the assumption that differences in gene expression between samples are minimal or balanced. This yields expression ratios relative to the collective ratio of all other genes in a given sample, which is artificially set at one (no change) and results in similar numbers of up- and downregulated genes within each individual time-point (Figure 2D).

The assumption of no global change is incorrect for studies of SP (Figure 1E). We have previously reported a method for normalizing microarray data that can deal with global or unbalanced shifts in mRNA populations (van de Peppel et al., 2003). A mixture of *in vitro* synthesized external RNA controls is spiked into total RNA samples, prior to mRNA isolation and normalization is based on equilibrating the signals from the 1344 external control spots present on the arrays. Because the control RNAs are spiked equivalently into total RNA, their signals represent microarray features for which no change can confidently be expected. An additional rescaling factor is based on total RNA yield per cell. Together this results in mRNA expression ratios for each gene (Figure 2E), relative to the expression level of that gene in exponentially growing cells, which was the reference sample.

External control normalization yields the expected global changes in mRNA levels: overall down-regulation in quiescent cells, upregulation to exponential phase levels, followed by down-regulation during entry into quiescence again (Figure 2E). Ribosomal protein genes (RPGs) demonstrate the difference between the two normalization methods, in particular for understanding mechanisms of transcription regulation. When normalized by the standard approach on all genes, RPG mRNAs appear only to fluctuate once, during the diauxic shift (DS) (DeRisi et al., 1997) (Figure 2F). It is unlikely that RPG mRNA levels do not change between SP exit and rapid exponential growth, given their central role in proliferation. When normalized through the external controls, RPG mRNA levels are indeed found to reflect cellular proliferation (Figure 2G), rising gradually during the lag and rapid exponential growth phases, dropping prior to DS, rising again slightly during the period of slow post-diauxic growth and dropping again during the transition to SP. As with previous microarray studies examining inactivation of components of the general transcription machinery (Holstege et al., 1998) or mRNA decay (Wang et al., 2002), external control normalization more accurately determines changes in mRNA levels during global events (van de Peppel et al., 2003). The results of both methods are presented for comparison with previously published microarray data. Further analyses of gene-specific changes are based on the external control normalized data.

Rapid and broad transcription response upon exit from quiescence

Cluster diagrams of all yeast genes during the quiescence exit and entry cycle demonstrate intricate patterns of regulation (Figure 2D,E). Figure 3 shows a selection of 20 groups of genes with distinct patterns of regulation. Because of the relatively high temporal resolution and the external control normalization, the data is rich in regulatory events and provides a framework for studying several aspects of growth phase related changes.

One remarkable feature is the rapid and broad response observed immediately upon diluting quiescent cells in fresh medium. At the 3-minute time-point (representing 6 minutes due to the time taken for harvesting), over 2500 genes are induced to levels higher than in the SP starting cells. Of these, 769 transcripts are induced between 2 and 35-fold (Figure 4A and Supplemental Table S3). Figure 3C-E shows selected groups of such rapid exit genes. Some genes are transiently induced prior to both rapid and slow growth phases (Figure 3C), suggesting a requirement for setting up proliferation regardless of the carbon source. Another group of transcripts is transiently induced mainly prior to rapid exponential growth (Figure 3D) and a third group of transcripts is induced upon SP exit and maintained at high levels throughout rapid growth (Figure 3E). The latter includes many genes involved in ribosomal RNA processing ($p=9{\cdot}10^{-13}$). Of the 91 most rapidly and transiently induced transcripts (at least 4-fold higher than in SP within 3 minutes, Supplemental Table S4), 23 code for transporters ($p=5{\cdot}10^{-6}$) and 16 are transcription regulators, indicating which factors are pivotal for reprogramming transcription in preparation for proliferation.

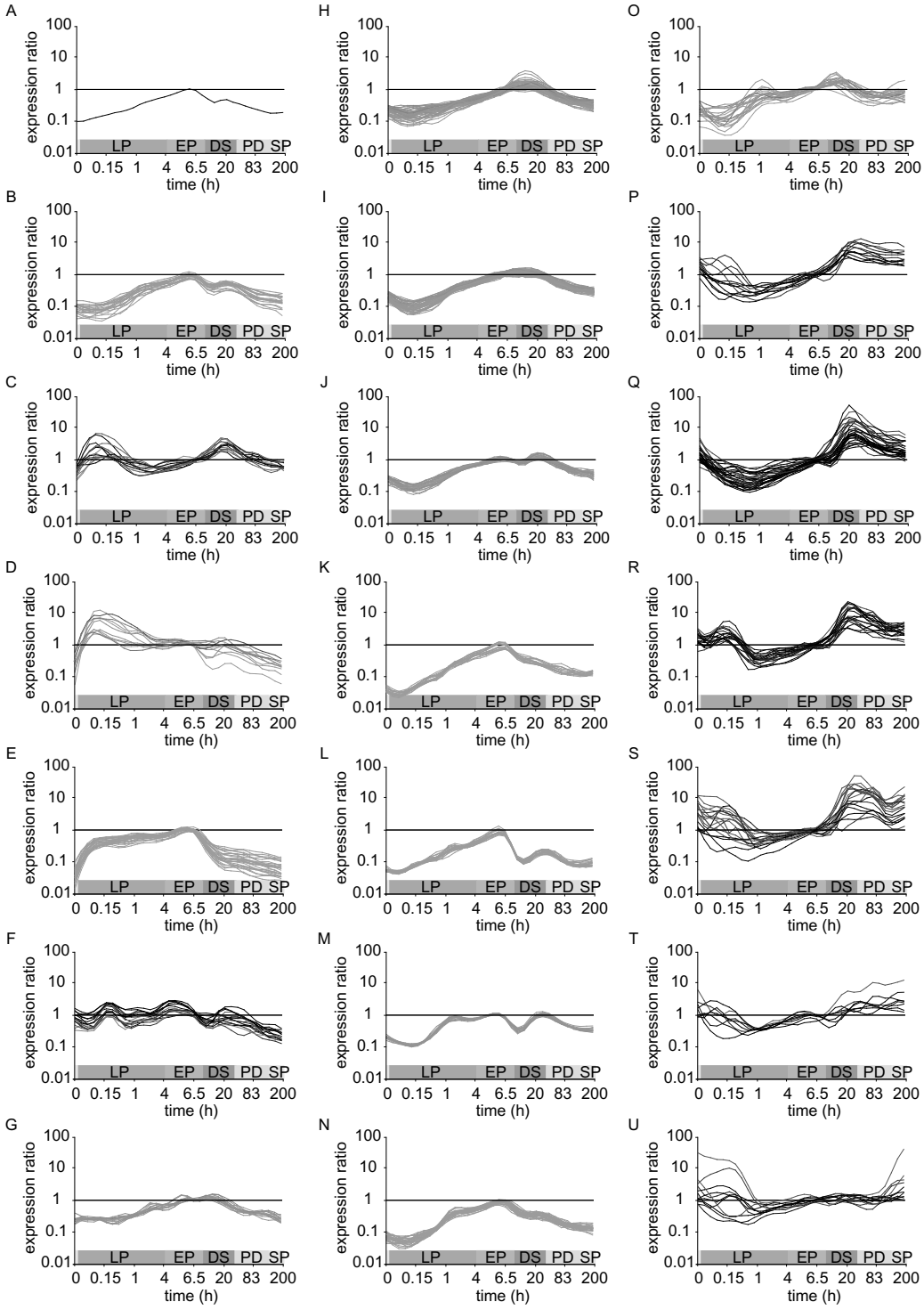
Stationary-phase enriched transcripts

In SP, the vast majority of transcripts are down-regulated in comparison to rapidly proliferating cells (Figure 2E). The 460 transcripts enriched in SP (Supplemental Figure S2 and Supplemental Table S5) are of interest as these should facilitate studies of SP and may shed light on the events regulating the transitions between proliferation and quiescence (Gray et al., 2004; Herman, 2002). Previously determined SP marker genes have largely been identified by analysis of SP end-points. The time-course allows examination of the temporal induction of SP transcripts. Figures 3P-3U show six groups of genes enriched in SP. A surprising finding is that the vast majority of SP enriched transcripts are already upregulated late during DS, at 20 to 30 hours, coinciding with the second DS peak in poly(A) levels (Figure 1E). This indicates that care needs to be taken in assigning specific quiescent roles to SP enriched transcripts, as the majority of these are already induced many days prior to the culture becoming stationary.

Similarly to previous studies, the strain studied here carries auxotrophic markers for modification of specific genes in subsequent analyses. To determine whether this influences SP enriched transcripts, a control experiment was performed with the parental prototrophic strain (Winston et al., 1995). The majority of SP enriched transcripts are identical regardless of the strain used (Supplemental Figure S2). However, two functional categories of genes upregulated in SP in the auxotrophic strain are not as significantly enriched in the prototrophic strain. These are genes involved in energy coupled proton transport, mainly ATP synthase components, as well as genes involved in vitamin metabolism, including the vast majority of SNO, SNZ and thiamine metabolism genes. Many of these have previously been associated with SP (Braun et al., 1996; Martinez et al., 2004). The auxotrophy dependent differences shows that SP is not a fixed state, but that the repertoire of genes upregulated in SP depends in part on metabolic processes. This indicates that SP cells may be able to respond to environmental changes without leaving SP.

The general transcription machinery is maintained during stationary phase

The late induction of a few SP enriched transcripts (Figure 3S-U) also indicates that some transcription machinery is active in SP


A
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L
M
N
O
P
Q
R
S
T
U
expression ratio
100
10
1
0.1
0.01
LP
EP
DS
PD
SP
0
0.15
1
4
6.5
20
83
200
time (h)

and is capable of responding to environmental changes. The possibility of active transcription is further supported by the maintained levels of many SP transcripts over several days (Figure 3P-R). These observations contradict evidence that generally required components of the transcription machinery such as the TATA binding protein (TBP) disappear in SP (Walker et al., 1997). Here, the time-course experiment was started with a nine-day old SP culture. The extremely rapid induction of a surprisingly large number of genes upon exit from SP (Figure 4A), further indicates that components of the transcription machinery are maintained during prolonged quiescence.

To investigate the general transcription machinery in SP, protein levels of key components such as RNA polymerase II (Pol II), TBP and Mediator were determined. Figure 4B shows that TBP is present in SP, at levels similar to ML cells. The apparent discrepancy with previous reports may be due to differences in the time points analyzed or in protein extraction methods. The method applied here makes use of chromatin enriched extracts and reduces the risk of losing proteins stably bound to DNA, which is removed during protein extraction (Experimental Procedures). Besides TBP, the Pol II components Rpb1p and Rpb3p, as well as the Srb4p component of the generally required Mediator complex are also present (Figure 4B). Apart from TBP, the protein levels of these subunits are

Figure 3. Many distinct patterns of gene expression during quiescence exit and entry

(A) The average expression ratio of all genes across the time-course.
(B) Genes that most closely follow the average transcript behavior.
(C) Rapid SP exit genes, transiently induced prior to both periods of proliferation.
(D) Rapid SP exit genes, transiently induced only prior to proliferation on glucose.
(E) Rapid SP exit genes, induced prior to proliferation and maintained at high levels during exponential growth.
(F) Transcripts that fluctuate several times during the time-course. These are mainly genes with uncharacterized molecular function and/or biological process.
(G) A group of uncharacterized/hypothetical ORFs induced at the end of lag phase.
(H) Genes that escape the general DS repression and which are induced very early in DS, at 10 to 11 hours.
(I) Similar to H, but with less prominent induction.
(J) Transcripts that peak at the end of rapid exponential growth and late in the DS.
(K) Transposon TyB Gag-Pol genes. The tight co-regulation is likely due to their overall identity.
(L) Transposon TyA Gag genes.
(M) Subtelomeric Y' helicases and homologs.
(N) A cluster of various functionally related genes induced late in lag-phase and including negative regulators of the Swe1p kinase, SPT6 and SPT16, genes involved in bud site selection and replication.
(O) Transcripts that show two transient peaks, late in lag phase and very early during DS. Half of these genes are involved in mating.
(P) SP enriched transcripts, induced late in DS and remaining high thereafter. This includes many of the stress and aerobic respiratory genes enriched in SP.
(Q) SP enriched transcripts, more transiently induced late in DS, but still remaining elevated in SP compared to ML. This also includes many of the stress and aerobic respiratory genes enriched in SP.
(R) SP enriched transcripts, as Q, but with an additional induction early in lag phase, although not immediately upon exit. This suggests that these genes may be required for transitions to any new carbon source.
(S) SP enriched transcripts as Q and R, but that show a second induction upon SP entry, after the post-diauxic growth phase.
(T) SP enriched transcripts that start to rise late in lag phase and continue to do so into SP. This group includes previously characterized SP genes such as SNO1, SNZ1 and SNZ2.
(U) SP enriched transcripts showing induction upon the transition between slow post-diauxic growth and SP. Groups B-O were obtained by clustering across the entire time-course. Groups P-U were clustered based on their behavior only in the latter part of the time-course. The genes depicted in each cluster are listed in Supplemental Table S2. More genes with similar behavior can be found in Supplemental Table S1.

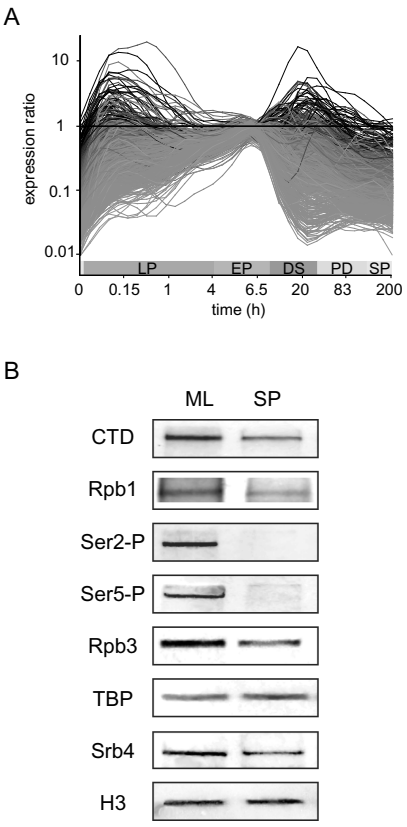

Figure 4. The general transcription machinery is maintained during quiescence

(A) Rapid and broad transcription activation upon exit from stationary phase (SP). The expression ratios throughout the time-course for the 769 genes induced more than two-fold within the first three minutes of SP exit are depicted. The genes are listed in Supplemental table S3.

(B) Western blot analysis of mid-log (ML) and stationary phase (SP) protein extracts for RNA polymerase II subunits Rpb1p, its C-terminal domain (CTD) and Rpb3p. Ser2-P and Ser5-P refer to specific phosphorylation sites within the heptapeptide repeat of the CTD. TBP refers to TATA-binding protein and Srb4p is a subunit of the Mediator complex. Extracts from equivalent amounts of cells were loaded and this was verified using an antibody specific for the invariant part of Histone H3 (H3).

lower than in ML cells (approximately 50%), but significantly higher than may be expected from the overall lack of transcriptional activity in SP (Figures 2E and 3A).

The general transcription shut-down in SP was further investigated by analyzing the phosphorylation status of the heptapeptide repeat of the carboxy-terminal domain (CTD) of the largest subunit of Pol II (Figure 4B). Phosphorylation of the CTD repeat at specific sites is indicative of active Pol II, either in the process of initiating transcription (Serine 5) or during elongation (Serine 2) (Dahmus, 1996; Komarnitsky et al., 2000). The low amount of phosphorylated Pol II CTD in SP, compared to the amount of the subunit bearing the CTD (Figure 4B), supports a model whereby the general transcription machinery is maintained but generally inactive in SP.

RNA polymerase II is present upstream of genes poised for rapid exit from quiescence

The presence of components of the generally required transcription machinery poses the question of where Pol II is located during SP. This question should also be viewed in light of the rapid and broad response upon SP exit and the proposal that quiescent cells can respond to environmental changes without leaving quiescence. Pol II location was therefore investigated by genome-wide chromatin immunoprecipitation (ChIP on chip), using a strain carrying a TAP-tagged version of the Rpb3p subunit. The microarray used for ChIP analysis carries 14178 PCR fragments representing all genes, hypothetical ORFs, dubious ORFs and intergenic regions (Harismendy et al., 2003). Genomic location profiles for Pol II were generated for SP and ML. For each condition, ChIP was performed on two independent cultures, each in dye-swap technical duplicate, resulting in four ChIP profiles for each condition (Figure 5A and 5B).

Previous genome-wide analysis of less frequently bound transcription factors located on a limited number of chromosomal regions, results in their appearance as a small group of features with distinct binding ratios compared to the rest of the genome (Harbison et al., 2004). This is in contrast to globally binding factors such as

RSC and TFIIB, or frequently occurring features such as histone modifications (Kurdistani et al., 2004; Ng et al., 2002). An external control normalization approach has not yet been developed for genome-wide location analysis. For globally bound factors, statistically significant ChIP enriched locations can only be interpreted as relatively higher enriched compared to the entire genome, which is artificially set at a binding ratio of one (no change relative to ChIP input material). Analysis of Pol II ChIP profiles reveals 1353 enriched regions for SP and 3215 for ML (Figure 5A,B). These numbers compare well to the estimated two-fold lower abundance of Pol II in SP cells and suggest that most of the inactive Pol II in SP is DNA bound.

Where is Pol II bound in SP? Interesting differences are observed between SP and ML, with regard to the distribution of Pol II between gene coding regions (GCRs) and intergenic regions (IGRs) (Table 1). In these analyses, only confidently assignable GCRs and promoter bearing IGRs are taken into account. Dubious open reading frames (ORFs) and IGRs between convergent genes are unassigned as these are unlikely to represent GCRs or promoter bearing IGRs respectively. In addition, due to the relatively low resolution of such experiments, signals from adjoining IGR-GCR pairs require filtering to prevent leakage of ChIP signals between such pairs from confounding the analyses. Taking this into account, 615 out of the 1353 Pol II ChIP enriched features in SP are promoter bearing IGRs. This represents a significant enrichment compared to the genome-wide distribution of such features (32%, $p=5 \cdot 10^{-29}$), and is also significantly different to the enrichment of IGRs in ML (28%, $p=3 \cdot 10^{-17}$ for t-test of difference between average IGR binding ratio in ML versus SP). Conversely and as expected, the Pol II ML ChIP enriches more significantly for GCRs (1481 out of 3215 enriched features, $p=1 \cdot 10^{-13}$ compared to genome-wide distribution of 41%). Pol II in SP is therefore more predominantly located on IGRs, whereas Pol II in ML is more predominantly located on GCRs. This agrees well with the presence of largely inactive components of the transcription machinery in SP (Figure 4B). The more prevalent location of Pol II on IGRs in SP compared to ML indicates that the inactive transcription machinery that is preserved in SP, is maintained on DNA in the vicinity of promoters.

Although Pol II in SP is more predominantly bound at IGRs, Pol II is found at some GCRs in SP. To verify some of the ChIP results and to more precisely map Pol II location, we chose two genomic regions for higher resolution conventional ChIP analysis. For finding the SP Pol II location within a GCR, the *PUT4* gene was analyzed. *PUT4* mRNA is also found upregulated by the expression analysis (Figure 3S). The higher resolution ChIP analysis confirms the location of Pol II on the GCR of *PUT4* in SP and lack of Pol II in ML (Figure 5C). The presence of Pol II all along the GCR is suggestive of an actively transcribing enzyme and agrees with the conclusion that transcription is occurring in SP for a limited set of genes. Of the GCRs that are significantly enriched in the SP Pol II ChIP, 86 belong to the class of genes upregulated in SP (Supplemental Figure S2 and Supplemental Table S5, $p=3 \cdot 10^{-16}$). This further supports the assertion that transcription is ongoing for many of the SP upregulated genes and is in agreement with the idea that quiescent cells can respond to changes in the environment. The fact that Pol II is not detected on all GCRs of SP upregulated genes, likely reflects the method used for ChIP normalization as well as limits of genome-wide ChIP sensitivity, especially for low level transcription. In agreement with the latter, the microarray expression intensities are on average two-fold higher for the group of SP expressed genes for which Pol II is determined to be significantly located on the corresponding GCRs (data not shown).

Pol II in SP is more predominantly located at IGRs, such as the IGR upstream of *SUT1* (Figure 5D). Conventional location analysis of this region at higher resolution, confirms the ChIP microarray result, showing Pol II binding upstream of *SUT1* in SP. *SUT1* is one of the many genes not expressed in SP, but immediately upregulated upon exit from quiescence (Figure 3D). Of the 615 IGRs significantly bound by Pol II in SP, 452 are upstream of genes induced within

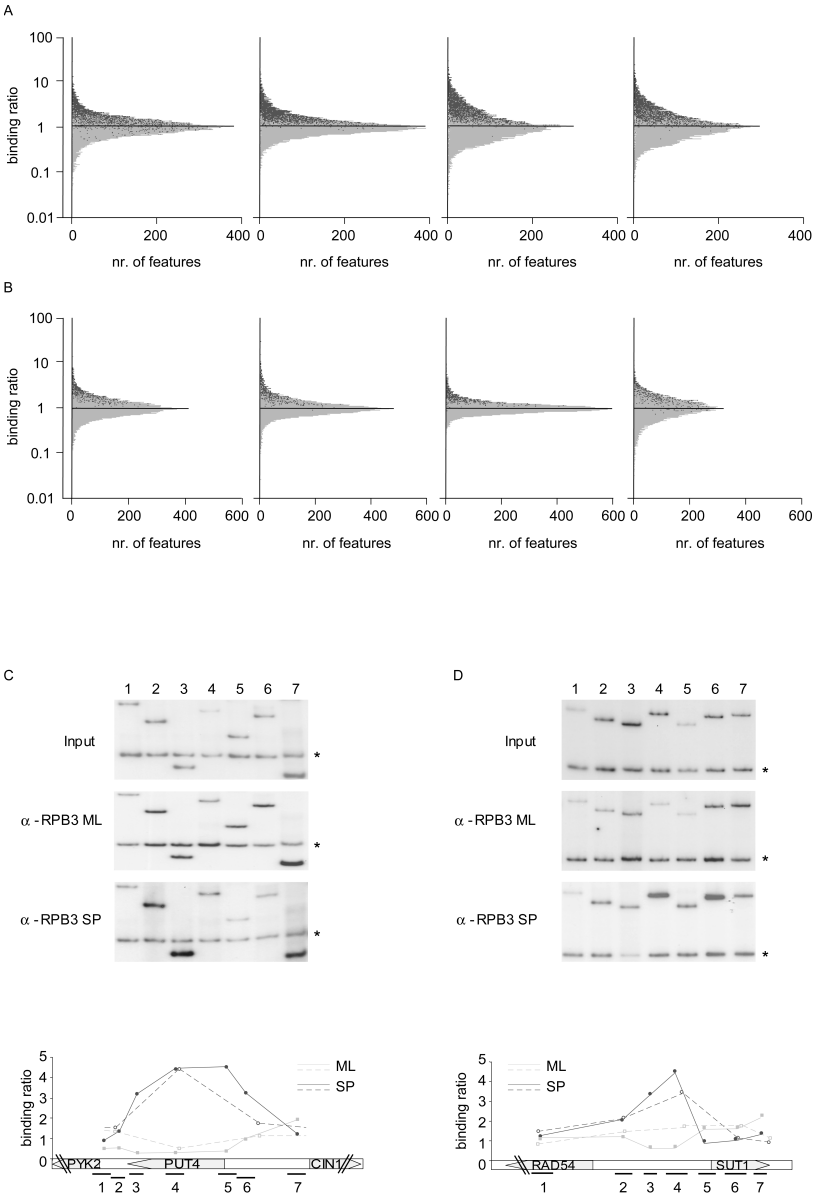

Figure 5. Genome-wide location analysis of RNA polymerase II

Histograms of Pol II binding ratios of all genomic sequences for ML (A) and SP (B). Each histogram is the result of a single microarray hybridization. The vertical axis represents the normalized binding ratios. In red are genomic features selected as significantly enriched in each collection of four hybridizations.

Conventional ChIP analysis of *PUT4* (C) and *SUT1* (D), analyzed by PCR in the linear range of amplification, loaded on a 7% polyacrylamide gel and quantified using a phospho-imager as described under Experimental Procedures. Relative positions of the PCR products are indicated below the graphs. The asterisk represents the position of the internal normalization control for PCR. The binding ratios depicted on the graphs are from the microarray (dashed lines) and conventional ChIP (solid lines), for ML (blue) and SP (red), respectively.

Table 1. Occupancy of Genomic Features by RNA Polymerase II in Mid-log and Stationary Phase

	Number of				
Occupancy in	Total Features	GCR [a]	IGR [b]	Unassigned	Filtered out
mid-log phase	3215	1481 (46%)	898 (28%)	700 (22%)	136 (4%)
stationary phase	1353	443 (33%)	615 (45%)	286 (21%)	9 (1%)
genome	14178	5757 (41%)	4504 (32%)	3917 (27%)	

[a] GCR - Gene Coding Regions
[b] IGR - Intergenic Regions

three minutes upon SP exit ($p=3\cdot10^{-30}$). Together with other data presented here this leads to the proposal that the transcription machinery is maintained in SP, largely in an inactive state, but that Pol II is poised for immediate response by being held upstream of many genes required for changes in environmental conditions and reproliferation.

Discussion

Current models of eukaryotic transcription activation consider that for most genes, Pol II recruitment is the rate-limiting step which is controlled through chromatin remodeling and other coregulatory events (Orphanides and Reinberg, 2002; Roeder, 2005). Finding Pol II maintained upstream of so many repressed genes in SP is therefore provocative, especially because quiescence is a frequently occurring cellular condition. The status of Pol II in quiescence may be related to the poised state of Pol II on the well-characterized heat-shock genes (Lis and Wu, 1993) which have always been considered as rather exceptional in this respect. The preferential location of Pol II in SP, upstream of rapid exit genes, strongly suggests that the same Pol II molecules that are maintained on intergenic promoter-bearing regions, are responsible for transcribing the corresponding downstream genes during exit. Although further investigation is required, this forms a plausible mechanism, likely evolved to permit a rapid response upon encountering nutrients in a competitive environment.

It will be of interest to discover which factors determine selective Pol II binding, as well as to understand the mechanism behind the general transcriptional shut-down in SP. One mechanism is suggested by the diminished phosphorylation status of the CTD of largest subunit of Pol II (Figure 4B). The general repression may however also simply be due to lack of available nucleoside precursors or DNA topology changes (Choder, 1991) causing stalling of activation immediately after Pol II recruitment. The analyses presented here show that any model of general repressive mechanisms will need to take into account that individual genes can escape repression and respond to environmental changes without cells leaving quiescence. Response of individual genes to environmental changes has also been reported for mammalian models of quiescence (Yusuf and Fruman, 2003). In this sense, the ability of quiescent yeast cells to respond to subtle environmental changes, apparently without leaving G0, further harmonizes models of eukaryotic quiescence.

The immediate increase in transcript levels upon SP exit has recently been suggested to be caused by an mRNA storage and release mechanism (Martinez et al., 2004). The relatively abundant level of general transcription machinery reported here indicates that such a mechanism does not necessarily need to be evoked. We further note that the time-course contains many additional observations that can form starting

points for other mechanistic analyses. The inability to generate cDNA from the 11 to 15 hour mRNA samples coincides with a previously reported drop in translation (Fuge et al., 1994), suggesting that this may be linked. Two peaks in poly(A) levels during the DS transition as well as the specific transcripts upregulated suggest that this phase is more than a single transition to a different carbon source. The set of regulators immediately and transiently upregulated upon SP exit contain several regulators of pseudohyphal differentiation, suggesting additional cross-talk between the dimorphic switch and nutrient survival programs (Madhani 1998). Finally, the high temporal resolution of externally normalized transcript levels for the complete cycle of quiescence and proliferation forms a framework for computational analyses of gene-specific regulatory events, similar to analyses of the active cell cycle of *S. cerevisiae* (e.g. Simon et al., 2001).

Experimental procedures

Yeast strains and culture conditions

BY4741 (*MATa; met15*Δ0; *ura3*Δ0; *his3*Δ1; *leu2*Δ0) (Research Genetics) was used for the time-course. The strain with TAP-tagged Pol II subunit Rpb3, MGD353-13D-RPB3-TAP (*MATa; ade2; arg4; leu2-3,112; trp1-289; ura3-52; YIL021W::TAP-K.l.URA3)* (Gavin et al., 2002) was obtained through EUROSCARF and used for ChIP and immunoblotting. Strains were cultured in YEP with 2% glucose at 30°C. For expression-profiling, cultures were grown to SP (OD_{600}12.0; 9-day culture) and diluted to OD_{600} 0.5 in fresh medium. Samples were collected by 2 min centrifugation at 30°C and frozen in liquid nitrogen. The first time point was from the starting SP culture and subsequent samples were taken at 0 min, 3 min, 6 min, 9 min, 12 min, 15 min, 25 min, 35 min, 60 min, 90 min, 2.5 h, 3.4 h, 3.9 h, 4 h, 4.25 h, 4.75 h, 5.25 h, 5.75 h, 6.5 h (ML reference sample for time points 0 – 6.5 h), 7.25 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 18 h, 20 h, 26.75 h, 36 h, 51 h, 62 h, 83 h, 107 h, 130 h, 178 h and 212.25 h. The ML reference sample for 7.25 – 212.25 h was an identical 6.5 h sample, grown in parallel. Cell number per ml was determined by hemocytometer, counting more then 500 cells in triplicate.

RNA isolation and cDNA labeling

Total RNA was prepared by hot phenol extraction. Nine external controls were added as a single mixture to total RNA, and cDNA was generated with 5 µg mRNA in the presence of 2-aminoallyl-dUTP (Sigma), all as previously described (van de Peppel et al., 2003). After Cy3 and Cy5 fluorophore (Amersham Biosciences) coupling, free dyes were removed using Chromaspin-30 (BD Biosciences Clontech) columns, and the efficiency of dye incorporation was measured using a spectrophotometer (UVmini 1240, Shimadzu). Each microarray hybridization was set up with 300 ng of cDNA for reference and time-point samples, labeled at 2-4%.

Expression-profiling

Microarray production, hybridization and quantification were performed using a 70-mer oligo collection as previously described (van de Peppel et al., 2003). Each gene is represented twice and the arrays additionally contained 2838 control features for external control normalization and QC. After image quantification and local background subtraction, all negative values were replaced with the standard deviation of the local background. Print-tip lowess (Yang et al., 2002) with a span of 0.4 was applied to normalize on genes or controls. After normalization, all signals lower than 50 were replaced with 50. Data normalized on external controls were subsequently scaled to further reflect absolute changes per cell using the ratio of total RNA per cell. A cubic spline was applied to smooth the data, using the smooth.spline function inside the statistical package R (http://www.r-project.org, smoothing parameter 0.4). After averaging of duplicate spots for each gene on the array, data was visualized and clustered using Expression Profiler (Kapushesky et al., 2004) for Figure 2 and GeneSpring 6 (SiliconGenetics) for Figure 3.

Poly(A) dot-blot analysis

Total RNA (3.5 µg per sample) was spotted in duplicate with a Genesis RSP liquid handler (Tecan) onto nylon membranes (GeneScreen). Membranes were prehybridized at 42°C in 1xSSC, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.5% SDS and 200 µg/ml of denatured haring sperm DNA. An oligo-dT 40-mer was labeled with γ-[^{32}P]ATP using T4 polynucleotide kinase. The membranes were hybridized at 42°C with the labeled probe (approximately 10^6 cpm/ml) in prehybridization buffer for 12-20 h. The membranes were washed once at 42°C in prehybridization buffer (15 min) and three times at room temperature in 0.1XSSC and 0.2% SDS (20 min). Imagene 4.0 (Biodiscovery) was used for quantification and average

values and standard deviations from two membranes were calculated. The signal was a linear function of the amount of input RNA in the assay. ***E.coli*** tRNA which was used as a negative control, showed no detectable signal.

Chromatin immunoprecipitation and genome-wide location analysis

Cells were collected at ML and SP, crosslinked with formaldehyde (1%) for 20 min at room temperature, quenched with 300 mM glycine and spun down at 4°C for 10 min at 4000 rpm. Pellets were washed in cold TBS pH7.5 (10 mM Tris pH7.5, 150 mM NaCl) and FA lysis buffer (50 mM Hepes pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF, protease inhibitor cocktail) and frozen in liquid nitrogen. ChIP extracts and IP were performed as previously described (Andrau et al., 2002). For chromatin extracts (CE), 300 ml of ML or 60 ml of SP cells were used, ending with 1.6 ml of CE resuspended in lysis buffer. IP of TAP-RPB3 was performed with 200 µl CE for ML or 300 µl CE for SP samples, incubated for 2 hours with 20 µl of IgG beads on a rolling boat, prior to reversal of cross-links and DNA purification. IP and input DNA (obtained with reverse cross-link of 5 µl of CE) were eluted in 30 µl. For LM-PCR amplification 10 µl (IP) or 1 µl (input) was used, labeled and hybridized following a previously described protocol for the same arrays encompassing 14178 PCR products for all yeast genomic elements (Harismendy et al., 2003). The amount of labeled material in each hybridization was 200 to 400 ng for each channel, with label incorporation of 2 to 4%. Four hybridizations for each condition (SP and ML) were performed using samples from two independent cultures, with independent extracts in dye-swap.

Conventional ChIP was performed using an internal POL1 control primer pair in both input and IP radioactive PCR reactions. Relative binding ratios were calculated as compared to POL1 and to input level in the linear range of amplification all as described (Andrau et al., 2002). Primer sequences are available upon request.

Genome-wide location data analysis

After image quantification, data was normalized over all features using print-tip lowess with a span of 0.4. The SAM algorithm (Tusher et al., 2001) in the siggenes package of R (http://www.r project.org) determined features significantly bound by Pol II, using an equivalent false discovery rate (less than 0.053) for both ML and SP conditions. For comparing genomic location with mRNA expression data, oligo sequences were mapped onto the yeast genome obtained from SGD (Christie et al., 2004) on August 20, 2004 using the program SSAHA (Ning et al., 2001). Trends in Poll II location were determined using only features corresponding to 1) proper ORFs (defined here by their annotation as “verified” or “uncharacterized” in SGD (Christie et al., 2004)) and 2) intergenic regions upstream of one or two ORFs on either strand (i.e. containing at least one promotor). Any PCR features that did not fall unambiguously into one of these four categories were ignored (3917 out of 14178 features, mostly PCR products between convergent genes). To correct for the spreading of ChIP signals to adjacent features as a result of the size of the sonicated DNA fragments (here 300 to 500 bp), the sets of significantly bound features were adjusted by removing, from each pair of adjacent features, the one having the lowest binding ratio.

Unless otherwise specified in the text, all p-values for comparisons of overlapping sets of genes or features are based on a hypergeometric test with multiple testing correction.

Immunoblotting

Cross-linked CE extracts were boiled in sample buffer for 10 to 15 min before loading on SDS polyacrylamide gels (10 to 17.5%). Proteins were transferred onto nitrocellulose membrane and incubated with the following antibodies: polyclonal affinity purified anti-yTBP, anti-Histone H3 ab1791 (Abcam), monoclonal anti-CTD 8WG16, anti-Ser2 and anti-Ser5 of the CTD (H4 and H5 from Berkeley Antibody Co.), anti-Rpb1 (y-80, Santa Cruz Biotechnology) and Srb4 (yC-20, Santa Cruz Biotechnology). Peroxidase-anti-peroxidase (PAP, Sigma) was used to detect the TAP tag of Rpb3.

Accession numbers

MIAME compliant protocols and datasets in MAGE–ML are accessible from ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), with the following accession numbers.

Array:

A-UMCU-4, UMC Utrecht *S. cerevisiae* 16k array, version 1.2

Protocols:

P-UMCU-5, Total RNA isolation

P-UMCU-6, Messenger RNA isolation

P-UMCU-7, Amino-allyl labeling

P-UMCU-8, Microarray production

P-UMCU-9, Hybridization

P-UMCU-10, Scanning protocol

P-UMCU-11, Image analysis
P-UMCU-27, Yeast stationary phase culture conditions
Experiments:
E-UMCU-12, Stationary phase time-course
E-UMCU-13, Pairwise comparison stationary phase vs mid-log

Supplemental Data

Supplemental Data include two additional figures, five additional tables, and Supplemental References, and seven additional figures and can be found with this article online at http://www.molecule.org/cgi/content/full/18/2/ ¡ ¡ ¡/DC1/.

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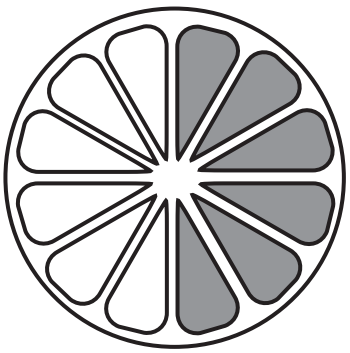
Chapter 3

Functional analysis of genes required for entry into and exit from stationary phase in *S. cerevisiae*

Marijana Radonjic, Theo Bijma and Frank C.P. Holstege



Functional analysis of genes required for entry into and exit from stationary phase in *S.cerevisiae*

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Summary

Quiescence is a common cellular state in all organisms, yet it remains largely unexplored. In yeast, quiescence is induced in response to starvation as a strategy that facilitates long-term survival under harsh conditions. We have previously used DNA microarray expression profiling to describe the changes in mRNA levels that accompany entry into and exit from stationary phase (SP) in the yeast *S. cerevisiae*. Here, we analyse phenotypes and functions of genes selected from the genome-wide expression data to determine processes and genes that are essential for long-term survival and for the exit from quiescence upon re-feeding. In total, phenotype analysis of gene deletion mutants identifies 50 strains with strong to intermediate SP-associated growth defects. The analysis of functional categories of SP-responsive genes reveals that the primary processes in the initiation of reproliferation are the facilitation of protein synthesis and the activation of gene-specific transcription regulators, and that entry into quiescence requires activation of cellular stress response and major changes in energy metabolism. Thirty percent of the analysed genes have not been previously associated with any biological role, which underscores the importance of these assays in the functional characterisation of the genome. Taken together, the results reveal many genes essential for yeast quiescence and provide a comprehensive description of the metabolic and regulatory processes responsible for entry and exit from this important and poorly explored cellular state.

Introduction

The resting state, also known as quiescence or G0, is the most common state of eukaryotic cells (Lewis, 1991). Quiescent cells are non-dividing, but they retain the potential to resume division upon receiving stimuli for reproliferation. Mammalian cells become quiescent after terminal differentiation and can be inappropriately induced to divide, as occurs during development of cancer and early stages of neurodegenerative diseases (Raina et al., 1999; Sherman and Goldberg, 2001; Yoshikawa, 2000). Understanding the regulation of quiescence is therefore important for cancer and ageing studies (Gershon and Gershon, 2000).

In yeast, quiescence can be induced by starvation. Nutrient depletion causes growth arrest and the yeast culture enters stationary phase (SP). In such SP cultures, most of the cells are in a quiescent state. SP is the result of multiple transitions. Upon depleting glucose, cells adjust metabolism for utilization of other carbon sources during the non-proliferative diauxic shift (DS). This is followed by slow postdiauxic growth (respiration), until complete carbon source depletion results in cells entering quiescence. Yeast quiescence represents a survival program involving morphological and physiological changes that protect cells during harsh environmental conditions (Werner-Washburne et al., 1993). The resistance of quiescent cells to stress is common for all microorganisms and causes difficulties in treatment of human microbial diseases with antibiotics. More insight into SP would therefore benefit strategies for fighting pathogenic microorganisms. In addition, *S. cerevisiae* is widely used in the food and biotech industry and better understanding of

stationary phase would also be beneficial for these fields (Schuller and Casal, 2005).

Despite the importance of understanding quiescence for biomedicine and industry, yeast stationary phase (SP) remains largely unexplored. In part, this is due to the properties of quiescent yeast cells. The thick cell wall obstructs lysis (de Nobel et al., 2000). Analysis of mRNA and proteins is difficult due to low biochemical activity during quiescence (Choder, 1991; Fuge et al., 1994). Understanding SP has also been complicated by different definitions, different methods of inducing quiescence, insufficient expression markers and lack of mutants with quiescence-associated phenotypes. Also, distinction between SP mutants involved in entry, maintenance and/or exit from quiescence has received little attention in previous studies.

The development of genome-wide techniques has started to facilitate an understanding of stationary phase. Several DNA microarray studies have investigated mRNA expression changes that occur in yeast cells during different growth phases (DeRisi et al., 1997; Gasch et al., 2000; Martinez et al., 2004; Radonjic et al., 2005). Here, we have employed a previously generated genome-wide mRNA expression dataset (Radonjic et al., 2005) to select SP-responsive genes for functional and phenotype analyses. The aim of this work was to determine which relevant processes occur during SP exit and entry and to identify novel mutants with SP phenotypes. The phenotype assays include mutants for genes that are induced during SP, identifying genes required for SP entry and maintenance, as well as mutants of genes induced upon SP exit, identifying genes required for exit from SP. Of 73 analysed mutants, we found 50 genes with a SP-related growth defect. The rate of phenotype discovery (68%) shows that genome-wide expression screens are a useful tool for identification of genes that are essential for particular conditions.

Results

Biological processes required for exit from stationary phase

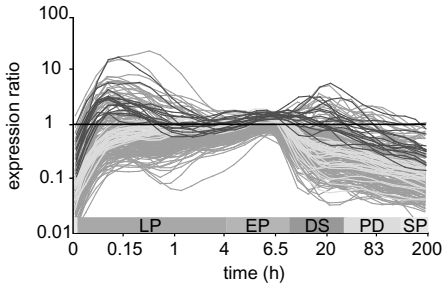
Exit from stationary phase (SP) in *S.cerevisiae* is characterized by an extremely rapid transcription response. Over 2500 genes are induced after only three minutes upon adding fresh medium to the SP culture (Radonjic et al., 2005). The functions of immediately transcribed genes may indicate critical events responsible for the reprogramming of cellular metabolism in preparation for growth. To identify pivotal biological processes that occur during the transition from quiescence to proliferation, we examined functional categories (Harris et al., 2004; Robinson et al., 2002) of 201 genes that were induced more than four-fold within the first 3 minutes upon exit from SP (Radonjic et al., 2005) (Figure 1A and 2A).

The most significant functional category for the members of rapidly induced genes is related to ribosome biogenesis and assembly (Figure 2A). Ribosome biosynthesis is necessary for the boost of protein production, which is indispensable for the establishment of cellular growth (Moss, 2004). All the genes from this category are rapidly induced upon exit from SP and their expression is further stabilized until the mid-log phase (Figure 1A), which confirms the necessity of these genes for both the SP exit and for the maintenance of exponential growth.

Many genes induced upon SP exit have a regulatory function. This includes transcription factors that regulate carbon utilization by repressing glucose repressible genes (*NRG1, MIG1, MIG2*) or by activating glycolysis (*TYE7*). Other identified regulators control growth through PKA and Rho1 PKC signalling pathways (*RGS2, BAG7, PDE2*) and the cell cycle (*RCK1, TYE7*). All the genes from the category of transcription regulators are induced transiently upon SP exit (Figure 1A), indicating that they are required for the initiation, but not for the maintenance of growth.

Other genes induced upon stationary phase exit include those with a function in cellular metabolism. This involves various transporters

A



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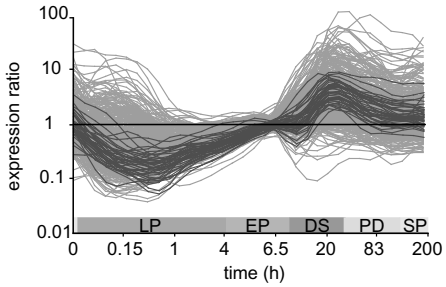


Figure 1. mRNA expression profiles of genes selected for functional analysis

(A) Genes induced more then 4 fold in the three minute time-point after stationary phase (SP) exit compared to the SP starting sample (Radonjic et al., 2005). Representatives of the functional categories "ribosome biogenesis and assembly" and "DNA dependent transcription" are shown in yellow and red, respectively. The remainder of SP exit genes is shown in gray. The coloured bar at the bottom of the graphs represents the various culture periods: LP, lag phase; EP, exponential phase; DS, diauxic shift; PD, post-diauxic phase; SP, stationary phase.

(B) Genes induced in stationary phase (Radonjic et al., 2005). Representatives of the functional category "respiration" are shown in red and the rest of the SP induced genes are shown in gray. All genes shown in (B) are induced during SP at least three fold more than in exponential phase, relative to expression of all genes in the genome.

(*HXT1, HXT2, HXT4, PHO84, PHO89, TPO2, TPO3, ENA2, ENA1, MEP1, MEP2, FCY2, THI7, AQY1*), genes with a role in amino acid biosynthesis (*LYS4, LYS9, LYS12, GLY1, CYS4*) and energy derivation (*PFK27, OYE2, DLD3, ERO1*). Within the 201 analysed genes, we found 64 that were not previously associated with any biological process or molecular function. Expression profiles of these genes imply their involvement in one or more aspects of the stationary phase exit and make them attractive candidates for further phenotype analysis. Finally, 54 out of 201 genes are classified as essential ($p=5 \cdot 10^{-4}$), which underlines the importance of the ability of the cells to successfully exit quiescence when nutrients are replenished.

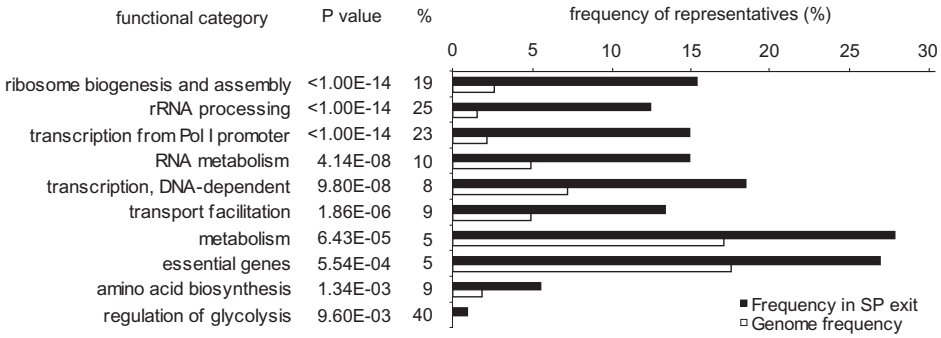
Entry into stationary phase is accompanied by metabolic changes and stress response

The next group of genes selected for functional analysis were stationary phase induced genes. We investigated functional categories of these genes to determine the most important processes required for quiescence entry and maintenance. The analysis included 460 genes upregulated at the nine-day stationary phase culture of *S. cerevisiae* (Radonjic et al., 2005) (Figure 1B and 2B).

SP induced genes have functions in various aspects of yeast metabolism (Figure 2B). The majority of these metabolic genes are involved in energy derivation. Over-representation of categories related to mitochondrial and peroxisomal function, tricarboxylic acid cycle (TCA) and oxidative phosphorylation suggests that energy is largely derived by respiration and oxidation of organic compounds. This is in agreement with the other studies of similar starvation endpoints (Boer et al., 2003; Martinez et al., 2004; Wu et al., 2004). In addition, all genes belonging to the categories of glyoxylate, propionate and vitamin metabolism were also identified within the set of SP induced genes.

Increased resistance to stress is an important property of quiescent cells (Werner-Washburne et al., 1993). Analysis of the functional categories among the SP upregulated genes reveals an expected overrepresentation of the stress response genes. This includes genes involved in resistance to heat shock (*HSP12, HSP26,*

A



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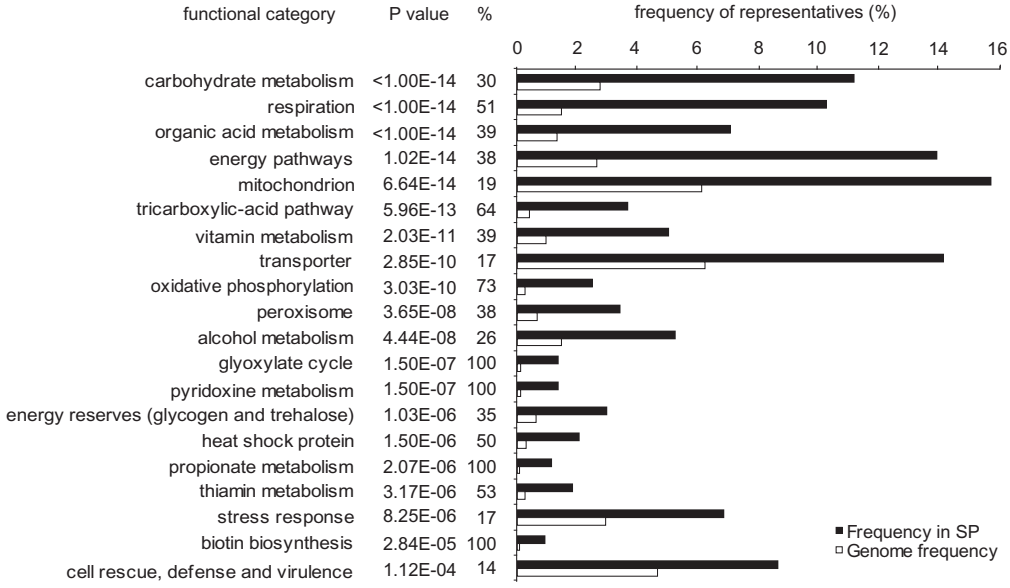

Figure 2. Functions of genes induced during stationary phase entry and exit

Functional categories overrepresented in the group of transcripts rapidly induced upon stationary phase exit (A) and in the group of stationary phase enriched transcripts (B). The functional category is shown on the left, followed by the p-value as determined by a hypergeometric test. Percentage values signify the fraction of queried genes within a category relative to all genes in that category. The black bar indicates how many of the queried genes are assigned to a category, and the white bar, how many genes in the genome belong to that category. Functional categories were derived from MIPS and GO databases.

HSP30, HSP42, HSP78, HSP104, SSA3, SSA4, YDC1, NTH1), oxidative stress (*GPX1, HYR1, TRR2, CTT1, TRX3, YCL033C*), osmotic shock (*GCY1, HOR7, SIP18*), copper (*CUP1, CUP1-2, CRS5*), and DNA damage (*DDR2*). The genes that regulate the metabolism of glycogen and trehalose are also induced, consistent with the notion that the quiescent cells generate energy reserves to survive prolonged starvation periods (Francois and Parrou, 2001). In addition to its role

as the reserve carbohydrate, trehalose functions as an important protectant against a variety of stresses (Wiemken, 1990).

Regulatory factors induced in stationary phase are of particular interest for understanding the transcriptional mechanisms underlying quiescence. A total of 42 (putative) transcription factors and components of signal transduction pathways are upregulated in SP. These include established regulators of stress and metabolism such as *MSN4, HAP4, ADR1, XBP1, MTH1, SIP4, DAL80, CUP2, ROX1* and *MET28*, 10 uncharacterised genes homologous to regulatory factors and genes implicated in cell cycle control such as the *PCL10* and *CLB3* cyclins. Of the 460 analysed SP-induced genes, 178 have no prior established molecular function or biological process. This underscores the uncharacterised nature of *S. cerevisiae* quiescence, indicating that SP provides good starting condition for assigning function to many unknown genes.

Mutants of SP-responsive genes exhibit stationary phase phenotypes

Elevated levels of expression during stationary phase (SP) exit and entry may indicate a requirement for successful survival and recovery from SP. To investigate this, we selected 22 genes with rapid induction upon SP exit, and 51 genes induced in SP for phenotype analysis (Table 1). Mutant strains bearing deletions of one of these 73 selected genes were grown to stationary phase at 30°C and subsequently screened for viability and for the rate of recovery from SP at 25, 30 and 37°C (Table 1, Figure 3) (Experimental procedures). Cells were grown to SP at an optimal temperature (30°C) to prevent additional stress which may affect entry into and maintenance of quiescence. Viability and rate of recovery from SP upon replating were monitored at both optimal and suboptimal temperatures to compare our findings with previous studies and discriminate between mutants with “real” and temperature sensitivity–coupled SP phenotypes.

Out of 22 deletion strains, selected mainly from the rapidly and transiently induced regulatory genes, 13 exhibit a slow recovery (SR) phenotype specifically during exit from SP (Table 1). In eight of these 13 mutants, the SR phenotype is accompanied by loss of viability in stationary phase. Examples of SP exit mutants showing survival defects are depicted in Figure 3A, 3B. Slow recovery genes include: *DIA1* and *SOK2*, regulators of pseudohyphal and invasive growth (Christie et al., 2004; Ward et al., 1995); *MIG1*, a regulator of glucose metabolism (Lutfiyya et al., 1998) and *PRS3*, proposed to link cell cycle progression with nutrient limitation (Binley et al., 1999; Jorgensen et al., 2002).

The phenotype analysis of strains bearing deletions in one of the 51 SP upregulated genes results in the identification of 34 strains with a reduced SP survival (Table 1, Figure 3D). Of these, 16 lose viability upon replating at any tested temperature and 18 have reduced survival exclusively at 37°C. Inability to recover from SP at 37°C indicates that the SP phenotype of these strains is related to temperature sensitivity.

Genes required for SP survival are mainly involved in metabolism, including transporters (*PUT4*, *JEN1*, *YLR004C*, *FUN34*, *PDR10*, *BIO5*) and various metabolic enzymes (*ACH1*, *SNO1*, *THI4*, *MLS1*, *INO1)*. Furthermore, many of the genes necessary for SP survival act as regulators. These include: *ADR1* and *SIP4,* carbon source-responsive transcription factors (Lesage et al., 1996; Vincent and Carlson, 1998; Young et al., 2003); *YPL054W* and *YGR067C,* putative transcription factors (Bohm et al., 1997; Epstein et al., 2001; Treger et al., 1998); *RDI1, YPT53C* and *YBR203W,* involved in signaling (Koch et al., 1997; Masuda et al., 1994; Singer-Kruger et al., 1994) as well as *RIM4* and *SPR3,* regulators of sporulation (Fares et al., 1996; Soushko and Mitchell, 2000). Among the other genes with phenotypes in these assays are *HBT1*, a target of Hub1p ubiquitination, implicated in regulation of cell polarity (Dittmar et al., 2002), *PBI2*, regulator of proteolysis (Betz, 1975; Slusarewicz et al., 1997), as well as the relatively uncharacterised histone acetyltransferase *HPA2* (Sterner and Berger, 2000). In addition, eight of the 34 genes with SP phenotypes have no previously established function.

Table 1. Analysis of stationary phase survival and rate of recovery from stationary phase

Deletion mutant			Replating temperature		
Systematic Name	Gene name	Description	25°C	30°C	37°C
Genes induced upon SP exit					
YOR051C	YOR051C	Protein that purifies with the nuclear pore complex	SR +	SR +	SR +
YMR316W	DIA1	Protein involved in invasive and pseudohyphal growth	++	SR +	++
YHL011C	PRS3	Role in nutrient limitation induced cell cycle regulation	SR ++	SR +	SR ++
YGL035C	MIG1	Transcriptional repressor involved in glucose repression	SR ++	SR +	SR ++
YML123C	PHO84	High-affinity inorganic phosphate/H+ symporter	SR ++	SR ++	SR ++
YOR052C	YOR052C	Protein containing an AN1-like zinc finger domain	SR ++	SR ++	+++
YOR028C	CIN5	Transcription factor of the basic leucine zipper family	++	SR +++	SR +++
YOR032C	HMS1	Probable myc family transcription factor involved pseudohyphal differentiation	SR ++	SR +++	+++
YGR123C	PPT1	Protein serine/threonine phosphatase of unknown function	SR +++	SR +++	SR ++
YGL209W	MIG2	Zinc-finger protein involved in glucose repression of SUC2	SR +++	SR +++	SR +++
YGL162W	SUT1	Involved in sterol uptake	SR +++	SR +++	SR +++
YMR016C	SOK2	Transcription factor of meiosis, pseudohyphal differentiation and sporulation	SR +++	SR+++	SR +++
YGL158W	RCK1	Serine/threonine protein kinase with similarity to Cmk1p, Cmk2p, and Cmk3p	++	SR+++	SR +++
YOR134W	BAG7	GTPase-activating protein (GAP), activates Rho1p	++	++	++
YOR107W	RGS2	G protein signaling regulator for inactivation of the cAMP signaling pathway	++	++	++
YMR291W	YMR291W	Serine/threonine protein kinase of unknown function	+++	++	++
YIL130W	GIN1	Putative zinc finger transcription factor	+++	+++	+++
YHR136C	SPL2	Putative inhibitor of Pho80p-Pho85p cyclin-dependent protein kinase	+++	+++	+++
YER064C	YER064C	Putative global transcriptional regulator	+++	+++	+++
YER037W	PHM8	Protein involved in phosphate metabolism	+++	+++	+++
YDR281C	PHM6	Protein predicted to have a role in phosphate metabolism	+++	+++	+++
YDR043C	NRG1	Transcriptional glucose repressor and suppressor of snf mutations	+++	+++	+++
Genes induced in SP					
YLR004C	YLR004C	Member of the allantoate permease family	+	+	+
YKL217W	JEN1	Pyruvate and lactate/H[+] symporter	NT	+	+
YNL015W	PBI2	Protease B inhibitor 2, also has unrelated activity to vacuolar fusion	NT	+	+
YOR348C	PUT4	High affinity proline permease, also transports alanine and glycine	NT	+	+
YBR203W	YBR203W	F-box domain protein for ubiquitin-conjugation	NT	+	+
YDL135C	RDI1	Rho GDP dissociation inhibitor	++	++	++
YDR216W	ADR1	Transcription factor, regulation of ADH2 and peroxisomal genes	NT	++	++
YBL015W	ACH1	Acetyl-CoA hydrolase, involved in acetate utilization and sporulation	NT	++	++
YDR070C	YDR070C	Protein of unknown function	NT	++	++
YDL223C	HBT1	Target of Hub1p ubiquitination, bud site selection and shmoo formation	NT	++	++
YKL163W	PIR3	Protein with similarity to members of the Pir1p/Hsp150p/Pir3p family	NT	++	++
YBR230C	YBR230C	Protein of unknown function	NT	++	++
YPR193C	HPA2	Histone and other protein acetyltransferase	++	++	++
YMR095C	SNO1	Putative pyridoxine (vitamin B6) biosynthetic enzyme	++	++	++
YGR144W	THI4	Thiamine-repressed protein, for growth in the absence of thiamine	++	++	++
YDL048C	STP4	Similarity to Stp1p: involved in tRNA splicing and amino acid uptake	NT	+++	+
YML122C	YML122C	Protein of unknown function	SR ++	SR ++	SR ++
YJL089W	SIP4	Activator of gluconeogenic genes, activated by Snf1p kinase	NT	+++	++
YGR043C	YGR043C	Protein that may be involved in signal transduction	NT	+++	++
YMR175W	SIP18	Interacts with phospholipid vesicles, induced by osmotic stress	NT	+++	++
YGR059W	SPR3	Sporulation-specific septin	NT	+++	++
YNL093W	YPT53	Rab family GTP-binding protein involved in endocytosis	NT	+++	++
YNR002C	FUN34	Induced by excess acetate	NT	+++	++
YPL054W	LEE1	Two zinc-finger protein	NT	+++	++
YHL024W	RIM4	Protein required for sporulation and formation of meiotic spindle	NT	+++	++
YGR146C	YGR146C	Protein of unknown function	NT	+++	++
YBR047W	YBR047W	Protein of unknown function	NT	+++	++

YNL117W	MLS1	Malate synthase 1, functions in glyoxylate cycle	NT	+++	++
YFR017C	YFR017C	Protein of unknown function	NT	+++	++
YOR328W	PDR10	Member of ATP-binding cassette (ABC) superfamily	+++	+++	++
YNR056C	BIO5	Transporter of KAPA/DAPA, biotin biosynthesis	+++	+++	++
YJL153C	INO1	Inositol-1-phosphate synthase	+++	+++	++
YGR067C	YGR067C	Protein with two tandem zinc finger domains	+++	+++	++
YLR311C	YLR311C	Protein of unknown function	+++	+++	++
YML128C	MSC1	Protein that affects meiotic homologous chromatid recombination	NT	+++	++++
YMR107W	YMR107W	Protein of unknown function	NT	++++	++++
YPR002W	PDH1	Protein possibly involved in propionate utilization	NT	++++	++++
YDR536W	STL1	Hexose transporter of the major facilitator superfamily	++++	++++	++++
YKR034W	DAL80	GATA-type zinc finger transcriptional repressor	NT	+++	+++
YJL103C	YJL103C	Putative Zinc finger transcription factor	NT	+++	+++
YPL230W	YPL230W	Putative Zinc finger transcription factor	NT	+++	+++
YLR178C	TFS1	CDC25-dependent nutrient-response cell cycle regulator	NT	+++	+++
YGR236C	SPG1	Protein of unknown function	+++	+++	+++
YBR045C	GIP1	Glc7p-interacting protein for spore wall deposition during meiosis	+++	+++	+++
YPL186C	UIP4	Protein of unknown function	+++	+++	+++
YBR240C	THI2	Zinc-finger regulatory protein for thiamine biosynthesis	+++	+++	+++
YJR122W	CAF17	Component of the CCR4 transcription complex	+++	+++	+++
YBL059W	YBL059W	Protein of unknown function	+++	+++	+++
YMR096W	SNZ1	Putative pyridoxine (vitamin B6) biosynthetic enzyme	+++	+++	+++
YPL058C	PDR12	Membrane transporter	+++	+++	+++
YBR067C	TIP1	Cold- and heat-shock induced mannoprotein of the cell wall	+++	+++	+++

SR slow recovery upon exit from stationary phase
\+ loss of viability in stationary phase
++ decreased viability in stationary phase
+++ viability in stationary phase equal to wild-type
++++ increased viability in stationary phase
NT condition not tested

A total of 50 genes are found with strong to intermediate phenotypes in these assays. The finding that mutants of genes with distinct expression patterns and diverse functions can exhibit SP-related phenotypes reflects the complexity of quiescence regulation.

False discovery rate for SP phenotypes in the gene deletion strain collection

The SP phenotype analysis was carried out using the deletion strain collection (Winzeler et al., 1999), which is a commonly used strain resource for the *S. cerevisiae* deletion studies. To test how often spurious mutations or errors in this collection give rise to loss of survival in SP, we selected 40 genes that showed no upregulation in stationary phase and with no previously known growth phenotypes (as reported in Saccharomyces Genome Database) and then subjected deletion strains of these genes to an identical phenotype analysis as reported for the SP induced genes (Table 2 and Figure 4). Of the 37 deletion strains that we were able to assay (Table 2), only one had the phenotype of loss of viability in stationary phase. In retrospect, this phenotype had previously been described and attributed to deletion of this gene, but in a different database (Costanzo et al., 2000; Csank et al., 2002). Out of the other 36 strains, none showed the phenotype. These numbers compare favorably to our original finding that 34 of the 51 deletion strains (selected for upregulation of the corresponding mRNA in stationary phase) have this phenotype. This control experiment indicates that the false-positive rate for this phenotype screen is less than 3% for the strains used here.

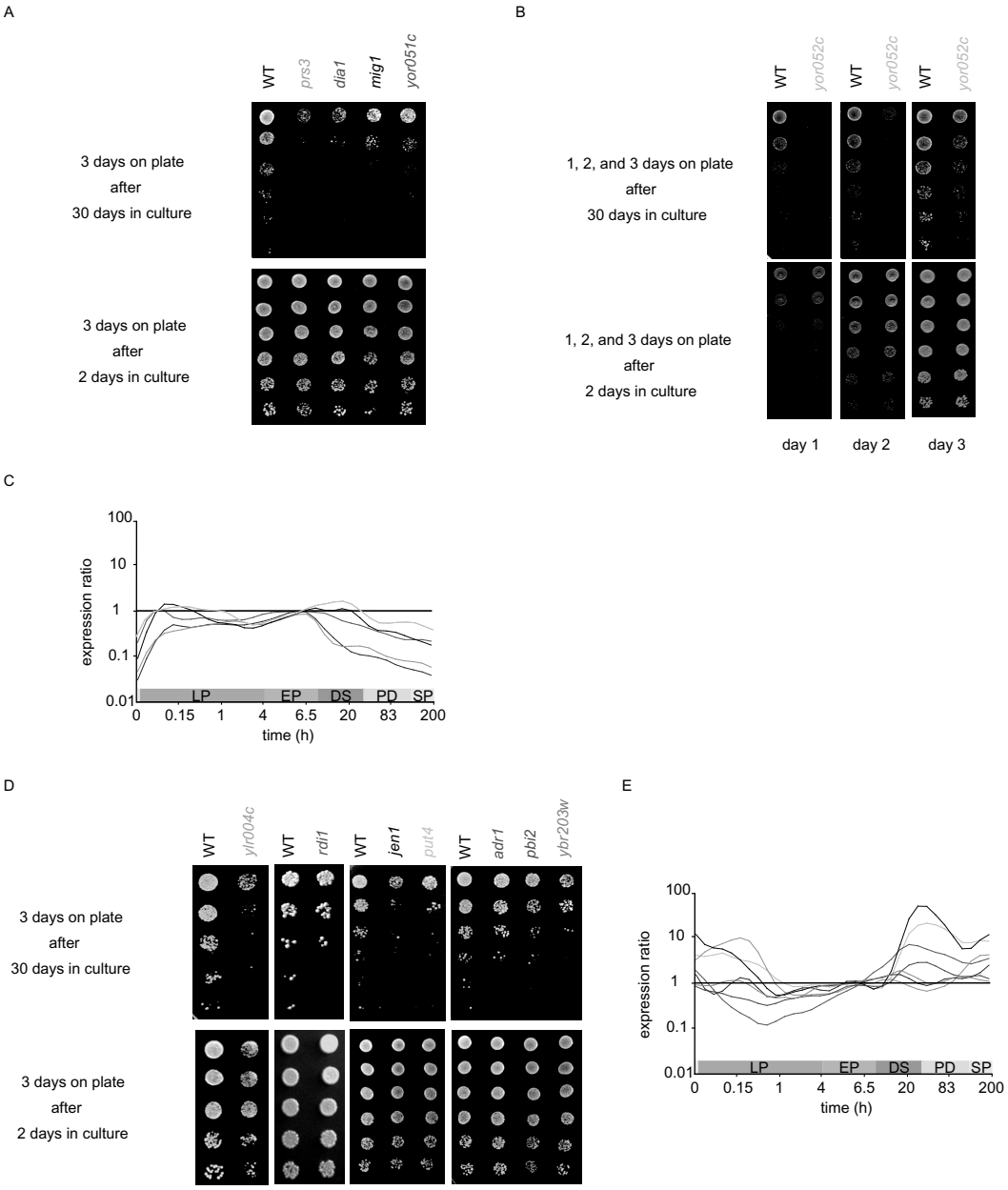

Figure 3. Stationary phase phenotypes

(A, B) Examples of phenotypes for mutants bearing deletion in stationary phase (SP) exit genes. Wild-type and mutant strains were grown for 30 days in YPD culture at 30°C. Cells from 30-day (top panels) or two-day (bottom panels) cultures were spotted in five-fold serial dilutions on YPD plates and incubated for 3 days at 30°C. Loss of viability in stationary phase is estimated from the number of colonies formed on the third day upon replating (A). Slow recovery from stationary phase is deduced from the size of colonies formed during first, second and third day upon replating (B).

Table 2. Phenotype analysis of strains from deletion strain collection

Deletion mutant	Systematic name	Gene name	Remark
1	YAL026C	DRS2, FUN38	
2	YAL013W	DEP1, FUN54	
3	YOR061W		
4	YOR355W		
5	YPL127C		
6	YEL014C		
7	YIL016W	SNL1	
8	YML075C	HMG1	both cultures contaminated
9	YJR010C-A	SPC1	
10	YJR032W	CPR7	
11	YBR189W	RPS9B, RPS13A, SUP46	both cultures contaminated
12	YGR038W	ORM1	
13	YGR106C		
14	YKR094C	RPL40B, UBI2	
15	YPL158C		overnight culture did not grow
16	YGR221C		
17	YKR074W		
18	YHR193C	EGD2	
19	YFR006W		
20	YKL207W		
21	YLR313C	SPH1	
22	YML060W	OGG1	
23	YPR068C	HOS1	
24	YPR126C		
25	YLR065C		
26	YLR179C		
27	YPL091W		loss of viability in SP
28	YBL079W	NUP170, NLE3	
29	YBR174C		
30	YBR187W		
31	YDL121C		
32	YDL236W	PHO13	
33	YDR257C	RMS1	
34	YDR279W		
35	YDR492W		
36	YDR503C	LPP1	
37	YGL050W		
38	YGL221C	NIF3	
39	YGL229C	SAP4	
40	YOR179C		

(C) mRNA expression profiles of genes with phenotypes shown in (A) and (B). The colour of mRNA expression graphs corresponds to the colour of gene names in (A) and (B). The bar at the bottom of mRNA expression graphs represents the various culture periods, with abbreviations according to Figure 1.
(D) Examples of phenotypes for mutants bearing deletions in SP induced genes, revealed by an identical analysis as in (A) and (B).
(E) mRNA expression profiles of genes with phenotypes shown in (D). The colour of mRNA expression graphs corresponds to the colour of gene names in (D).

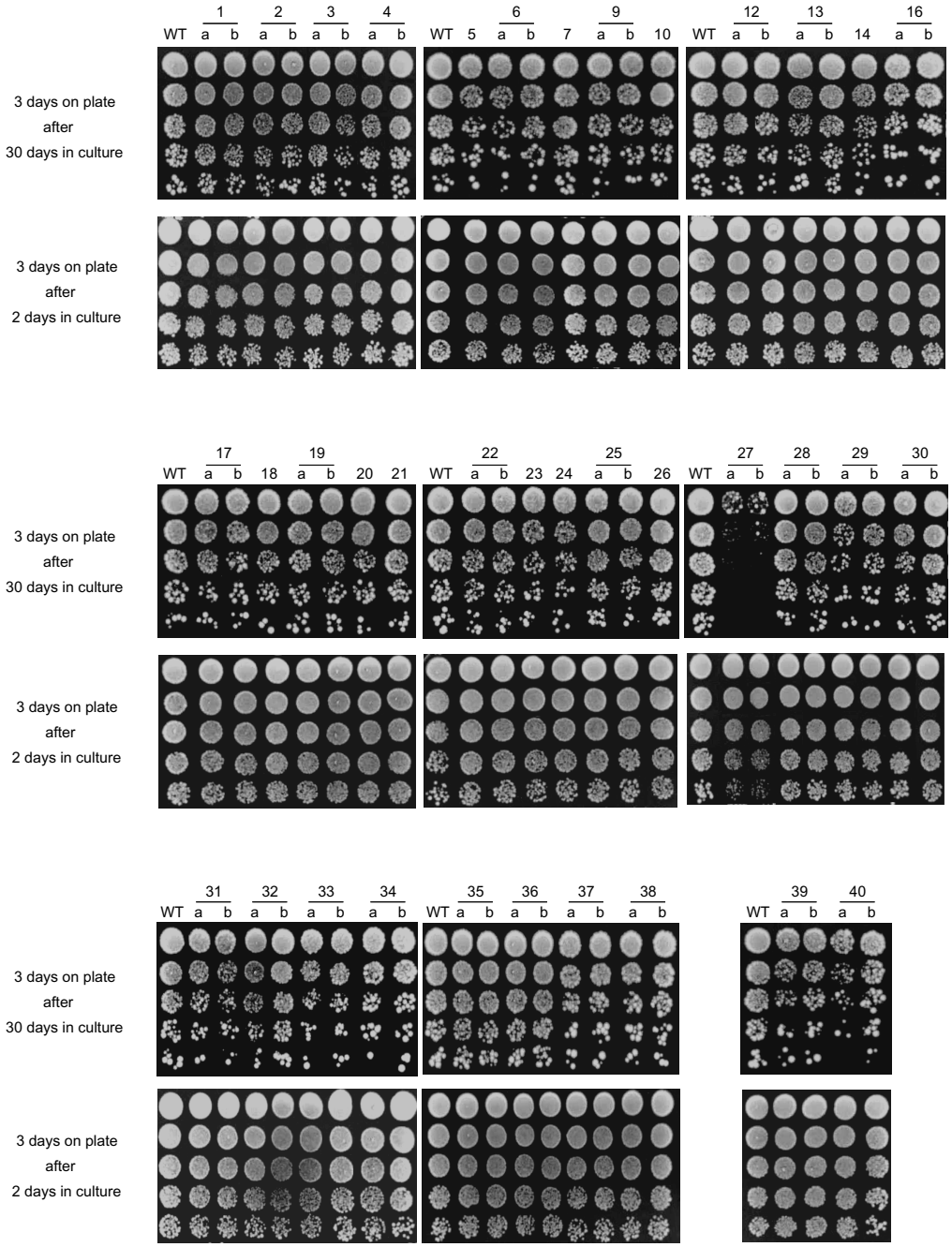

Figure 4. Determination of false discovery rate for SP phenotypes in the gene deletion strain collection
To determine how often spurious mutations in deletion strain collection give rise to SP survival phenotypes, 40 mutant strains listed in Table 2 were subjected to SP phenotype analysis as reported in the Experimental Procedures. Gene names corresponding to mutants 1 to 40 are given in Table 2. (a) and (b) refer to replicate experiments, i.e. independently picked colonies, grown independently.

Discussion

Genome-wide studies are a powerful means of identifying gene function. Here, we employ gene expression data (Radonjic et al., 2005), to determine the most important biological processes that occur during the exit and entry into stationary phase (SP). Furthermore, we find a SP related role for many of the SP expressed genes by phenotype analysis. The diversity of the identified genes suggests that yeast quiescence is a complex cellular state and that regulation of multiple cellular functions is required for successful completion of the quiescence cycle.

Functions of SP exit genes

Important processes developed to cope with the improved nutritional status upon exit from stationary phase are preparation for protein synthesis and activation of gene-specific transcription regulators. The ribosome biosynthesis genes are identified as the most significant category of SP exit induced genes, which is in agreement with previous studies (Martinez et al., 2004). The rapidly activated transcription regulators are particularly informative for understanding quiescence exit control. The nature of these regulators reveals the expression remodeling that takes place upon SP exit, leading to inactivation of the program required for the maintenance of SP (*ROX1, ROX3, NRG1, MIG1*), and to activation of cellular growth (*DRS1, FAL1, SNU13, NOP1, NSR1*).

Quiescence versus stationary phase

Functional categories of genes induced during various starvation conditions have been investigated in several expression studies (DeRisi et al., 1997; Gasch et al., 2000; Wu et al., 2004). Our analysis of stationary phase induced genes (Radonjic et al., 2005), identified activation of metabolic processes and stress response genes. Clearly, a distinction needs to be made between genes required for the quiescence growth arrest and genes required for the particular condition under which quiescence is induced and maintained. For example, induction of SNO and SNZ gene families has traditionally been considered a hallmark of stationary phase (Padilla et al., 1998). However, induction of such genes has been shown to be specific to stationary phase in auxotrophic strains (Radonjic et al., 2005), demonstrating that this activation is a consequence of specific requirements in amino acid metabolism and not the universal property of arrested quiescent cells. This is in agreement with the finding that these genes function in pyridoxine metabolism (Rodriguez-Navarro et al., 2002), which serves as the co-factor for amino acid biosynthesis enzymes. Most of the stationary phase enriched genes are indeed involved in cellular metabolism. Many are required for respiration and and are already induced at the end of the diauxic shift (Radonjic et al., 2005) (Figure 1B). These genes have specific metabolic roles and therefore, similar to the SNO/SNZ family, are likely to be activated as a consequence of subsequent changes in the culture environment such as pH, salt and other limiting nutrients, rather than directly due to quiescence.

The induction of most of the stationary phase transcripts during the diauxic shift (DS) (Radonjic et al., 2005) suggests that the program of quiescence may already be (partially) initiated during this phase. This hypothesis is supported by transient activation of the genes required for diverse survival programs early during DS (Radonjic et al., 2005). This includes the cell cycle and nutrient limitation regulators as well as the genes involved in mating, sporulation and pseudohyphal differentiation, indicating integrative control of various nutrient adaptation survival programs (Madhani and Fink, 1998). The model emerging from these data suggests that the quiescent program is induced much earlier in the glucose starvation experiments than previously assumed (Gray et al., 2004). Another implication of the model proposed above is that glucose starvation, the most frequently applied method of quiescence induction, is perhaps too complex to allow simple unraveling of purely quiescent regulatory mechanisms.

Stationary phase exit, entry and maintenance mutants

The analysis of mutant phenotypes is a powerful method for determination of gene function. Three subclasses of "stationary phase" (SP) mutants are proposed to exist, all of which may be defective in one or more transitions during the cell quiescence cycle (Gray et al., 2004). This distinction has not been addressed in previous mutant analyses. SP mutants were simplistically defined as those that lose viability when cultured to stationary phase, and were often confused with temperature sensitivity phenotypes. For example, in Martinez et al., 2004, SP phenotypes are investigated by growing mutants to SP at 37 instead of 30°C, which results in additional stress. All strains that lose viability were classified as "stationary phase essential", although only quarter shows any growth defect when grown to SP at 30°C. This demonstrates that the majority of such previously reported mutants lose viability as the consequence of temperature sensitivity, rather than only impaired SP survival.

Mutants that are defective in exit from SP are viable during quiescence but unable to generate colony forming units (CFU) upon replating (Gray et al., 2004). It is likely that strains with such phenotypes would already be classified as essential by previous analyses of the deletion collection (Winzeler et al., 1999), since successful manipulation of the stock cultures requires completion of the entire quiescence cycle. Mutants with severe SP exit phenotypes are therefore probably not present in the mutant collection and could not be investigated here. This agrees with the fact that 25% of the SP exit induced genes are indeed found to be essential. We propose that mutants that are capable of partially recovering from stationary phase, but which are unable to reach the wild-type rate of colony growth, represent a less severe manifestation of the lethal SP exit phenotype (Table 1, Figure 3A, 3B).

Although our phenotype study introduces several improvements, a downside of the commonly used replating assay is that it cannot distinguish between mutants defective in establishment of SP and mutants impaired for SP maintenance (Gray et al., 2004). Both of the defects are manifested by decreased viability in stationary phase, however, this lethality has different causes: entry mutants fail to establish quiescence and to acquire protective properties that are essential for survival, and maintenance mutants successfully enter quiescence but are unable to maintain long-term viability in that state. These two subclasses can be discriminated by the assessment of various quiescence properties of the mutants. It would be useful to examine DNA content of the cells during entry into stationary phase to determine which mutants lack the ability to arrest growth in G0 phase. Additionally, levels of stress resistance and diverse biochemical properties of the cells could be monitored as indicators of protective capacity. These studies will be required to make a distinction between SP entry and SP maintenance mutants and to determine in which particular phase of the quiescence cycle are the mutated genes involved.

RDI1 is a putative quiescence regulator

Our phenotype study implies a SP related role for 34 SP induced genes, including many regulators of growth and metabolism. Particularly interesting is the phenotype observed upon deleting *RDI1*, a proposed GDP dissociation inhibitor for Cdc42. Cdc42 is an evolutionary conserved Rho-type GTPase regulator of mammalian cell morphogenesis and cell division polarity in yeast (Johnson, 1999). The function of a Rho GDP dissociation inhibitor in yeast has remained elusive, despite the interest because of its interaction with the growth regulator Cdc42 (Masuda et al., 1994). Previous deletion analysis of *RDI1* did not show loss of viability in normal cultures or any defects in mating, sporulation, heat shock or budding (Masuda et al., 1994). However, over-expression of *RDI1* did result in inhibition of cell growth. Loss of SP survival upon *RDI1* deletion (Figure 3D), suggests that the Rho GDP dissociation activity keeps Cdc42 inactive, thereby preventing inappropriate proliferation during quiescence. To the best of our knowledge,

RDI1 represents the first gene that shows these reciprocal phenotypes, that is inhibition of growth upon over-expression in proliferating cells and loss of SP survival upon deletion. This leads to the proposal that Rdi1 negatively regulates proliferation during quiescence through its interaction with Cdc42. In accordance with the model of early quiescence induction proposed here, transient upregulation of *RDI1* mRNA also occurs at the beginning of the diauxic shift (Figure 3E).

The analyses described here discover regulators involved in entry into and exit from quiescence and identify many genes required for stationary phase survival. A substantial set of these genes has no prior established cellular role, including 64 of 201 rapidly induced genes upon SP exit and 178 of 460 genes upregulated in SP. These genes can now be classified as SP related, increasing significantly the number of currently annotated SP genes. Altogether, the data provide novel insights into the nature and the regulation of quiescence and opens new avenues for the further exploration of this relatively uncharacterised and important cellular state.

Experimental procedures

Yeast strains

S.cerevisiae BY4741 (*MATa*; *met15*Δ0; *ura3*Δ0; *his3*Δ1; *leu2*Δ0) (Research Genetics) was used as the wild-type reference strain in all experiments. The stationary phase phenotype analysis was carried out using the yeast knockout strain collection of the yeast gene deletion consortium (Winzeler et al., 1999). Deletion mutants were obtained through EUROSCARF. In addition, the mutant strain bearing deletion of *RDI1* gene was reconstructed for this study, by replacing the ORF with a kanamycin cassette in the BY4741 parental strain.

Stationary phase phenotype analysis

All strains were cultured in YEP medium supplemented with 2% glucose at 30°C. The cultures were grown under agitation (250 rpm) for 30 days and subsequently examined for viability by spot assay. Starting with equal amounts of stationary phase cells, five-fold serial dilutions were spotted on YPD plates and incubated for 3 days at 25, 30 and 37°C. Plates were screened for growth after the first, second and third day upon replating. The viability of strains was estimated by the number of colonies formed on a plate after the third day of incubation. Fewer colonies formed by mutant compared to wild-type strain identified mutants with decreased viability in the stationary phase. To discover mutants that are indispensable for reproliferation from the stationary phase, the plates were additionally checked for growth after the first and second day of incubation. This enabled the assessment of the slow recovery phenotype (SR) manifested by formation of smaller colonies by mutants compared to wild-type strain in the first days upon plating. Upon continued culture of the plates, these SR strains partially recover and show closer to wild-type growth on later days. For all mutants, the control spot assay was performed after 2 days of growth in the liquid culture. The aim of this control was to ensure that observed growth-related defects are specific to the stationary phase of growth.

Functional categorization of co-expressed genes

Genes co-regulated upon a stationary phase exit and entry (Radonjic et al., 2005) were queried for annotations of functional categories using FunSpec (Robinson et al., 2002). Biological Process and Molecular Function categories were derived from the Gene Ontology (GO) database (Harris et al., 2004) and MIPS database (Mewes et al., 2004). Obtained p-values were subjected to Bonferroni correction for multi-variant testing.

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Chapter 4

Integrative genomic analysis characterizes a novel regulator of the *S. cerevisiae* starvation response

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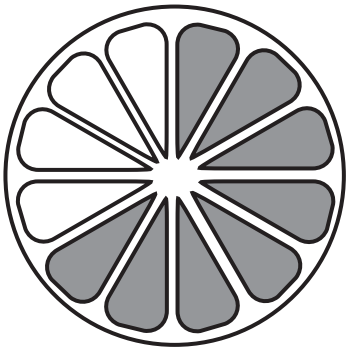

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Integrative genomic analysis characterizes a novel regulator of the *S. cerevisiae* starvation response

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Summary

Cells respond to environmental changes by altering expression of genes that allow adaptation to the new condition. These transcriptional changes are initiated by signals that converge on transcription factors, which act combinatorially to control many genes in multiple pathways. In the yeast *Saccharomyces cerevisiae*, the set of regulators that control the yeast starvation response has not been completely characterized. Previous genome-wide mRNA expression analysis identified an uncharacterized zinc-finger containing protein, Yjl103c, as being induced exclusively after exponential growth, suggesting a role in regulation of the post-exponential growth phases. Here we employed genome-wide location analysis (ChIP on chip), DNA microarray expression profiling, DNA motif searches and phenotype analysis to identify direct and indirect targets of Yjl103c and to discover processes that it regulates. These analyses reveal that Yjl103c acts as an activator of genes, many of which are regulators themselves, such as gene specific transcription factors Hap4, Adr1, Gis1, Gat2, Sfl1, Mcm1, Mga1, Sef1, and Yer103c, and global regulators Rba50, Snf11, and Htz1. The results reveal that Yjl103c modulates multiple processes during adaptation to nonfermentable carbon sources and entry into stationary phase, including gluconeogenesis, the cell wall integrity pathway, protein degradation, stress response, the tricarboxylic acid cycle (TCA), respiration and peroxisomal fatty acid beta-oxidation. Some of these processes are likely to be controlled directly by Yjl103c, for which we propose the name Gsm1 (Glucose Starvation Modulator), whereas others are indirectly regulated through Yjl103c targets. Taken together, these results functionally characterize a novel regulator of the starvation response and uncover its downstream regulatory network.

Introduction

Advances in high-troughput DNA-sequencing technology during the late 1980s have facilitated the development of genomics approaches to studying genes and their functions (Kumar and Snyder, 2001). Since the release of the first complete genome sequences, a variety of genome-wide experimental methods has been developed that exploit the huge body of sequence data and provide rapid, parallel surveys of thousands of genes in single assays. An important step forward in this field has been the development of DNA microarray-based technologies (Chee et al., 1996; Schena et al., 1995). Primarily employed in mRNA expression profiling (Lockhart et al., 1996), the use of microarrays has expanded to include a variety of applications, such as location analysis of DNA-binding proteins (ChIP on chip), alternate transcript and microRNA profiling, monitoring of nucleosome positioning and mapping chromatin modifications (Liu et al., 2005; Loden and van Steensel, 2005; Pokholok et al., 2005; Ren et al., 2000; Sun et al., 2004). Individually and particularly in combination, such large-scale analyses are starting to facilitate rapid functional

characterization of eukaryotic genomes.

The budding yeast *S. cerevisiae* is an important experimental organism for large-scale functional analyses. With its small genome and tractable genetics, this yeast has played a prominent role in the development of many methodologies for genome-wide functional assays (Grunenfelder and Winzeler, 2002). A number of genome-wide approaches have been employed to systematically determine *S. cerevisiae* gene functions, contributing to making it one of the most thoroughly studied eukaryotes (Giaever et al., 2002; Hughes et al., 2000; Ito et al., 2000; Uetz et al., 2000; Winzeler et al., 1999).

Despite these advances, one-third of yeast genes remains functionally uncharacterized and the roles of many characterized genes are still obscure (Hirschman et al., 2006). This is in part due to the fact that the vast majority of systematic functional analyses are performed during yeast exponential growth, when cells exhibit maximal biochemical activity. Although this approach is likely to detect and describe a wide spectrum of expressed genes, it largely overlooks those genes that are active under suboptimal conditions including post-diauxic growth phases and environmental stresses. Consequently, 45% of genes induced in the environmental stress response (ESR) and 40% of genes induced in stationary phase are completely uncharacterized (Gasch et al., 2000; Radonjic et al., 2005).

Finding an appropriate experimental condition is also fundamental for investigation of transcription factors (TF). One approach to identify TF target genes is to determine transcription factor location on DNA *in vivo* by chromatin immunoprecipitation (ChIP) (Orlando, 2000). However, the success of ChIP-based methods depends on the expression and cellular location of a particular TF under a given condition. If the TF is not expressed under the studied experimental condition, ChIP will not result in specific TF enrichment and its targets cannot be identified. This is exemplified by systematic analysis of 106 *S. cerevisiae* TFs under optimal growth conditions by microarray-coupled chromatin immunoprecipitation (ChIP on chip) (Lee et al., 2002). This study detected known targets for 50% of TFs, and for example, failed to detect targets of TFs that play a role in stress responses. The importance of studying TFs under appropriate activating conditions was recognized and addressed in a later study by Harbison and colleagues, who investigated TF binding patterns in both standard and alternative environmental conditions (Harbison et al., 2004). Many of the binding profiles differ considerably depending on growth conditions, generally with more promoters bound by the TFs under perturbed conditions. Therefore, studies of uncharacterized transcription regulators require environmental perturbations as a means to increase the range of transcription factors targets and to uncover new biological roles that cannot be detected under standard settings.

We have previously used DNA microarray expression profiling to describe the changes in mRNA levels that accompany entry into and exit from stationary phase (SP) in the yeast *S. cerevisiae* (Radonjic et al., 2005). This study revealed that out of 460 genes upregulated in SP, 40% are functionally uncharacterized. Investigation of putative regulators among the uncharacterized SP-induced genes discovered several candidates for further functional analysis. Here, we examine the putative regulatory role of Yjl103c, a protein with structural similarity to transcription factors but with unknown function (Issel-Tarver et al., 2002; Todd and Andrianopoulos, 1997). The initial observation that *YJL103C* mRNA increases upon glucose depletion prompted further investigation of the regulatory properties of this factor, including genome-wide location analysis (ChIP on chip), DNA microarray expression profiling of a deletion mutant, DNA motif searches and phenotype analyses. The combination of genome-wide assays performed under activating conditions for Yjl103c enables identification of direct and indirect targets of Yjl103c and reveals its involvement in regulation of multiple processes ongoing during adaptation to nonfermentable carbon sources and entry into stationary phase. In addition, the considerable number of transcription factors among Yjl103 target genes suggests a role for this factor in modulating the complex regulatory

network that controls the *S.cerevisiae* starvation response.

Results

Yjl103c is a putative regulator of the *S. cerevisiae* starvation response

Fermentative growth on glucose is an optimal phase of the *S. cerevisiae* life cycle, characterized by rapid cell proliferation (exponential growth phase, EP). When glucose is exhausted, cells temporary arrest growth during the diauxic shift (DS), while adjusting their metabolism for utilization of alternative (non-fermentable) carbon sources. This results in slow, post-diauxic growth (PD), which is driven by oxidative processes such as respiration and fatty acid oxidation. When all available carbon sources are depleted, cells cease proliferation and enter quiescence, forming a stationary phase (SP) culture (Gray et al., 2004).

The growth changes occurring during glucose depletion are accompanied by elevated stress resistance, major perturbations in metabolism and altered biochemical activity (Gray et al., 2004). Investigation of genome-wide mRNA expression in exponential and stationary phase of growth revealed a global transcriptional shut-down that occurs in SP, affecting 95% of the genome (Choder, 1991; Radonjic et al., 2005). The small group of SP-induced genes is largely uncharacterized. Based on the expression profile, SP-induced genes are proposed to be involved in one of the many processes occurring during adaptation to nonfermentable carbon sources and entry into stationary phase.

To discover novel gene-specific regulators of stationary phase, we examined uncharacterized SP induced genes for the presence of regulatory features in their protein structure, such as DNA binding domains. The product of one of such genes, Yjl103c, is a protein of unknown function that contains a Zn[2]-Cys[6] fungal-type binuclear cluster domain in the N-terminal region, probably involved in DNA recognition (Issel-Tarver et al., 2002). The expression

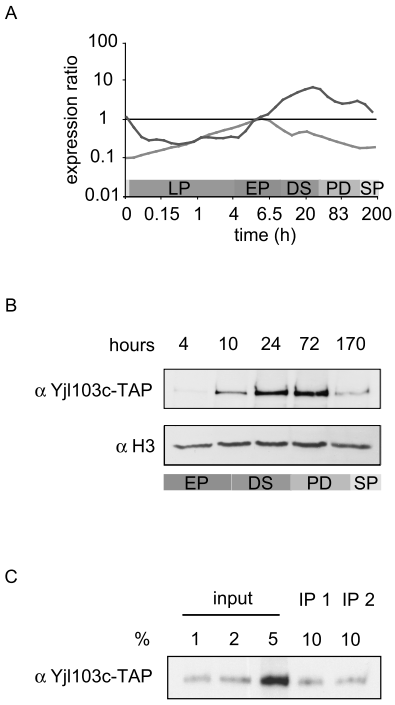
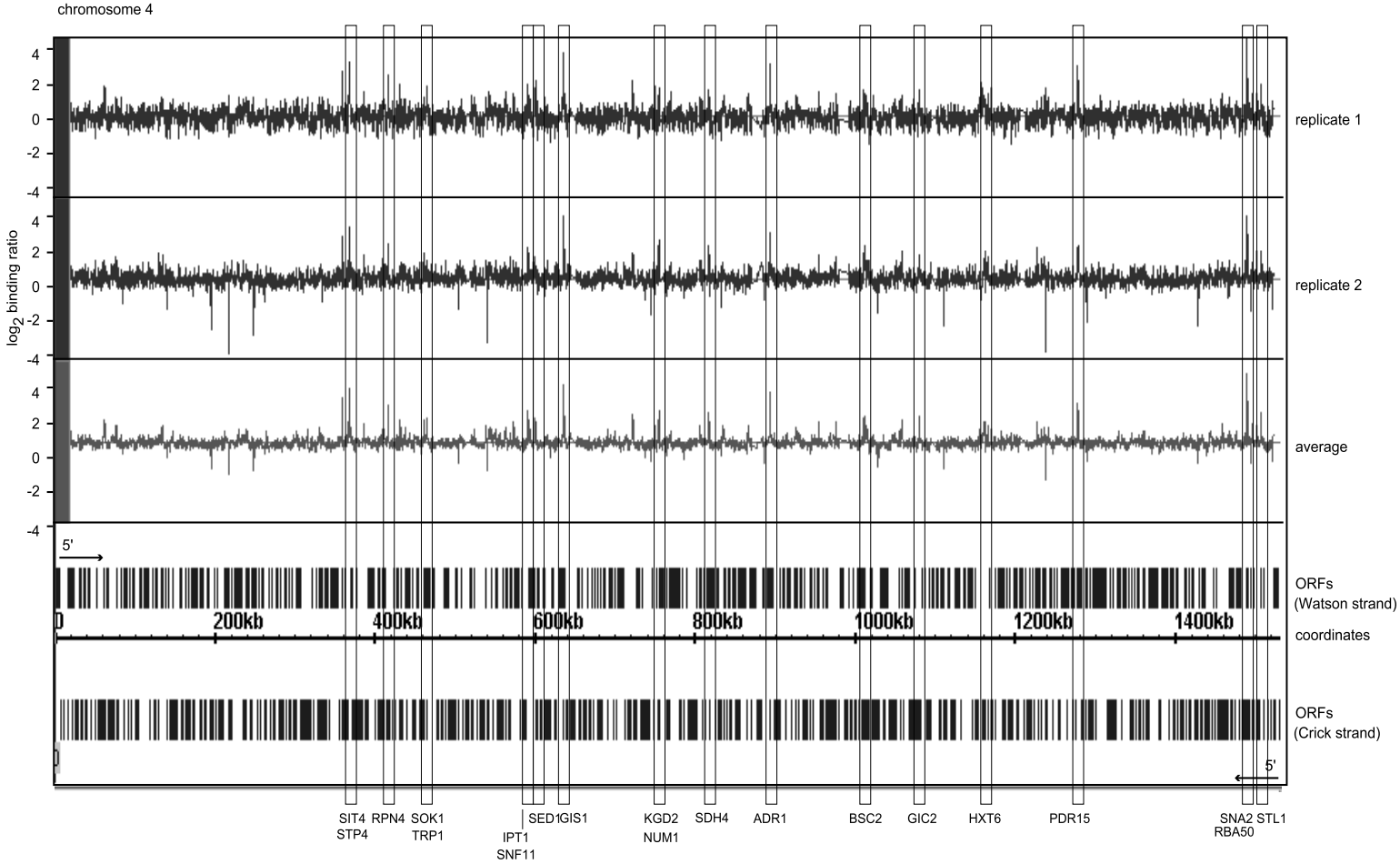


Figure 1. Yjl103c is expressed upon glucose depletion

(A) mRNA expression profile of *YJL103C* (red) during the entire *S. cerevisiae* growth cycle, including exit from stationary phase (SP), entry into SP and intervening phases. The gray line represents the average expression of all genes. Data are normalized through external controls (Radonjic et al., 2005). The coloured bar at the bottom of graphs illustrates the various culture periods: LP, lag phase; EP, exponential phase; DS, diauxic shift; PD, post-diauxic phase; SP, stationary phase.

(B) Yjl103c protein expression during the exponential and post-exponential growth phases. Western-blot analysis of chromatin enriched protein extracts was performed using an antibody against TAP-tagged Yjl103c. Extracts from equivalent amounts of cells were loaded and this was verified using an antibody specific for the invariant part of Histone H3 (H3). The coloured bar at the bottom of the graphs corresponds to the growth phases described in (A).

(C) Immunoprecipitation (IP) efficiency of Yjl103c from the chromatin extracts (CE) at the 24 hours time-point (see B). IP efficiency was monitored by western blot analysis comparing 1, 2 and 5% of input (CE) to 10% of IP extracts, and estimated to be approximately 20% of the input.


chromosome 4
log2 binding ratio
4
2
0
-2
-4
replicate 1
replicate 2
average
5'
0
200kb
400kb
600kb
800kb
1000kb
1200kb
1400kb
ORFs
(Watson strand)
coordinates
ORFs
(Crick strand)
5'
SIT4
STP4
RPN4
SOK1
TRP1
IPT1
SNF11
SED1
GIS1
KGD2
NUM1
SDH4
ADR1
BSC2
GIC2
HXT6
PDR15
SNA2
RBA50
STL1

pattern of *YJL103C* during the *S. cerevisiae* growth cycle demonstrates that mRNA levels of this gene increase immediately upon glucose depletion, reach maximum expression at the diauxic shift, decline during post-diauxic growth and partially recover during entry into stationary phase (Radonjic et al., 2005). Upon re-addition of glucose-rich medium to the culture, *YJL103C* is immediately repressed (Figure 1A). The mRNA expression profile of *YJL103C* suggests that this factor might be involved in processes ongoing during adaptation to nonfermentable carbon sources and entry into stationary phase. The presence of a putative DNA recognition motif indicates that Yjl103c may act as a DNA-binding transcription factor. A potential role for Yjl103c in the regulation of metabolism was also predicted by computational analysis of *YJL103C* mRNA expression in the diauxic-shift dataset provided by DeRisi and colleagues (Deng et al., 2005; DeRisi et al., 1997).

Yjl103c is present in chromatin extracts of glucose-starved cells

To investigate putative regulatory properties of Yjl103c, we considered it necessary to first determine possible activating conditions for this factor. Starting from the mRNA expression profile, we chose conditions similar to those of mRNA induction to analyse Yjl103c protein expression. This included five time-points of the growth cycle, covering exponential growth phase, diauxic shift, post diauxic growth, and stationary phase (Figure 1B). To ensure that the detected protein levels reflect the portion of Yjl103c that may be bound to DNA (and therefore more likely to be active), we investigated the presence of Yjl103c in chromatin-enriched extracts (see Experimental Procedures). During the exponential growth phase, Yjl103c protein is nearly undetectable (Figure 1B). The absence of Yjl103c from the chromatin extracts at this phase corresponds to mRNA repression (Figure 1A). Immediately upon glucose depletion, Yjl103c is detected in chromatin extracts, reaching a peak of expression during the diauxic shift (Figure 1B). Unlike its mRNA, Yjl103c protein remains highly expressed throughout post-diauxic growth and declines later, as the culture enters stationary phase. The high expression of both mRNA and protein levels of Yjl103c during the diauxic shift indicates that this phase represents good activating condition for further functional analysis of this factor.

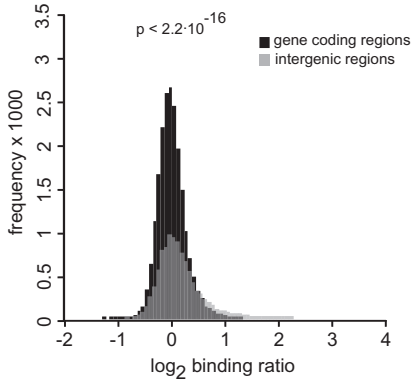
Genome-wide location of Yjl103c

To identify genes that might be regulated by Yjl103c, we investigated the genome-wide binding pattern of TAP-tagged Yjl103c using microarray-coupled chromatin immunoprecipitation (ChIP on chip) (Ren et al., 2000). The microarray used for ChIP analysis carries over 40,000 probes for the yeast genome, representing all genes, hypothetical open reading frames (ORFs), dubious ORFs and intergenic regions (Pokholok et al., 2005). The genomic location of Yjl103c was monitored during the diauxic shift (24 hour time-point) because of the high transcriptional

Figure 2. Pattern of Yjl103c binding to chromosome 4

An example of Yjl103c binding to an entire chromosome, visualized using the Integrated Genome Browser (IGB) (http://www.affymetrix.com/). Three binding tracks are shown, representing $\log_2$ binding ratios of Yjl103c for all chromosomal features. The two top graphs (black) are derived from the two replicate ChIP on chip experiments and the bottom graph (red) shows the average values of these replicates. The horizontal axis illustrates the chromosomal position of the array features along *S. cerevisiae* chromosome 4, and the blue blocks represent the array features themselves on Watson and Crick DNA strands. Framed peaks of binding ratios and their corresponding array features are selected as significantly enriched after applying statistical analyses and "distance from ORF" criterion (Experimental procedures). The open reading frames (ORFs) located downstream of these enriched features are shown. ORF pairs *SIT4* and *STP4*, *SOK1* and *TRP1*, *IPT1* and *SNF11*, *KGD2* and *NUM1*, and *SNA2* and *RBA50* have common enriched features, as these are positioned within sufficient proximity upstream of both ORFs.

A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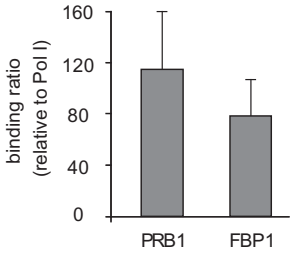
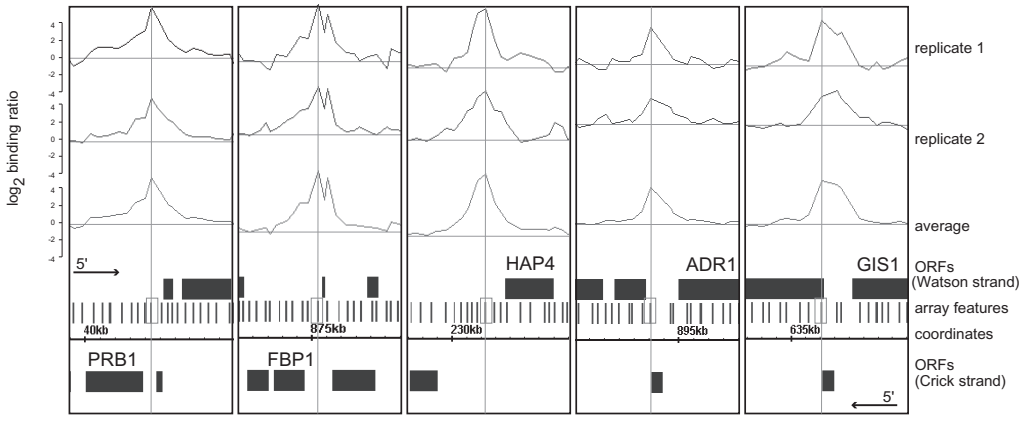
gene coding regions
intergenic regions
frequency x 1000
log2 binding ratio

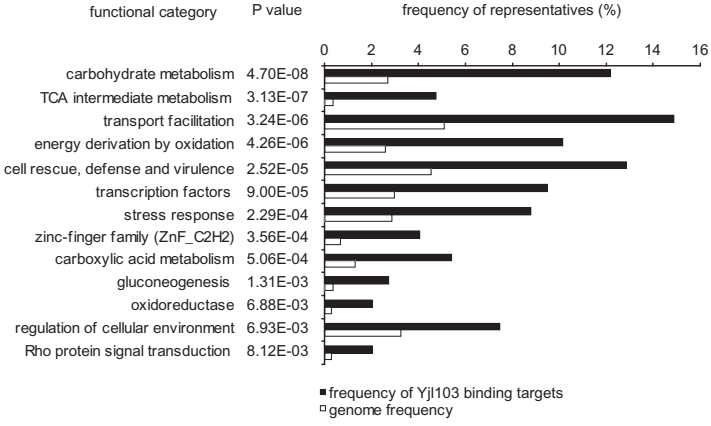
B


binding ratio
(relative to Pol I)
PRB1
FBP1

C


log2 binding ratio
replicate 1
replicate 2
average
HAP4
ADR1
GIS1
ORFs
(Watson strand)
array features
coordinates
ORFs
(Crick strand)
PRB1
FBP1

D


functional category
P value
frequency of representatives (%)
carbohydrate metabolism 4.70E-08
TCA intermediate metabolism 3.13E-07
transport facilitation 3.24E-06
energy derivation by oxidation 4.26E-06
cell rescue, defense and virulence 2.52E-05
transcription factors 9.00E-05
stress response 2.29E-04
zinc-finger family (ZnF_C2H2) 3.56E-04
carboxylic acid metabolism 5.06E-04
gluconeogenesis 1.31E-03
oxidoreductase 6.88E-03
regulation of cellular environment 6.93E-03
Rho protein signal transduction 8.12E-03
frequency of Yjl103 binding targets
genome frequency

and translational activity of *YJL103C* at this time. The efficiency of Yjl103c immunoprecipitation from chromatin extracts was approximately 20% (Figure 1C). Genomic localization profiles for Yjl103c were generated from immunoprecipitated samples originating from two independent cultures, in dye-swap, resulting in two ChIP profiles (Figure 2, Figure 3B). An example of the Yjl103c binding pattern across a chromosome is presented in Figure 2. Due to the normalization method, statistically significant ChIP enriched locations are those that are relatively higher enriched compared to the entire genome, which is artificially set at a binding ratio of one (no change relative to ChIP input material). Analysis of Yjl103c ChIP profiles reveals 257 enriched array features, representing 192 intergenic regions (IGRs) and 65 gene coding regions (GCRs). The preferential binding of Yjl103c to IGRs (Figure 3A) is an expected location pattern for gene-specific transcription factors and further indicates a putative regulatory role.

To confirm enrichment of Yjl103c on IGRs, we selected two regions (upstream of *PRB1* and *FBP1*) for a control ChIP assay using quantitative PCR (qPCR) (Figure 3B). The binding ratios obtained by qPCR reflect the observed ChIP-chip enrichments (Figure 3C), but with more extreme values. This is probably due to different sensitivities of the two methods, suggesting that binding ratios obtained by ChIP on chip might be an underestimation of the actual enrichment. The range of statistically significant binding ratios for ChIP on chip experiments was between 1.9 and 15.9.

To investigate targets of Yjl103c regulation, we applied a distance criterion to map those ORFs which are located downstream of the Yjl103c-bound IGRs within sufficient proximity (Experimental Procedures, Figure 3C). This identified a total of 151 ORFs that can be considered as possible targets of Yjl103c regulation (Figure 2, Figure 3). Analysis of overrepresented functional categories among the Yjl103c binding targets reveals a large overlap with functions of stationary phase induced genes (Radonjic et al., 2005), suggesting that Yjl103c might affect many processes required for entry into stationary phase (Figure 3D). This includes carbohydrate metabolism, processes involved in energy derivation from oxidation and stress response.

Interestingly, Yjl103c binding targets also include numerous transcription regulators and

Figure 3. Genome-wide location analysis of Yjl103c

(A) Distribution of $\log_2$ binding ratios (BRs) for gene coding regions (GCR) and intergenic regions (IGRs). The shift of IGR binding ratios towards higher values implies preferential binding of Yjl103c to IGRs. The p-value is determined by a t-test that assessed the probability of binding ratios for GCRs and IGRs being equal. After applying the significance cutoff, 257 features are selected as significantly enriched and 192 of those are annotated as intergenic regions.

(B) Confirmation of the ChIP on chip results by quantitative real-time PCR (qPCR). Binding ratios (IP / IP Pol I) for ChIP experiments were generated using real time PCR with specific primer sets corresponding to two Yjl103c bound regions (as determined by ChIP on chip), located upstream of *PRB1* and *FBP1*. A *POL1* reaction was performed concomitantly and later used as a relative ratio reference. The values shown are an average of two biological replicate experiments and each of these included 3 replicate PCR reactions performed in parallel and averaged. The standard deviation of values derived from the two biological replicate experiments is shown.

(C) Examples of Yjl103c binding. The binding of Yjl103c is represented equivalently as in Figure 2, with the focus on the 1-kilobase (kb) wide surroundings of the peak of Yjl103c enrichment. The vertical line is drawn to mark the array features with maximal binding ratios. Their corresponding target ORFs are labeled with gene names. The numbers on the horizontal axis represent the location of array features on their corresponding chromosomes.

(D) Functional categories enriched among Yjl103c binding targets. ORFs located downstream of Yjl103c binding sites were analysed for overrepresentation of functional categories derived from MIPS and GO databases. The functional category is shown on the left, followed by the p-value as determined by a hypergeometric test. The black bar indicates how many of the queried genes are assigned to a category and the white bar, how many genes in the genome belong to that category.

several components of intracellular signaling cascades. Examples of transcription regulators are *MSN2* and *MSN4*, activators of general environmental stress response; *YER130C*, also involved in stress response; *ADR1* and *HAP4*, glucose-responsive regulators; *GAT1* and *GAT2*, nitrogen depletion responsive factors; *GIS1*, activator of diauxic shift genes; *ROX1* and *SUT1*, regulators of hypoxic genes; *MCM1*, regulator of pheromone response; *MOT3*, repressor of multiple processes including pheromone response and hypoxia; *SEF1*, regulator of sporulation; *SFL1*, regulator of cell-surface assembly and pseudohyphal growth; *MGA1*, regulator of pseudohyphal growth; *RBA50*, global regulator interacting with RNA polymerase II; *SNF11*, component of SWI-SNF global transcription activator complex acting through chromatin remodeling; *SPT8*, member of histone acetyltransferase SAGA complex; and *HTZ1*, histone-related regulator of chromatin silencing. Components of intracellular signaling cascades include representatives of Rho (*RHO5, GIC2, PEA2*), Ras (*SHR5*), cAMP/PKA (*SOK1*) and protein kinase C/MAPK signaling pathways (*RHO5, HOG1*). These pathways are involved in regulation of many processes, including the maintenance of cell wall integrity, bud emergence, cell polarity, exocytosis and vesicle transport, pseudohyphal development and pheromone response. Location of Yjl103c on the upstream sequences of transcription and signaling factors, as well as on the upstream sequences of metabolic genes, suggests that this regulator might be modulating multiple processes both directly, and indirectly, through its downstream targets. Therefore, it is likely that Yjl103c affects a larger portion of genes than it actually binds, and that sum of direct and indirect targets may exceed the number of targets revealed by the location analysis.

Genome-wide mRNA expression changes caused by deletion of *YJL103C*

The location pattern of Yjl103c throughout the genome detects direct binding targets of this factor. However, this approach fails to identify the effect that Yjl103c has on putative target genes. To investigate the regulatory nature of Yjl103c, we performed genome-wide DNA microarray experiments, comparing the mRNA expression pattern of the *yjl103c* deletion mutant with the wild-type mRNA expression (see Experimental Procedures). The study included an equivalent condition as applied for the location analyses (24 hours, diauxic shift), and three additional time–points, including 4 hours (exponential phase), 72 hours (post-diauxic growth) and 144 hours (late post-diauxic growth/entry into stationary phase) (Figure 4A). The wider range of conditions for expression analyses was introduced to control the sensitivity of the experiment by monitoring expression changes at the non-activating conditions for Yjl103c (at 4 hours) and to increase the probability of identifying delayed effects (at 72 and 144 hours), including (1) regulation of genes that can be bound by Yjl103c at any condition (and thus being identified by ChIP at 24 hours), but are actually regulated at later stages (e.g. during entry into stationary phase), and (2) delayed secondary effects of Yjl103c regulation, which occur via its downstream targets.

The analysis of mRNA expression profiles over the entire set of examined conditions reveals that deletion of *YJL103C* causes downregulation of 430 genes and upregulation of 293 genes. As expected, expression changes at 4 hours (h) were minimal, and fold-changes measured at reliable signal intensities (higher then 100) were far below the statistical significance threshold (Figure 4A, 4 h). Interestingly, upregulation of genes in these assays seems to be mainly the feature of the 72 h time-point dataset, as 212 out of 293 genes induced by *YJL103C* deletion are detected at this time. At the two other post-exponential time points, the number of downregulated genes prevails over the number of upregulated genes.

Comparison of expression and location data reveals significant overlap between the group of genes bearing Yjl103c on their upstream sequences and genes which are downregulated in the *yjl103c* deletion mutant ($p < 1 \cdot 10^{-22}$) (Figure 4B, Table 1). Based on this comparison and on the analysis of functional categories (see below),

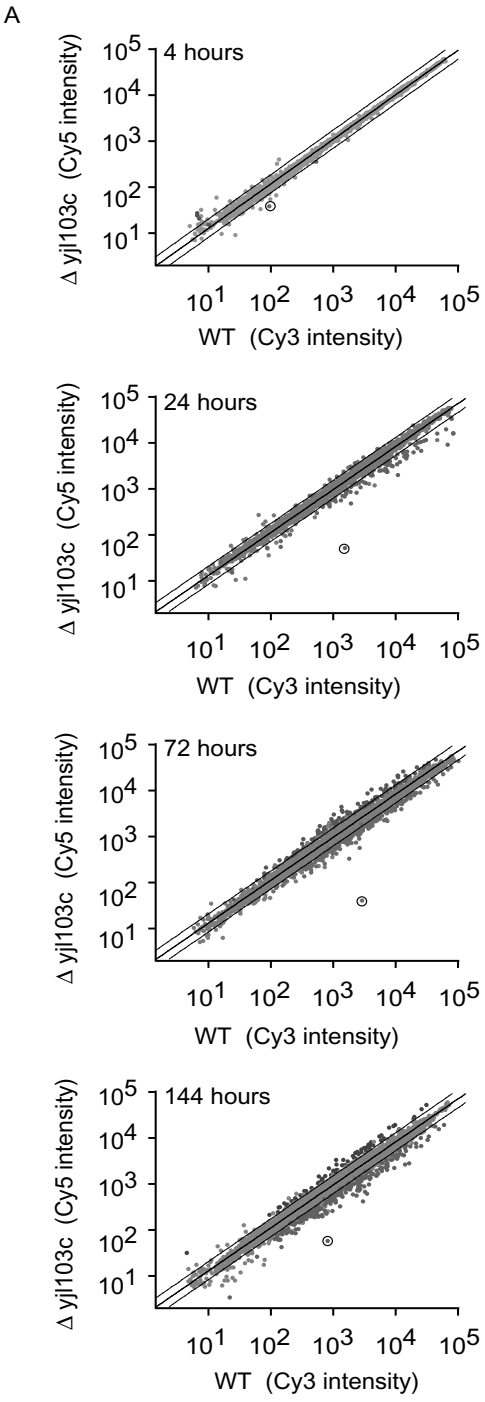


Figure 4. Genome-wide expression changes in the *yjl103c* deletion mutant

(A) Scatter-plots of average microarray gene spot intensities, comparing *yjl103c* deletion mutant samples (Cy5) with the wild type samples (Cy3) in four time points (4 hours, exponential phase; 24 hours, diauxic shift; 72 hours, post-diauxic phase; 144h, entry into stationary phase). The three lines represent 1.6-fold up, no change and 1.6-fold down boundaries, from top to bottom respectively. Colored in red and green are genes that were determined to show statistically significant mRNA expression changes in the analysis of the Δ*yjl103c* versus wild type replicate measurements per each time-point. The deleted gene, *YJL103c*, is marked with a circle.

(B) Overlap between Yjl103c targets identified by location and expression analyses. Venn diagrams show overlap between ORFs with altered mRNA expression in Δ*yjl103c* in any of the time points and ORFs located downstream of Yjl103c binding sites. The p-values for comparisons of overlapping sets of genes are based on a hypergeometric test with multiple testing correction.

we propose that Yjl103c mainly acts as a positive regulator of its binding targets. The 50 genes identified by both approaches can be more confidently designated as the direct targets of Yjl103c regulation under the studied conditions. Genes that are upregulated by *YJL103C* deletion show small and statistically insignificant overlap with Yjl103c binding targets (Figure 4B). This suggests that the upregulation of genes in *yjl103c* mutant at the post-diauxic growth is the

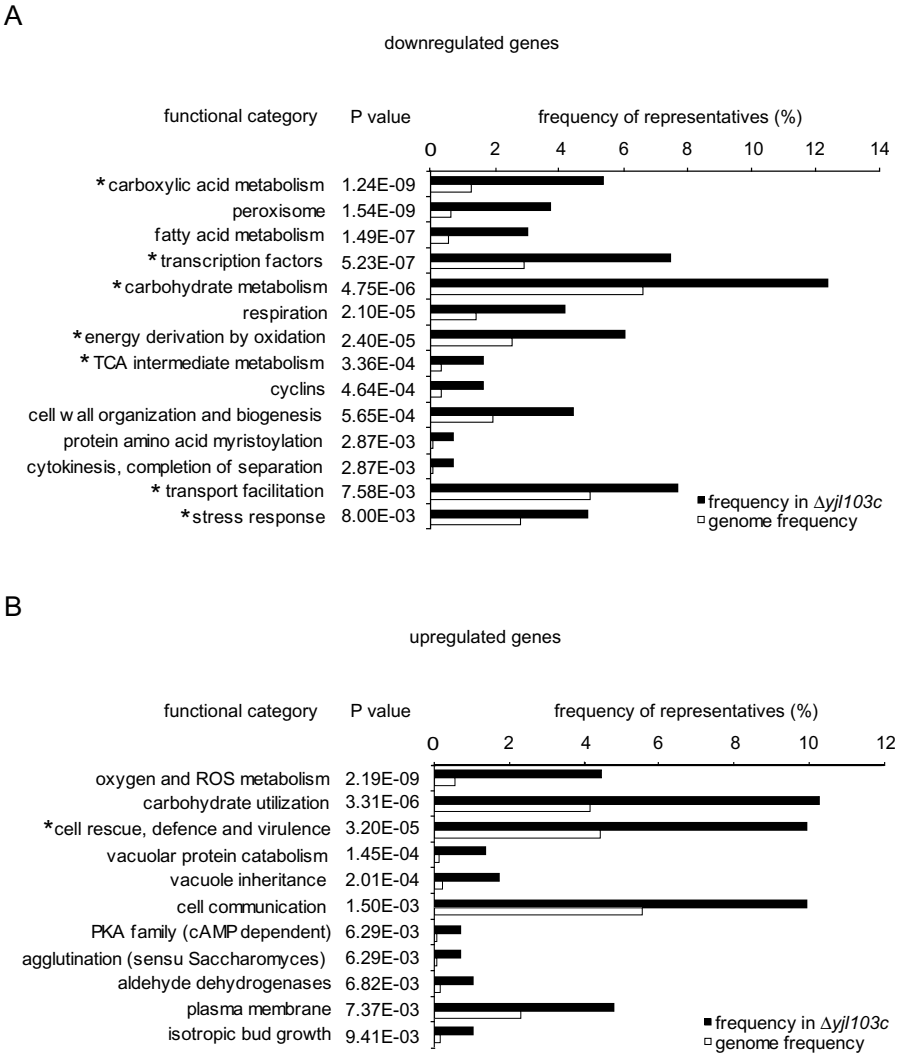


Figure 5. Functional categories of differentially expressed genes in *yjl103c* deletion mutant

(A) Genes downregulated in Δ*yjl103c* in any of the time points were analysed for overrepresentation of the functional categories derived from MIPS and GO databases, as described in Figure 3D. The asterisks demarcate functional categories that are also overrepresented among Yjl103c binding targets (Figure 3D).
(B) Equivalent analysis as in (A), but for genes upregulated in Δ*yjl103c* in any of the time points.

consequence of indirect Yjl103c effects. This indirect repressive function of Yjl103c could be achieved through regulatory factors that are identified among the direct Yjl103c targets.

The positive correlation between downregulated genes in *yjl103c* and Yjl103c binding targets is also demonstrated by the functional analysis of all 430 down-regulated genes in the *yjl103c* deletion strain (Figure 5A). At least half of the identified overrepresented functional categories are also significantly enriched among the 151 Yjl103c binding targets (Figure 3D). This includes the category of transcription regulators and the functional categories related to metabolism and stress response. However, certain categories are enriched exclusively in the expression dataset, suggesting that Yjl103c might have indirect effect on those processes. This is the case for the specific oxidative processes, such as peroxisomal fatty acid oxidation and respiration, regulatory processes controlled through cyclins and protein myristoylation and diverse cell wall related functions. The Yjl103c activation of the genes from these categories could be mediated through other activators, present among Yjl103c targets.

In contrast to the functions of downregulated genes in *yjl103c* mutant, only one functional category that is enriched among upregulated genes is also overepresented in the Yjl103c binding dataset (Figure 5B). The other identified categories suggest indirect involvement of Yjl103c in the repression of two catalytic subunits of cAMP-dependent protein kinase (PKA) and several other processes, such as metabolism of reactive oxygen species (ROS), vacuolar protein catabolism, cells communication and budding.

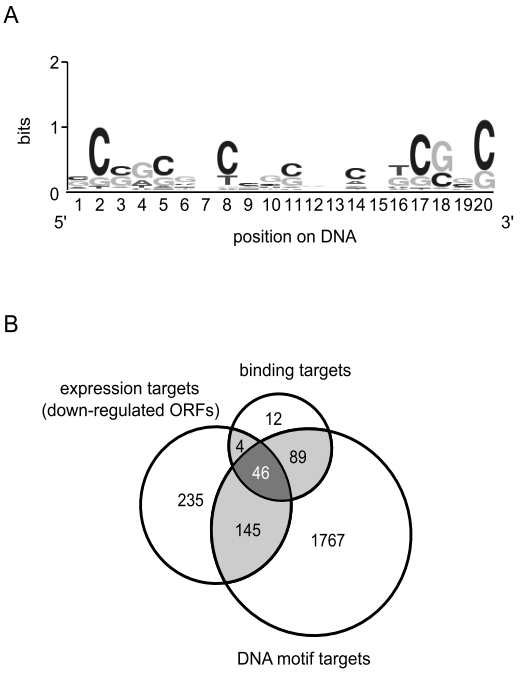


Figure 6. Yjl103c DNA binding motif

(A) Sequence logo of the DNA motif identified as the most specific among Yjl103c binding sequences. The motif was identified using MEME (Bailey and Elkan, 1994). The relative size of each letter represents the frequency of its appearance on the corresponding DNA position multiplied by the total bits of information.
(B) The overlap of ORFs that contain the motif shown in (A) in their upstream sequences with Yjl103c binding targets and genes downregulated in Δ*yjl103c*.

DNA binding specificity of Yjl103c

An important characteristic of gene-specific DNA binding transcription factors is their ability to recognize and bind specific DNA sequences (motifs) in the upstream sequences of their target genes. To identify the sequence motifs recognized by Yjl103c, we employed a number of motif discovery methods on the sequence regions around the enriched array features. Since we were for this purpose interested in binding of the protein to the motif *per se*, rather than in the potential regulatory action of such binding (as measured by the expression data), we restricted ourselves exclusively to the binding data during the motif discovery process. All methods pointed to a number of GC-rich motifs, similar to those recognized by the Gal4 family of transcription factors (Todd and Andrianopoulos, 1997). The most specific pattern was found using MEME (Bailey and Elkan, 1994), and is shown in Figure 6A. To the best of our knowledge, this pattern has not been found amongst known transcription factor binding sites. Importantly, although the

Table 1. Genes identified as Yjl103c targets by both genome-wide location and expression (exclusively downregulation) analyses

Systematic name	Gene name	Description	BR[a]	BR p-value	ER[b]	ER p-value
YKL109W	HAP4	Transcription factor, regulates respiratory functions	15.9	1.2E-08	1.7[2)]	1.6E-07
YDR216W	ADR1	Transcription factor, regulates peroxisomal genes	6.5	9.5E-04	1.5[2)]	1.2E-03
YMR043W	MCM1	Transcription factor, required for activation and repression at a variety of loci	2.2	3.8E-02	1.9[3)]	8.8E-03
YDR096W	GIS1	Transcription factor, regulates starvation, target of Ras/cAMP and Rim15	15.9	1.2E-08	1.6[2)]	5.9E-05
YMR136W	GAT2	Transcription factor, regulates nitrogen depletion response	3.2	7.1E-03	1.6[1)]	2.0E-03
YBL066C	SEF1	Transcription factor, involved in sporulation	2.3	1.1E-02	1.6[2)]	8.1E-05
YGR249W	MGA1	Transcription factor, similar to heat shock transcription factors	2.4	3.4E-02	1.6[2)]	1.6E-07
YER130C	YER130C	Transcription factor (putative), involved in stress response	2.4	4.3E-02	1.3[2)]	9.1E-03
YDR073W	SNF11	Transcription activator (global), SWI-SNF chromatin remodeling complex	2.7	4.0E-03	1.5[2)]	9.2E-03
YOR140W	SFL1	Transcriptional repressor interacting with Mediator, homologous to myc	2.7	4.0E-03	1.4[2)]	1.6E-03
YOL012C	HTZ1	Transcription regulator (histone-related), involved in silencing	4.7	4.4E-04	1.7[2)]	1.6E-07
YDR527W	RBA50	Transcription involved protein, interacts with RNA polymerase II	8.7	6.4E-03	1.4[2)]	2.7E-03
YMR135C	GID8	Regulator of cell cycle, overexpression shortens the G1 phase of the cell cycle	3.9	1.1E-03	1.6[2)]	3.2E-04
YOL110W	SHR5	Regulator of Ras function,acts through RAS localization and palmitoylation	5.2	1.6E-03	1.8[2)]	1.6E-07
YEL012W	*UBC8	Ubiquitin-conjugating enzyme that is able to ubiquitinate histones in vitro	2.2	3.0E-02	2.1[2)]	1.6E-07
YEL060C	PRB1	Serine protease with broad proteolytic specificity	15.7	3.5E-04	1.4[2)]	3.5E-03
YOL111C	MDY2	Protein with similarity to human ubiquitin-like protein GDX	7.8	1.2E-03	1.5[2)]	8.2E-04
YKL171W	YKL171W	Serine/threonine protein kinase, involved in proteolysis and peptidolysis	5.6	3.5E-04	1.7[2)]	1.6E-07
YKR093W	PTR2	Transporter of small peptides into the cell	3.3	3.5E-03	2.5[3)]	3.4E-03
YDR525W-A	SNA2	Protein involved in trafficking to the vacuole	8.7	6.4E-03	1.5[2)]	2.7E-04
YLR377C	FBP1	Gluconeogenic enzyme (fructose-1,6-bisphosphatase)	14.7	1.2E-08	4.0[2)]	1.6E-07
YKR097W	PCK1	Gluconeogenic enzyme (phosphoenolpyruvate carboxylkinase)	8.5	2.3E-03	3.8[2)]	1.6E-07
YDR077W	SED1	Protein involved in cell wall organization and biogenesis	2.2	4.1E-02	2.2[3)]	3.9E-03
YKL096W-A	CWP2	Protein involved in cell wall organization and biogenesis	2.5	7.1E-03	2.1[3)]	3.9E-03
YCL040W	GLK1	Glucose phosphorylation	3.0	3.7E-03	1.9[3)]	9.4E-03
YFL014W	HSP12	Stress induced heat shock protein, induced in stationary phase	4.3	5.6E-03	1.8[3)]	4.9E-03
YMR251W-A	HOR7	Stress induced protein involved in responsiveness to hyperosmolarity	2.5	7.9E-03	1.3[2)]	9.7E-03
YNL160W	YGP1	Stationary phase induced cell wall protein	2.4	1.7E-02	2.7[3)]	3.3E-03
YER150W	SPI1	Stationary phase induced protein	4.0	2.6E-03	1.6[2)]	1.6E-07
YIL125W	KGD1	alpha-ketoglutarate dehydrogenase (functions in TCA)	2.5	1.1E-02	2.2[3)]	3.9E-03
YDR148C	*KGD2	alpha-ketoglutarate dehydrogenase (functions in TCA)	2.8	1.1E-02	1.5[2)]	6.6E-05
YDR178W	*SDH4	succinate dehydrogenase complex (functions in TCA)	2.6	1.1E-02	2.3[3)]	3.4E-03
YOL084W	PHM7	Protein of unknown function, regulated by phosphate	6.3	8.7E-03	1.8[1)]	1.6E-04
YNR018W	YNR018W	Protein of unknown function	3.6	1.8E-03	1.5[2)]	4.5E-03
YIL057C	YIL057C	Protein of unknown function	2.0	3.0E-02	2.8[1)]	1.6E-07
YGR035C	YGR035C	Protein of unknown function	2.4	2.2E-02	1.5[2)]	8.1E-05
YLR152C	YLR152C	Protein of unknown function	3.2	2.3E-03	2.1[2)]	1.6E-07
YGL056C	SDS23	Spindle pole body protein	4.8	4.4E-04	1.9[2)]	1.6E-07
YGR250C	YGR250C	Protein with RNA recognition domains, similar to human polyadenylation factor	3.1	3.3E-02	1.8[3)]	5.0E-03
YOR348C	PUT4	High affinity proline permease, required for nitrogen starvation	2.6	4.9E-03	1.6[1)]	2.8E-04
YJR077C	MIR1	Phosphate transporter, required for growth on a non-fermentable carbons	2.4	8.7E-03	1.8[3)]	7.8E-03
YJR095W	*SFC1	Mitochondrial membrane succinate-fumarate transporter	3.4	2.3E-03	1.9[2)]	1.6E-07
YGL008C	PMA1	Major regulator of cytoplasmic pH	3.2	6.5E-03	4.5[3)]	1.0E-03
YEL017C-A	PMP2	Plasma membrane proteolipid associated with Pma1 (see above)	2.8	3.9E-02	1.7[3)]	9.1E-03
YHR050W	SMF2	Manganese transporter, may influence protein import	2.0	4.9E-02	1.9[2)]	1.6E-07
YJL153C	INO1	Inositol-3-phosphate synthase	2.5	6.1E-03	2.1[3)]	3.9E-03
YLL018C-A	COX19	Cytochrome oxidase	2.9	3.8E-03	2.3[3)]	3.9E-03
YJR121W	ATP2	ATP synthase involved in ATP generation during cellular respiration	3.5	2.1E-02	2.0[3)]	4.3E-03
YOR374W	ALD4	Glucose repressed aldehyde dehydrogenase	2.1	2.0E-02	2.3[1)]	1.6E-07
YEL046C	GLY1	Threonine aldolase, required for glycine biosynthesis	2.7	1.0E-02	1.9[3)]	8.4E-03

BR[a] Binding ratio of the feature with highest Yjl103c enrichement, located upstream of the ORF
ER[b] Expression ratio (fold change of downregulation, wt / Δyjl103c) in the time-point of the minimal expression
* Genes that do not contain identified Yjl103c DNA-binding motif in their upstream sequences
1) Expression fold change at 24 hours
2) Expression fold change at 72 hours
3) Expression fold change at 144 hours

expression data was not used as an input for motif discovery, the identified motif is present in upstream sequences of nearly half of the genes downregulated in the *yjl103c* deletion mutant, including 46 out of the 50 genes identified as Yjl103c targets by both location and expression data (Figure 6B).

Phenotype analysis of the *yjl103c* deletion mutant

Despite its apparent role in the transcription changes that occur during glucose starvation, deletion of *YJL103C* causes only a minor growth defect when cells are cultured to late stationary phase (Radonjic et al., unpublished data). Therefore, we performed phenotype analysis of *yjl103c* under more specific conditions, under which some of the Yjl103c target genes are also likely to be required. This included growth on non-fermentable carbon sources (glycerol, oleate and acetate), oxidative and osmotic stress (hydrogen peroxide; NaCl, sorbitol) and growth in the presence of either caffeine or DNA-damaging agent methyl methanesulfonate (MMS). The *yjl103c* and wild-type strains were cultured in rich, glucose-based media (YPD) to exponential phase, and subsequently, monitored for growth under the conditions listed above (Experimental procedures, Figure 7).

The *yjl103c* deletion strain exhibits growth defects when grown on glycerol and oleate, showing the requirement of Yjl103c for the optimal utilization of these non-fermentable carbon sources (Figure 7). The observed phenotypes indicate that Yjl103c plays important roles in respiration (glycerol phenotype) and peroxisomal beta-oxidation of fatty acids (oleate phenotype) (Hampsey, 1997; Hiltunen et al., 2003). The involvement of Yjl103c in regulation of these metabolic processes is in agreement with overrepresentation of the corresponding

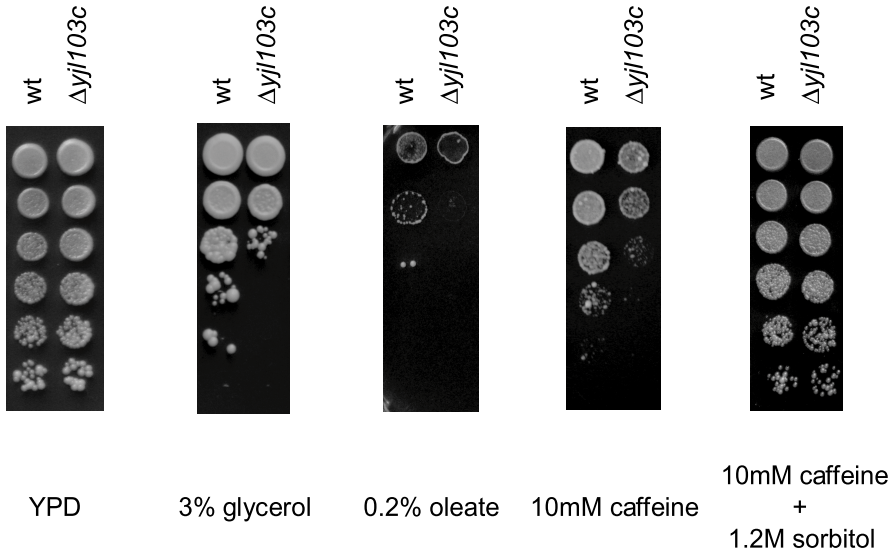


Figure 7. Phenotypes of *yjl103c* deletion mutant

Wild type and Δ*yjl103c* mutant strains were grown to exponential phase in YPD culture. Subsequently, cells from both cultures were spotted in five-fold serial dilutions on various plates and incubated for 3-7 days. Deletion mutant strain Δ*yjl103c* exhibits loss of viability when grown on glycerol or oleate as carbon sources, and in YPD supplemented with caffeine. The caffeine phenotype can be suppressed by addition sorbitol, pointing to involvement of Yjl103c in the PKC/MAPK regulated cell-wall integrity pathway.

functional categories among Yjl103c expression targets (Figure 5A). In contrast, growth on acetate as the sole carbon source did not result in the growth defect of *yjl103c* (data not shown). The fact that cells can utilize acetate regardless of Yjl103c activity suggests that Yjl103c is not essential for the maintenance of the tricarboxylic acid cycle (TCA) (Hampsey, 1997).

In addition to growth defects on some of the non-fermentable carbon sources, *yjl103c* also exhibits a growth defect when grown in the presence of caffeine (Figure 7). Caffeine is a purine analog that affects many cellular proceses, such as cAMP signaling and MAP kinase cell integrity pathway (Hampsey, 1997; Nickas and Yaffe, 1996; Tortora et al., 1982). To investigate more precisely which processes are disrupted by *YJL103C* deletion, we additionally monitored *yjl103c* growth in the medium supplemented with caffeine and the cell wall stabilizing agent, sorbitol. When included in the growth medium, sorbitol causes high osmolarity of the environment, which suppresses cell lysis defects caused by disruption of PKC/MAPK-regulated cell wall integrity pathway. Therefore, addition of sorbitol can remediate the caffeine sensitivity phenotype that is caused by mutations in components of the PKC/MAPK cell integrity signaling cascade (Nickas and Yaffe, 1996). In agreement with enriched functional categories among targets derived from the expression data (category "cell wall organization and biogenesis"), deletion of *YJL103C* causes sensitivity to caffeine that is rescued upon addition of sorbitol (Figure 7), indicating involvement of Yjl103c in regulation of cell wall integrity signaling. This function of Yjl103c is also in agreement with enrichment of "Rho protein signal transduction" category among its binding targets, as Rho acts upstream of PKC/MAPK in regulation of cell wall integrity (Claret et al., 2005). In contrast to growth in the presence of caffeine, growth of *yjl103c* in the media supplemented with MMS resulted in only modest growth defect and exposure to oxidative and osmotic stress did not have any effect on *yjl103c* growth (data not shown), showing expendability of Yjl103c for these processes.

Discussion

Regulatory properties of Yjl103c are typical for the Zn(II)2Cys6 protein family

The identification of downstream targets of regulatory factors is required to understand cellular responses to environmental and developmental cues. Which genes are regulated by gene-specific transcription factors is partly determined by the DNA binding domains of these proteins. This domain allows the transcription factor to bind to specific DNA motifs in the vicinity of target genes, ensuring the specificity of the transcriptional response (Kadonaga, 2004). The factor investigated in this study, Yjl103c, contains a fungal-specific six-cysteine (Zn(II)2Cys6) binuclear cluster DNA binding domain. This DNA binding domain has been identified by sequence analysis in 56 *S. cerevisiae* genes, exemplified by the well-characterized regulator *GAL4* (Todd and Andrianopoulos, 1997). The analyses performed here reveal several features of YJL103c regulation that are characteristic for members of the Zn(II)2Cys6 protein family (Todd and Andrianopoulos, 1997). First, all proteins of this family with determined function are regulatory proteins, with the exception of CBF3b from *S.cerevisiae,* which forms part of the kinetochore and functions in chromosome segregation (Lechner, 1994). The ability of Yjl103c to bind intergenic DNA regions, the expression changes caused by its deletion and the growth defects of its deletion mutant show that Yjl103c acts as a gene-specific transcription factor, similarly to other Zn(II)2Cys6 proteins. Second, many Zn(II)2Cys6 proteins are involved in metabolic processes which are also targeted by Yjl103c regulation (Figure 3, Figure 5). Third, Zn(II)2Cys6 proteins are typically transcriptional activators, though some of them may act as both activators and repressors. The difference in the overlaps between Yjl103c binding targets and genes which mRNA expression changes in *yjl103c* mutant reveals that downregulated genes are much more likely to be direct targets of Yjl103c then the upregulated genes. Therefore, Yjl103c mainly acts as an activator of its direct

targets, as the majority of Zn(II)2Cys6 proteins. Finally, the identified DNA binding motif of Yjl103c resembles those of other members of Zn(II)2Cys6 family. DNA binding domains of this family, like those of other DNA binding domain classes, interact with similar DNA binding sites. Most of Zn(II)2Cys6 proteins recognize and bind to DNA motif that contains conserved terminal GC-rich trinucleotides spaced by an internal variable sequence of defined length. The related pattern also occurs in Yjl103c DNA binding motif (Figure 6). The listed similarities of Yjl103c regulatory properties with other members of Zn(II)2Cys6 protein family exemplify the confirmation of the structurally predicted features of a transcription factor by its functional analyses.

Combining genome-wide approaches under activating conditions

The discovery of whole genome sequences and development of DNA microarray technologies enable more exhaustive methods for finding transcription factor targets. These methods usually include one of the following three approaches: (1) microarray expression profiling, identifying genes that exibit significant changes in mRNA levels upon inactivation of a transcription factor; (2) DNA motif discovery, finding the TF targets by searching for the corresponding recognition motif within the genome sequence itself; and (3) genome-wide location analysis of TF's DNA binding sites (ChIP on chip), identifying the sites of TF enrichment *in vivo*. Of 173 transcription factors identified in *S. cerevisiae*, 76 have remained poorly characterized (Chua et al., 2004). One of the reasons for failure of systematic studies to identify targets for these uncharacterized TFs is choosing an inappropriate experimental condition, under which a given TF is not active. The other difficulties originate from pitfalls of each individual genome-wide method, when solely employed. In this study, we address both of these problems: we investigate Yjl103c during diauxic shift, identified as (at least) one of the activating conditions for this factor and we perform several genome-wide analysis in parallel, which allows discrimination between direct and indirect targets of Yjl103c, enables assessment of Yjl103c regulatory effect (activator or repressor) and enhances the confidence of Yjl103c regulatory effect for targets discovered by more than one method.

How do the results of the different methods compare? The binding targets of Yjl103c, discovered by ChIP on chip, significantly overlap with genes downregulated in *yjl103c* deletion mutant, revealing 50 targets identified by both methods (Figure 4B, Table 1). However, many of the binding targets do not change their expression under investigated conditions. This is may be due to functional redundancy of TFs. Upon deletion of *YJL103C*, other factor(s) may be taking over its role, rescuing the phenotypes (including the expression changes) of *yjl103c*. Another possibility is that Yjl103c binds, but does not regulate some of its targets under non-activating conditions (in this case glucose starvation during diauxic shift). This would allow discovery of certain TF's targets by location analysis, but not their expression changes, which occur only under specific activating conditions. It is likely that investigation of expression changes under settings other than glucose starvation may result in the overlap with different set of Yjl103c binding targets. The potential of TFs to bind their targets whenever they are available, rather than when it is needed, has been proposed to be a general mechanism (Chua et al., 2004). Lastly, another possibility that we cannot exclude is that Yjl103c may act in tandem with another TF, and that their strong protein-protein interaction causes contamination of enriched sequences with targets of the Yjl103c partner.

In addition to targets identified solely by location analyses, there are many expression changes that cannot be directly explained by the Yjl103c binding pattern. This is expected. The representation of numerous transcription regulators among Yjl103c binding targets suggest that Yjl103c probably controls more genes that it actually binds, through indirect effects. The third approach to target identification, the presence of an Yjl103c DNA binding motif, correlates well with the binding targets (which is expected since these were used for defining the search

space), but also has good correlation with independent, expression data (Figure 6). Genes that carry Yjl103c DNA binding motif in their upstream sequences represent nearly half of genes downregulated in *yjl103c* deletion mutant ($p=2 \cdot 10^{-10}$) and 92% of genes discovered by both expression and location analyses. The high significance of discovery by all three approaches qualifies such identified genes as definite Yjl103c targets (Table 1).

The differences between location and expression data suggest that beside the post-exponential growth phases, Yjl103c may be also activated under other conditions. This assumption is supported by phenotype analysis of *yjl103c* deletion mutant. When grown under the equivalent conditions to those used for genome-wide analyses, *yjl103c* exhibits only a slight growth defect observed late in stationary phase. However, under more specific environmental settings, such as growth on glycerol, oleate, or in the presence of caffeine, *yjl103c* has more difficulties to maintain wild-type growth rates (Figure 7). It is likely that genome-wide analyses performed under these conditions would improve assessment of Yjl103c regulatory features.

Yjl103c modulates multiple processes involved in the glucose starvation response

The response of cells to glucose starvation, as occurs during *S. cerevisiae* post-exponential growth phases, includes changes in multiple processes, such as adaptation for utilization of non-fermentable carbon sources, changes in means of energy derivation and acquisition of various stress resistance properties. Yjl103c seems to be involved in modulation of many of these adaptive processes, acting either directly, or indirectly, through regulators that are identified among its targets. Based on the function of Yjl103c, we propose the name *GSM1* (glucose starvation modulator) for this factor. One possible scenario is that Gsm1 contributes to fine-tuning of intracellular signaling. This factor is enriched on upstream sequences of Rho signaling pathway components (involved in regulation of cell-wall integrity via PKC/MAPK) and therefore, potentially regulates gene expression through modulation of this signaling cascade. This is supported by the PKC/MAPK pathway-specific caffeine sensitivity of *gsm1* deletion mutant.

Analysis of Gsm1 target genes identified by both expression and location analyses (Table 1) reveals that Gsm1 directly regulates at least five processes, including gluconeogenesis, cell wall organization and biogenesis, protein degradation, response to stress and TCA cycle. Importantly, among the genes detected by expression and location analyses, the category of regulators is the most frequently represented. The processes that are known to be controlled by these regulators and are also represented among functional categories of genes with altered expression in *gsm1* deletion mutant, can be considered as probable indirect targets of Gsm1 regulation. This includes: respiration (regulated via Hap4), peroxisomal fatty acid beta-oxidation (Adr1), activation of general diauxic shift response (Gis1), nitrogen depletion response (Gat2), pheromone response-associated processes (Mcm1), and cell cycle regulation (Gid8). Indirect regulation of transcription by Gsm1 may also be achieved in a more global way, for example through Rba50, a protein that interacts with RNA polymerase II, Snf11, a global activator and chromatin remodeling factor, and Htz1, histone-related protein that regulates silencing.

The precise functional relationships between Gsm1, its particular target regulators and the actual processes that they control remain to be investigated in detail. We currently do not have any knowledge about regulation of *GSM1*, except that it is a glucose-repressed gene. The analyses of the regulatory mechanisms operating upstream of this factor would enable its more precise positioning in the regulatory network activated upon glucose depletion. Our results uncover the effects downstream of Gsm1, including the transcription regulators controlled by this factor, and provide a foundation for further mapping of the regulatory interactions within the complex network that underlies the yeast starvation response.

Experimental procedures

Yeast strains and culture conditions

S. cerevisiae YPH499 (*MATa ura3-52 his3-Δ200 ade2-101uaa trp1-Δ63 lys2-801uag leu2-Δ1*) was used as the wild type strain for expression and phenotype analysis. Deletion mutant Δ*yjl103c* and TAP-tagged Yjl103c (Yjl103c-TAP) were constructed in this study in the YPH499 genetic background (Sikorski and Hieter, 1989). Δ*yjl103c* was made by replacing the *YJL103C* ORF with a kanamycin cassette in the YPH499 parental strain. Yjl103c-TAP was constructed by introducing the carboxy-terminal TAP-tag epitope by transformation of a PCR product carrying a K.l. *TRP1* cassette with the TAP epitope as described (Puig et al., 2001). PCR oligonucleotides are available upon request.

Strains were cultured in YEP with 2% glucose (YPD) under agitation (250rpm), at 30°C. For the phenotype analysis, cultures were grown to exponential phase (4 hours) prior to replating. For expression and location analyses, cells were cultivated for longer periods (up to 144 hours), allowing cells to deplete glucose and experience diauxic shift, post-diauxic growth and entry into stationary phase.

mRNA expression profiling

For expression profiling, four independent Δ*yjl103c* and four independent wild type cultures were grown to entry into stationary phase. Samples of cells from exponential phase (4 hours), diauxic shift (24 hours), post-diauxic growth (72 hours) and entry into stationary phase (144 hours) were collected by 2 min centrifugation at 30°C and frozen in liquid nitrogen. RNA isolation, cDNA labeling, microarray hybridizations, images quantification and normalization on genes were performed as previously described (Radonjic et al., 2005). Time-points 4, 24 and 72 hours are represented by 8 measurements per ORF (four hybridizations, two dye-swaps), and time-point 144 hours is represented by 4 measurements per ORF (two hybridizations, one dye-swap). To identify differentially expressed genes, pairwise comparisons for each time point were performed in R (http://www.r-project.org), using the MAANOVA package (Wu et al., 2003.), which is part of the BioConductor suite (http://www.bioconductor.org) (Gentleman et al., 2004) for microarray analysis. A Benjamini-Hochberg multiple-testing correction was employed to identify differentially expressed genes, considering significant p-values lower than 0.01. Data was visualized using GeneSpring 7 (Agilent Technologies).

Chromatin immunoprecipitation (ChIP)

Crosslinking of proteins to DNA and preparation of chromatin extracts (CE) and ChIP extracts were performed as previously described (Radonjic et al., 2005). DNA obtained from input (CE) and IP samples was amplified using T7-based linear amplification protocol as previously described (Liu et al., 2003), with following modifications. The first round of T7 amplification included terminal transferase reaction as follows: 2 µl 5x TDT buffer (Roche), 0.5 µl $CoCl_2$ (15 mM), 0.5 ul 8% 100 µM ddCTP in 100 µM dTTP mix, 1 µl Linear acrylamide, sample (10 ng) and water to final volume of 9 µl were subjected to PCR program (3 minutes at 95°C, 5 minutes at 4°C and 20 minutes at 37 °C). Samples were purified using QIAGEN MinElute KIT, and subsequently subjected to Klenow fill in reaction. 7.5 µl of sample was combined with 1µl 10x NEB buffer 2, 0.5 µl dNTPs (5 mM total) and 0.5 µl T7 Primer (5 µM), and subjected to following PCR program: 3 minutes at 95°C, 2 minutes at 35°C (ramp rate 1 °C/sec), 5 minutes at 25°C (ramp rate 0.5°C/sec). 0.5 µl Klenow (NEB) enzyme was added during the 5 min incubation at 25°C. Samples were further incubated for 90 minutes at 37°C and subsequently purified using QIAGEN MinElute KIT. Subsequent in vitro transcription (IVT) mix contained 2 µl of Buffer 10x, 2 µl of T7 ATP (75 mM), 2 µl of T7 CTP (75 mM), 2 µl of T7 GTP (75 mM), 2 µl of T7 UTP (75 mM), 2 µl of T7 Enzyme mix and 1 µl of Linear acrylamide. IVT mix was added to each dsDNA sample (7 µl), incubated for 2 hours at 37°C and purified using QIAGEN Rneasy KIT, after which samples were subjected to second round of amplification. During first strand synthesis, RNA was combined with 1 µl of random primers (1µg/µl) and water to 10 µl, incubated for 10 minutes at 70°C and further kept at 48°C. First strand mix included 4 µl of first strand buffer (5 x), 2 µl of DTT (0.1 M), 1 µl of RNAse inhibitor (40U/µl), 1 µl of dNTPs (20mM each), 1 µl of Linear acrylamide (0.1 µg/ul) and 1 µl of Superscript III (Invitrogen). 10 µl of first strand mix (preheated to 48°C) was combined with 10 µl RNA/random primer, incubated at 48°C for 2 hours and at 94°C for 2 minutes, and placed on ice for 5 minutes. Samples were further purified using QIAGEN MinElute KIT, and subsequently subjected to Klenow fill in reaction. 15 µl of sample was combined with 2 ul of 10x NEB buffer 2, 1 µl of dNTPs (5 mM total) and 1 µl of T7 Primer (5 uM) and subjected to following PCR program: 95°C for 3 minutes, 35°C (ramp rate 1°C/sec) for 2 minutes and 25°C (ramp rate 0.5°C/sec) for 5 minutes. 1 ul of Klenow (NEB) enzyme was added during the 5 minute incubation at

25°C, and samples were further incubated at 37°C for 90 minutes. After purification using QIAGEN MinElute KIT, IVT reaction was performed. IVT mix contained 2.08 µl of Reaction buffer (10 x), 2 µl of T7 ATP (75 mM), 2 µl of T7 CTP (75 mM), 2 µl of T7 GTP (75 mM), 0.6 µl of T7 UTP (75 mM), 2.1 µl of 5-(3-aminoallyl)-UTP (50 mM) and 2 µl of T7 Enzyme mix. 12.8 µl of IVT mix was added to each dsDNA sample (8 µl) and incubated for 4 hours at 37°C, after which samples were purified using QIAGEN Rneasy KIT. 6 µg of cRNA samples were labeled according to previously described cDNA labeling protocol (Radonjic et al., 2005), using 4 µl of Cy dye per sample. The amount of labeled material in each hybridization was 4 µg for each channel, with label incorporation of 2 to 4%. The hybridization buffer contained following ingredients: herring sperm DNA (750 ng), tRNA (40 µg), CoT (10 µg), Na-MES (pH 6.9) (50 mM), NaCl (500 mM), EDTA (6mM), N-Lauroylsarcosine (0.5%), formamide (30%), and water to 500 µl. After 3 minutes of boiling, samples were hybridized to the microarrays using Agilent protocol (http://www.chem.agilent.com) and incubated overnight in Agilent hybridization oven at 40°C. The microarray slides were washed with stirring for 5 minutes, in buffer 1 (SSPE 6x, sarcosine 0.005%) and subsequently in buffer 2 (SSPE 0.06x). The hybridisations were performed using Agilent DNA microarrays containing 44256 features, which consist of 60-mer oligonucleotide probes. The array covers 12 Mb of the yeast genome (85%), excluding highly repetitive regions, with an average probe density of 266 bp. Intergenic regions are represented by 14256 probes, and ORFs are represented by 27185 probes. The remaining 2849 features included blank spots and controls (Pokholok et al., 2005). Genomic localization profiles for Yjl103c were generated for immunoprecipitated samples originating from two independent cultures, in dyeswap technical duplicate, resulting in two ChIP profiles.

Genome-wide location data analysis

After image quantification, data was normalized over all features using print-tip lowess with a span of 0.4. To determine features significantly bound by Yjl103C, the MAANOVA package (Wu et al. 2003) was used as described for expression data, again with a Benjamini-Hochberg multiple-testing correction. Genes with a p-value lower than 0.05 and the binding ratio higher than 1.0 are considered significantly enriched by Yjl103c. For comparing genomic location with mRNA expression data, oligo sequences were mapped onto the yeast genome obtained from the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) (Christie et al., 2004) on August 20, 2004 using the program BLAT (Kent, 2002). A distance criterion was applied to identify ORFs that are located downstream of enriched array features (oligos) within sufficient proximity, and which were therefore considered Yjl103c binding targets. These included ORFs with translation start sites positioned within 800 basepairs (bp) downstream and 200 bp upstream of the oligos. If the distance criterion for one oligo was met by more than one ORF, multiple ORFs would be assigned to be downstream of such an oligo.

Quantitative real time PCR

Quantitative PCR experiments were performed as previously described (Andrau et al., 2006). The primers used for the real time PCR are: PRB1<<SOM1_LEFT (GAAAGCCCTGCTACTCTGCTAAC) and PRB1<<SOM1_RIGHT (CAGATCAGGTCTGTGC CTACTATG), located upstream of *PRB1* gene, and FBP<<tK(UUU)L-SEC61_LEFT (CGCCCTTAACA TTACCTAGCTTTA) and FBP<<tK(UUU)L-SEC61_RIGHT (ATTATTCTTAGTAGTCGCGGTCGT), located upstream of the *FBP1* gene.

DNA motif discovery

To establish which sequence motifs may be bound by YJL103C, the following procedure was followed: oligo features that had a binding ratio p-value lower than 0.01, as well as a binding ratio larger than 1.0 were taken; oligos further than 800 bp upstream of the translation start site of any open reading frame (ORF) classified in SGD as “Verified” or “Uncharacterized”, or more than 200 bp into any such ORF, were discarded, as were oligos that are not unique across the genome. Sequences consisting of 400 bp around the midpoint of the genomic location of these oligos were taken (overlaps were merged), and scanned with MEME (version 3.5.3) (http://meme.sdsc.edu/meme/) (Bailey and Elkan, 1994) for a motif with a width of between 6 and 20 bp. A 5th order Markov model of all *S. cerevisiae* upstream sequences was used as the background against which to perform the motif discovery. The best motif obtained has an information content of 13.7 bits and a E-value of 1.7^{10-119}. During the process of motif discovery, we experimented with a number of different settings for the length and number of starting sequences, as well as the preferred motif length. We chose the settings that resulted in a pattern that gave maximum discrimination, in an AUC

sense [Area Under Curve, the curve being the ROC (=Receiver Operator Characteristic) curve], between motif occurrences close to the binding sites, and motif occurrences throughout the whole genome.

Analyses of functional categories

Genes downstream of Yjl103c binding sites and genes which mRNA expression changes in Δ*yjl103c* were queried for annotations of functional categories using FunSpec (Robinson et al., 2002). Functional categories were derived from the Gene Ontology (GO) database (Harris et al., 2004) and MIPS database (Mewes et al., 2004).

Immunoblotting

Cross-linked cell extracts (as prepared for the ChIP experiments) and IP-ed protein fractions were boiled in sample buffer for 10 to 15 min before loading on SDS 10% polyacrylamide gels. Proteins were transferred onto nitrocellulose membrane and incubated with Peroxidase-anti-peroxidase antibody (PAP, Sigma) to detect the TAP tag of Yjl103c.

Phenotype analysis

Deletion mutant and the wild-type strain of *S. cerevisiae* were cultured in a YEP media supplemented with 2% glucose (YPD), at 30°C. The cultures were grown under agitation (250 rpm) for 4 hours until exponential phase and subsequently washed and examined for viability by spot assay on the plates with various substrates. Starting with equal amounts of exponential phase cells, five-fold serial dilutions were spotted on plates and incubated for 3 to 7 days at 30°C. The tested conditions included plates with following media: YPD; YEP + 3% glycerol; YEP + 0.2% oleate; YEP + 2% potassium acetate; YPD + 1.5 mM hydrogen peroxide; YPD + 1M NaCl; YPD + 1.2M sorbitol; YPD + 10mM caffeine; YPD + 10mM caffeine + 1.2M sorbitol; and YPD + 0.02% methyl methanesulfonate (MMS). In the glycerol, oleate, NaCl and caffeine supplemented media, both deletion and wild type strains were growing slower then in YPD, and were therefore screened for phenotypes after 5 to 7 days of growth on the plates.

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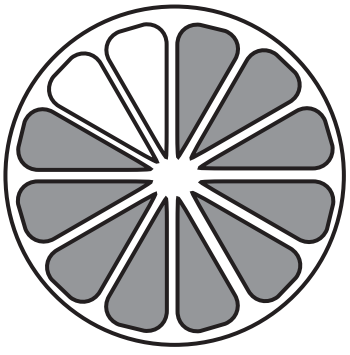
Chapter 5

General discussion



The project presented in this thesis was initiated in order to unravel largely uncharacterized processes underlying the transcription regulation of eukaryotic quiescence. Transcription regulatory mechanisms were investigated globally (Chapter 2 and Chapter 3) and at the gene specific level (Chapter 3 and Chapter 4). The findings shed new light on several aspects of quiescence and how it should be studied. A number of new questions are raised, which may direct the further exploration of this common but relatively uncharacterized cellular state.

Poised RNA polymerase II facilitates rapid transcription induction upon the exit from quiescence

Early studies of gene regulation identified activators and repressors, proteins that bind to specific DNA sequences upstream or downstream of promoters and stimulate or repress the initiation of transcription. Activators typically consist of two domains: one that binds specific DNA sequences and one that recruits the generally required transcription apparatus (Lee and Young, 2000). The current "recruitment" model of transcription activation proposes that transcriptional activators, by means of protein-protein interactions, recruit the transcription machinery to DNA and that this concomitantly results in activation (Ptashne, 2005). Activation of transcription by recruitment in eukaryotes includes assembly of at least 100 proteins, most of which exist as complexes, such as those responsible for chromatin-remodeling, which affect the chromatin template and facilitate further recruitment of other complexes. Other important protein complexes include co-activators such as the Mediator complex, which integrates signals from gene specific transcription factors at promoters and mediates the recruitment of other components of the general transcription machinery (Bryant and Ptashne, 2003; Cosma et al., 1999). These complexes interact with and recruit RNA polymerase II, leading to initiation of transcription. Thus, the activator, through DNA binding and subsequent recruitment of other proteins, triggers a cascade of events that results in expression of a specific gene (Figure 1).

Gene expression during yeast quiescence is largely abolished, with mRNA levels at one-sixth of the levels detected during exponential growth (Choder, 1991; Radonjic et al., 2005). However, the amount of RNA polymerase II (Pol II) in quiescence does not reflect this high degree of transcriptional shut down: it is only 50% lower compared to the exponential phase (Chapter 2). This unexpected preservation is also observed for other components of the general transcription machinery, such as the TATA binding protein (TBP), which is present in similar amounts in both quiescence and exponential growth phase. Questions that arise from these findings are: where, how, and why is the general transcription machinery preserved in quiescent cells under conditions of global transcriptional shut-down? The analyses of phosphorylation status of the carboxy-terminal domain (CTD) of the largest Pol II subunit and the genome-wide Pol II location analysis show that majority of the Pol II molecules preserved in quiescence are transcriptionally inactive and reside on the intergenic regions (Chapter 2). The "recruitment" model of transcription activation does not fit these observations, as it assumes the presence of the general transcription machinery only when it is specifically recruited to initiate transcription, which occurs in quiescence at the most for 5% of the genes. Our findings, in combination with the mRNA expression data, provide a possible explanation for the Pol II preservation on specific locations during quiescence. Namely, most of the genes located downstream of Pol II binding sites exhibit rapid induction upon exit from quiescence, suggesting that the same Pol II molecules that are maintained on intergenic promoter-bearing regions, are responsible for transcribing the corresponding downstream genes during exit. Therefore, the transcription initiation of the "rapid exit" genes would not require *de novo* recruitment of the Pol II, as this molecule is already present in the vicinity of the genes promoters and poised for the rapid gene induction (Figure 1). This mechanism could be evolutionary developed to enable the more rapid response of the transcription machinery to improved nutrient status, providing the advantage

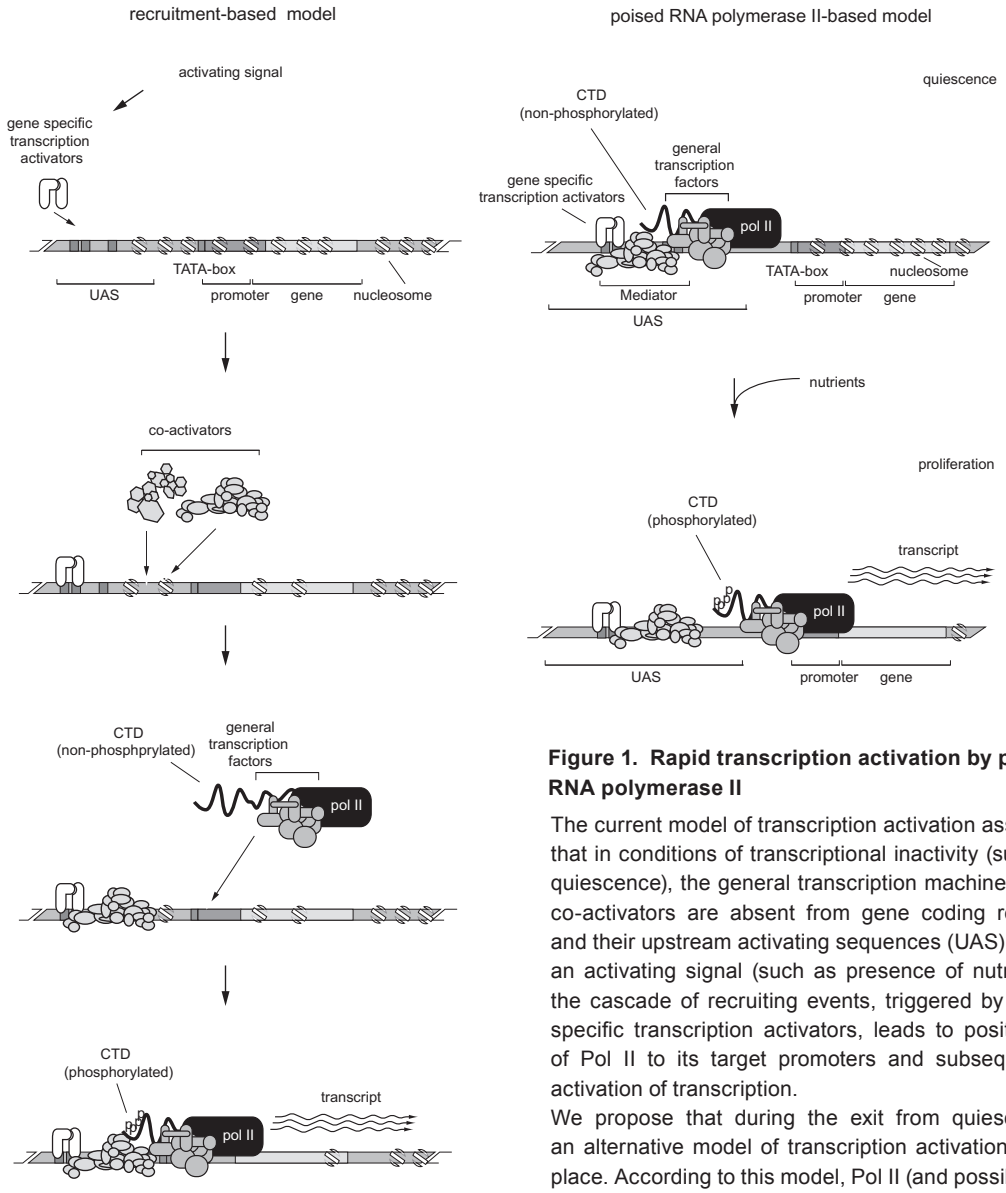


Figure 1. Rapid transcription activation by poised RNA polymerase II

The current model of transcription activation assumes that in conditions of transcriptional inactivity (such as quiescence), the general transcription machinery and co-activators are absent from gene coding regions and their upstream activating sequences (UAS). Upon an activating signal (such as presence of nutrients), the cascade of recruiting events, triggered by gene-specific transcription activators, leads to positioning of Pol II to its target promoters and subsequently, activation of transcription.

We propose that during the exit from quiescence, an alternative model of transcription activation takes place. According to this model, Pol II (and possibly the Mediator complex and the gene specific activators) are already stored on upstream regulatory sequences of repressed genes which are required for the rapid exit from quiescence. This positioning of inactive Pol II in the proximity of its target genes allows fast relocation to the promoters of the target genes, where transcription can be activated. This model does not require the recruitment step, and therefore, enables more rapid transcription response to activating signals. It is still unknown what is the role of gene-specific transcription activators in this type of transcription activation; it is possible that they as well as the Mediator bind and mark genes required for response to environmental changes, or that they have specific roles in signaling Pol II to relocate to target promoters under activating conditions. It also remains to be determined what is the subunit composition and location of Mediator during quiescence and the exit. After the activation of transcription by a poised Pol II molecule, it is possible that *de novo* recruitment events take place to enable amplification of an already initiated transcription response.

in a competitive environment.

The status of Pol II in quiescence resembles two other examples of gene activation by mechanisms alternative to the recruitment model. First, the set of genes involved in nitrogen metabolism in bacteria is transcribed by specific form of RNA polymerase, which spontaneously binds to DNA and forms a stable inactive complex (Rombel et al., 1998). The second example includes heat shock and several other genes in Drosophila, where RNA polymerase II is bound (and paused after initiating a short transcript) to heat shock promoters under non-activating conditions together with at least two general regulatory factors, including TBP (Lis and Wu, 1993). In addition, heat shock promoters are also poised for a rapid change in transcription by assembly into an accessible chromatin structure that is depleted of histones and hypersensitive to nuclease digestion (Nacheva et al., 1989). Similar to Drosophila heat shock-genes, human c-myc gene also carries a paused RNA polymerase on its 5' end of the gene (Krumm et al., 1992). It remains to be determined to which extent these mechanisms are related to one observed in yeast quiescence, however, it is evident that there is an emerging class of eukaryotic genes transcribed by Pol II whose regulation is not confined to the level of the recruitment of the general transcription machinery. Poised Pol II in Drosophila heat shock-genes has always been considered an exception to recruitment–coupled activation. Our findings of poised Pol II on hundreds of genes during yeast quiescence indicate that this may in fact be a much more general mechanism. Yeast quiescence can serve as a simple model to explore the recruitment-independent control of genes.

One question that remains open is which factors facilitate re-location of Pol II from actively transcribed genes (where they reside during exponential growth phase) to intergenic regions upstream of the rapid exit genes (where Pol II is stored in an inactive mode during quiescence). One type of factor that could be involved in selective Pol II binding are the co-activators, such as the Mediator complex. The role of Mediator in anchoring inactive Pol II to upstream sequences of the rapid exit genes is suggested by the fact that several Mediator components bind to these sequences during exponential phase (Andrau et al., 2006). The similarity between the genomic location of Pol II in quiescence and the Mediator subunits in exponential phase (EP) is even higher than between Mediator and Pol II in EP, despite the best established role of Mediator as a Pol II recruiter associated with sites of active transcription (Kornberg, 2005). These findings suggest that during EP and SP, Mediator location on some IGRs serves to mark genes required for response to environmental changes and also serves as a binding platform for inactive Pol II during prolonged periods without proliferation.

The positioning of co-activators is therefore likely to be the main, and probably sufficient mechanism for anchoring inactive Pol II to upstream sequences of the rapid exit genes. The additional factor that could possibly be involved in selective Pol II location is the DNA binding sequence that this molecule binds. This sequence would evolve specifically in the upstream regulatory regions of the genes which rapid activation upon re-feeding provides an evolutionary advantage. The identification of such DNA binding motif is facilitated by recent development of the high-resolution DNA microarrays for DNA bound protein location analysis (Pokholok et al., 2005). Mapping the Pol II binding pattern in quiescence using these arrays should enable detection of the more precise genomic locations and provide the input sequences for DNA motifs search. However, the high-resolution, conventional ChIP assay of the *SUT1* region (Chapter 2) suggests that this DNA specificity of Pol II may not be necessary. This analysis reveals that Pol II location corresponds to the binding sites of Rox1 transcription factor (which is a *SUT1* regulator), suggesting that Pol II probably binds via gene-specific transcription activators, and therefore does not require its own specific binding sites.

In addition to the sequence specificity of Pol II binding, it would be of interest to determine the Pol II interacting proteins during different cellular stages, to investigate the phosphorylation status of the CTD and to examine possible role of

chromatin modifying complexes in the specific accessibility of sequences upstream of rapid exit genes. Some of these studies are underway and are expected to further unravel the underlying mechanisms of rapid transcription activation by poised RNA Polymerase II.

A survival program is induced early during the diauxic shift

In *S. cerevisiae*, quiescence is most commonly induced by allowing a liquid culture of haploid cells to deplete an essential nutrient. This gradual nutrient depletion may last over ten days and involves multiple transitions in cellular states. The growth of a culture to stationary phase causes at least three distinct changes: (1) rapid proliferating (fermenting) to non-proliferating, (2) non-proliferating to slow proliferating (respiring), associated with the diauxic shift of the culture, and (3) slow proliferating (respiring) to quiescence, associated with entry of the culture into stationary phase. It has been previously suggested that the first two transitions (triggered by the lack of good carbon source) may be necessary precursor for the third transition (triggered by lack of any carbon source) and for the consequent long-term survival (Gray et al., 2004). This is supported by the fact that post-diauxic cells acquire many, but not all, characteristics of quiescent cells.

To investigate the relationship between transient arrest early in the diauxic shift and the permanent arrest in quiescence, and to answer the question of at what point the program of quiescence is established, we employed the time-course DNA microarray study described in Chapter 2 to analyze the temporal patterns of induction for genes upregulated in stationary phase. Supported by many observations (see below), we propose that a survival program preparatory to quiescence may be induced twice in glucose starvation experiments, first quite early, at the start of DS, and then in quiescence.

A first surprising finding is that the vast majority of SP enriched transcripts are already upregulated late during DS, at 20 to 30 hours, coinciding with the second DS peak in global mRNA levels (Chapter 2, Figure 1E). This includes most of the aerobic respiratory, stress and transporter genes upregulated in SP (Chapter 3), some of the previously reported SP genes and a few genes associated with sporulation and meiosis. Only a few SP enriched transcripts are more gradually or extremely late induced (Chapter 2, Figure 3T, 3U). Induction of most SP enriched transcripts late during DS is a first observation (I) that suggests that the program of quiescence may already be (partially) initiated during this phase. This agrees with the relatively long 30-hour period without any proliferation during the DS (II) and is also supported by several other observations. Rim15 is a protein kinase that integrates signals from several nutrient-sensory pathways and is required for G0 entry (Pedruzzi et al., 2003). It is provocative that *RIM15* mRNA is upregulated at 10 hours, at the onset of DS (Chapter 2, Figure 3H). Weak transient induction of *RDI1*, a proposed inhibitor of proliferation through its interaction with Cdc42 (Chapter 3), also occurs at the beginning of DS. Several other proposed regulators of SP and nutrient limitation are also transiently upregulated at this time (III). These include *MSN1*, an activator of *SNF1* nutrient limitation control (Estruch and Carlson, 1990) and *SRB11*/Cyclin C, also involved in regulating the response to nutrient limitation (Chang et al., 2001; Holstege et al., 1998; Kuchin et al., 1995). A fourth observation (IV) is that several cell-cycle regulatory genes show a similar induction, including *CDC15*, a MAPKKK required for exit from mitosis (Fankhauser et al., 1995) and *MPS1*, which also has a checkpoint function (Weiss and Winey, 1996). Another observation (V) suggesting that a survival program is initiated early in the DS, is the significant number of mating genes upregulated at this time($p=3\cdot10^{-14}$, Chapter 2, Figure 3O). Sporulation of diploid cells is one of the strategies that *S. cerevisiae* employs to survive harsh conditions. It makes sense that optimal preparation for the possibility of mating (and subsequently, sporulation) occurs immediately after sensing limited glucose at the start of DS. It is also noteworthy (VI) that the growth phase dependent morphological changes, reflected by the number of cells per OD600, already stably occur early in the DS

(Chapter 2, Figure 1B).

The model that emerges from these data suggests that a survival program preparatory to quiescence is induced quite early during glucose starvation, at the start of DS. Upon glucose limitation, immediately prior to the onset of DS, global mRNA levels drop dramatically as part of a program of metabolic and proliferation reduction (Chapter 2, Figure 1E). As glucose levels drop further, proliferation ceases completely and at this point translation drops (Fuge et al., 1994). We suggest that our inability to produce labeled cDNA from most of the poly(A) transcripts between the 11- and 15-hour time points could be related to this (VII). Some mRNAs escape repression and are induced early in the DS at 10 to 11 hrs (Chapter 2, Figure 3H, I, O). The nature of these genes suggests that a survival program is already being set up in response to glucose limitation and that this involves several strategies, including a transient activation of mating genes, other cell cycle related changes as well as altered nutrient sensing. The model of glucose depletion leading to a program with various survival strategies agrees with the finding that invasive growth on agar is also induced by glucose depletion (Cullen and Sprague, 2000) and suggests that the integrated control of mating and dimorphic switch (Madhani and Fink, 1998) may extend to quiescence. We propose that upon glucose limitation, early during DS, a survival program is started that integratively controls three alternative strategies, mating to allow spore formation, the dimorphic switch for foraging and thirdly, quiescence. The strain used and the environment offered in our experiment exclude mating and invasive growth as survival mechanisms, and therefore, in this culture, cells go into quiescence.

Although further experiments are required to unequivocally determine when quiescence is initiated, there are many indications that this may occur twice in glucose starvation experiments. Cessation of proliferation is one clear property of quiescent cells. This occurs twice during the glucose starvation experiment, first in DS and then in SP. As described above, the first cessation of proliferation is initially accompanied by upregulation of a group of genes indicative of diverse survival strategies and only much later by those genes required for aerobic respiration. As has been pointed out before (Gray et al., 2004; Herman, 2002), the multiple transitions that occur after the rapid exponential growth phase emphasize that care needs to be taken in properly assessing at which point samples are being taken during glucose starvation and that many so-called “stationary phase” experiments are actually performed at distinctly variable points in early DS, late DS, post-diauxic growth or SP. Another important implication is that glucose starvation, the most frequently applied method of quiescence induction, is perhaps too complex to allow simple unraveling of purely quiescent regulatory mechanisms. It would be of interest for further quiescence studies to establish an experimental condition that would allow more direct transition between quiescence and proliferation, and importantly, allow acquisition all of quiescence characteristics. So far, the attempts of rapid quiescence induction (such as abrupt starvation, using mutants with impaired respiratory potential or inactivation of the TOR pathway) have been unsuccessful in establishing proper quiescence state, and therefore, this challenge still remains to be overcomed.

General remarks

The study of *S. cerevisiae* stationary phase reveals many novel aspects of quiescence, some of which are possibly conserved among all eukaryotes. This is probably true for the mechanism of rapid transcription activation upon exit from quiescence by poised RNA Polymerase II, as suggested by fundamental conservation of transcriptional regulatory mechanisms in eukaryotic organisms and the similar Pol II states in Drosophila and humans. In addition to Pol II mechanisms of transcription regulation, many components of signaling pathways that are likely to have a role in yeast quiescence (such as Rdi1 and Rim15) are also conserved.

The stationary phase of *S. cerevisiae* is a complex state, established through multiple adaptations, including those that do not occur

in multicellular eukaryotes. Reprogramming of metabolism from fermentation to respiration to quiescence is one example of a *S. cerevisiae*-specific feature of the growth cycle, suggesting that many of the metabolic changes associated with quiescence in yeast may not be evoked during quiescence in other eukaryotes. However, many of processes induced during transitions to stationary phase in yeast are surprisingly similar to those observed in quiescent cells of worms, flies and mammals (Longo, 1999; Longo and Fabrizio, 2002; Stuart and Brown, 2006; Zhang et al., 2006). In all of these organisms, metabolism during quiescence is generally aerobic and supported by lipid fuels (Carey et al., 2003; Storey, 2002). As a result of these oxidative processes, reactive oxygen species (ROS) accumulate in the cell, provoking oxidative stress response and affecting longevity. Due to the conservation of these metabolic pathways, *S. cerevisiae* quiescence is regarded as highly informative model for the study of life span regulation. Another similarity between yeast quiescence and equivalent state in multicellular eukaryotes are changes in glucose metabolism, mediated through insulin signaling pathways in complex organisms (Stuart and Brown, 2006). The importance of synchronizing G0 arrest and specific metabolic processes in establishment of quiescence is supported by the fact that in worms and mammals, the same pathway that regulates cell cycle arrest also controls changes in redox regulation and transcription of key metabolic enzymes, such as glucose-6-phosphatase and phosphoenolpyruvate carboxyl kinase (Burgering and Kops, 2002). These two enzymes are also induced during yeast starvation response and are identified as direct targets of Yjl103c regulation (Chapter 4).

It remains debatable to which extent the metabolic changes associated with yeast quiescence can be extrapolated to changes observed in other eukaryotes. Nevertheless, understanding of these processes, especially those contributing to elevated stress resistance, is important for elucidation of fungal physiology and development of strategies for fighting pathogens. Taken together, the results presented in this thesis uncover many novel aspects of yeast quiescence and provide a framework for further studies of the fungal-specific as well as the evolutionary conserved regulatory mechanisms controlling the quiescence state.

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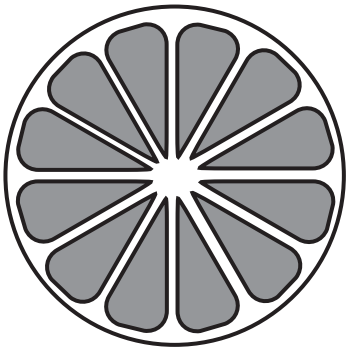
Summary



Summary

Current knowledge of eukaryotic quiescence is obscure. Recent developments of DNA microarray techniques facilitate studies of this common cellular state, providing tools for monitoring genome-wide regulatory effects occurring during the transition between quiescence and proliferation. We have employed several approaches to studying the transcription regulation of quiescence in *S. cerevisiae*, both globally and at the gene-specific level.

First, in **Chapter 2**, we investigated global mRNA levels in cells passing through the entire yeast growth cycle, including exit from stationary phase (quiescence), exponential growth, diauxic shift, post diauxic (respiratory) growth and entry into quiescence. The growth phase-associated changes in poly(A) mRNA levels reveal two major drops, corresponding to glucose depletion (during late exponential phase) and quiescence. The mRNA levels at these points are almost six-fold lower than in exponential phase, revealing global transcription shut-down. Changes in mRNA levels also include three peaks of mRNA accumulation, corresponding to (I) exponential (fermentative) growth on glucose, (II) specific activation of survival programs following glucose depletion, and (III) reactivation of slow growth during post-diauxic (respiratory) phase. The subsequent analysis of genes induced during the second, small peak of mRNA accumulation leads to a model of two-step quiescence induction; it suggests that upon glucose limitation, early during DS, a survival program is started that integratively controls three alternative strategies, mating to allow spore formation, the dimorphic switch for foraging and thirdly, quiescence **(Discussion)**. Therefore, survival program preparatory to quiescence may be induced twice in glucose starvation experiments, first quite early, at the start of diauxic shift, and then immediately prior to quiescence itself.

Our second approach to study quiescence transcription regulation was to investigate gene-specific mRNA expression changes using DNA microarrays **(Chapter 2)**. This resulted in the collection of expression profiles for all yeast genes during the ten-day 34-time-point experiment. To accurately determine mRNA changes per cell, a unique normalization method was applied that is based on external controls and total RNA yield per cell. In contrast to commonly used normalization methods, this accurately determines mRNA changes per cell during global mRNA shifts. The collection of expression profiles provides a starting point for investigation of many gene-specific processes, as is exemplified by work described in **Chapters 3 and 4** (see below).

One important observation derived from the mRNA expression time-course analysis **(Chapter 2)** is the induction of over 2500 genes to levels higher than in quiescence, only 3 minutes upon adding nutrients to the stationary phase culture. The extremely rapid induction of a surprisingly large number of genes upon exit from quiescence indicates that components of the transcription machinery are maintained during prolonged quiescence. In addition, the late induction of a few transcripts enriched in quiescence indicates that some transcription machinery is active in SP and is capable of responding to environmental changes.

The discoveries made by genome-wide expression profiling prompted further investigation of the role of RNA polymerase II in transcriptional control of growth condition-related changes. To correlate the observed changes with the activity of the general transcription machinery, we examined the RNA polymerase II (Pol II) in exponential and stationary phase of growth **(Chapter 2)**. This revealed an unexpected high preservation of inactive Pol II and some of the components of general transcription machinery during quiescence. In addition, genome-wide location analyses (ChIP on chip) showed that this inactive Pol II binds preferentially to the regulatory regions upstream of genes induced immediately upon exit from quiescence. This leads to the proposal that during quiescence, Pol II is stored in the vicinity of promoters of genes required for quiescence exit, likely enabling their rapid activation upon sensing of nutrients. According to this model, transcription activation

of the “rapid exit” genes does not require *de novo* recruitment of the Pol II, and therefore, enables more rapid response of the transcription machinery to improved nutrient status. In addition to binding to the intergenic regions, Pol II is also located on a small number of genes, most of which are induced during quiescence. This agrees with the fact that a small proportion of Pol II is active during quiescence and transcribes stationary phase-induced genes.

In **Chapter 3**, we analyse the phenotypes and functions of genes selected from the genome-wide expression data to determine processes and genes that are essential for long-term survival and for the exit from quiescence upon re-feeding. In total, phenotype analysis of gene deletion mutants identifies 50 strains with strong to intermediate stationary phase-associated growth defects. This includes many regulators, such as Rdi1, the GDP dissociation inhibitor proposed to negatively regulate proliferation during quiescence through its interaction with Cdc42.

The analyses of functional categories of SP-responsive genes reveal that the primary processes in the initiation of reproliferation are a boost in protein synthesis, achieved through activation of ribosome biosynthesis genes, the activation of gene-specific transcription regulators, and the initiation of growth by activation of PKA and Rho signaling pathways as well as cell cycle regulators. On the other hand, entry into quiescence requires activation of the cellular stress response and major changes in energy metabolism, enabling adaptation to deriving energy from oxidative processes. This includes genes involved in mitochondrial and peroxisomal function, tricarboxylic acid cycle (TCA) and oxidative phosphorylation. Also, many (putative) transcription factors and components of signal transduction pathways are induced during quiescence, suggesting the active control of this state. In addition to discovering the most important processes and genes associated with quiescence exit and entry, these functional analyses also provide a starting point for the functional characterization of hundreds of genes with unknown function.

To shift the focus of our study from global to gene-specific gene regulation, we used the collection of time-course expression profiles **(Chapter 2)** to select putative gene-specific transcription regulators of the starvation response. One protein that satisfied several criteria for a putative regulatory role is Yjl103c. In **Chapter 4**, we employ multiple genome-wide approaches to discover genes and processes controlled by Yjl103c. This includes identification of Yjl103c binding targets using genome-wide location analyses (ChIP on chip), identification of the nature of Yjl103c regulation (activator or repressor) using genome-wide expression studies of a *yjl103c* deletion mutant and determination of the DNA sequence motif likely recognized by Yjl103c. These analyses reveal that Yjl103c acts as an activator of genes, many of which are regulators themselves. These include the gene specific transcription factors Hap4, Adr1, Gis1, Gat2, Sfl1, Sef1, Mga1, Mcm1 and Yer130c, global regulators such as Rba50, a protein that interacts with RNA polymerase II, Snf11, a global activator and chromatin remodeling factor, and Htz1, histone-related regulator of chromatin silencing.

The genome-wide functional analyses reveal that Yjl103c controls multiple processes, either directly or indirectly. Processes such as gluconeogenesis, cell wall organization and biogenesis, protein degradation, tricarboxylic acid cycle and response to stress are likely to be directly controlled by Yjl103c, whereas respiration, peroxisomal fatty acid beta-oxidation, general diauxic shift response, nitrogen depletion response, pheromone response and cell cycle are probably controlled through gene-specific transcription factors found among Yjl103c targets.

The effect of *YJL103C* deletion was investigated by phenotype analysis of *yjl103c* mutant under specific environmental settings **(Chapter 4)**. This mutant exhibits growth defects when grown on glycerol or oleate as sole carbon sources, consistent with its role in regulation of respiration and peroxisomal fatty acid beta-oxidation (as suggested by expression analyses and the presence of Hap4 and Adr1, regulators

of these processes, among Yjl103c targets). In addition, *yjl103c* has reduced fitness in the presence of caffeine, a phenotype that can be rescued by a cell-wall stabilizing agent. This phenotype is typical for components of PKC/MAPK signaling pathway that regulates cell-wall integrity, suggesting that Yjl103c potentially regulates gene expression through modulation of this signaling cascade. Binding of Yjl103c to upstream sequences of Rho signaling pathway components, which act upstream of PKC/MAPK signaling cascade, further supports the Yjl103c role in regulation of the cell-wall integrity pathway.

In summary, our genome-wide investigation of transcription regulation during *S. cerevisiae* quiescence (I) provides a comprehensive description of global and gene-specific mRNA expression changes associated with quiescence exit and entry, (II) shows the importance of applying appropriate normalization for microarray data (III) unravels a novel mechanism of rapid transcription activation upon exit from quiescence by poised RNA polymerase II, (IV) leads to the proposal of a two-step model for quiescence induction, (V) identifies genes and processes necessary for quiescence exit and entry, and (VI) functionally characterizes a novel gene-specific transcription regulator of the starvation response and uncovers its downstream regulatory network. In addition to these findings, this work raises several new questions and provides a framework for further exploration of the quiescent state.

Samenvatting

Onze huidige kennis van quiescence (cel rust) in eukaryotische cellen is nog steeds minimaal. De recente ontwikkelingen van DNA microarray technieken vergemakkelijken studies van deze cellulaire staat en zijn hulpmiddelen om genoom-brede regelgevende gevolgen te analyseren die plaatsvinden tijdens de overgang van quiescence naar proliferatie (cel deling). In dit proefschrift beschrijven wij het gebruik van verschillende genoom-brede methodes om globale en genspecifieke transcriptie regulatie mechanismen te analyseren tijdens quiescence in *Saccharomyces cerevisiae* (bakkergist).

In **Hoofdstuk 2**, onderzochten wij eerst de globale mRNA veranderingen in gistcellen die door een volledige groei-cyclus gingen: uitgang van quiescence, exponentiële groei / fermentatie, diauxic shift, post-diauxic groei / respiratie en de ingang in quiescence. De groeifase geassocieerde veranderingen in poly(A) mRNA laten twee belangrijke dalingen zien welke corresponderen aan de uitputting van glucose (tijdens de late exponentiële groei) en quiescence. De mRNA niveaus op deze punten zijn bijna zes keer lager dan tijdens de exponentiële groei en wijzen erop dat er een globale transcriptie stop plaatsvindt.

Verder hebben wij met behulp van DNA microarrays, mRNA expressie niveaus geanalyseerd tijdens de gehele groei-cyclus van *S. cerevisiae* (**Hoofdstuk 2**). Dit resulteerde in een verzameling van expressieprofielen voor alle gistgenen van 34 tijdspunten over een periode van 10 dagen.

Om mRNA veranderingen per cel nauwkeurig te kunnen bepalen, werd een unieke normalisatiemethode toegepast die gebaseerd is op externe controles en de totale opbrengst van RNA per cel. In tegenstelling tot de algemeen gebruikte normalisatiemethodes, bepaalt deze methode mRNA veranderingen per cel tijdens globale verschuivingen van alle transcripten. De verzameling van expressieprofielen is een uitgangspunt geweest voor het onderzoek van vele genspecifieke processen, zoals verder beschreven is in de **Hoofdstukken 3** en **4**.

Één belangrijke observatie uit de mRNA expressieprofielen is de activering van >2500 genen op 3 minuten na toevoeging van voedingsstoffen aan rustende cellen. De uiterst snelle activering van deze genen suggereert dat de verschillende componenten die nodig zijn voor transcriptie, aanwezig zijn tijdens quiescence. Bovendien wijst de late inductie van een aantal transcripten, die verrijkt zijn in quiescence, erop dat de algemene transcriptie machine actief is in stationaire fase.

Verder hebben wij gevonden dat RNA polymerase II (Pol II), het enzym dat verantwoordelijk is voor de transcriptie van genen, al aanwezig is op de promoter van de te activerende genen. Dit is strijdig met modellen van genactivering waarin transcriptie van genen wordt gelimiteerd door de hoeveelheid Pol II dat wordt gerekruteerd op de promoters van de te activerende genen. In rustende cellen was het enzym al gebonden op de beginplaatsen van honderden genen die onmiddellijk aangeschakeld werden zodra cellen met de celdeling begonnen. Dergelijke rekrutering staat een cel toe om snel te antwoorden en beginnen met groeien wanneer veranderingen plaatsvinden in het milieu waar de cel zich in bevindt. Bovendien werd Pol II ook gevonden op de coderende gedeeltes van een klein aantal genen die geactiveerd zijn tijdens quiescence wat akkoord gaat met het feit dat Pol II actief blijft tijdens quiescence.

In **Hoofdstuk 3** hebben wij vervolgens fenotypes en functies geanalyseerd van genen, die geselecteerd zijn uit de genoom-brede expressie analyse, welke essentieel zijn voor lange termijn overleving en uitgang van quiescence. In totaal hebben wij 50 deletie mutanten geïdentificeerd die een stationaire fase geassocieerde groeidefect hebben.

In **Hoofdstuk 4** hebben wij een transcriptiefactor (Yjl103c) gekarakteriseerd die mogelijk nodig is voor de regulatie en activering tijdens verhongering van gist cellen. Deze transcriptiefactor was geïnduceerd direct na uitputting van glucose. Dit is gedaan door genoom-brede analyse van expressieprofielen van een deletie mutant en de identificatie van DNA bindingsplaatsen van deze transcriptie factor. Deze analyses laat zien dat Yjl103c een activator is van genen die zelf ook regulatoren zijn zoals genspecifieke transcriptie factoren maar ook globale regulatoren. Ook hebben wij een DNA bindingspatroon gevonden in de promoter van genen die geactiveerd en gebonden worden door Yjl103c. De functie van de geïdentificeerde genen en fenotypering van de *yjl103c* suggereert dat Yjl103c betrokken is bij de regulatie van verschillende processen die nodig zijn voor een juiste reactie tijdens de verhongering van *S. cerevisiae*. Sommige van deze processen zijn direct gereguleerd door Yjl103c (zoals gluconeogenese, celwand organisatie en biogenese, eiwit degradatie), waarbij andere indirect gereguleerd worden door regulatoren die targets zijn van Yjl103c.

Het in dit proefschrift beschreven genoom-brede onderzoek van transcriptie regulatie tijdens *S. cerevisiae* quiescence (I) geeft een beschrijving van globale en genspecifieke mRNA expressie veranderingen geassocieerd met de in- en uitgang van quiescence; (II) toont het belang van een goede normalisatiemethode voor microarray data; (III) beschrijft een mechanisme van snelle transcriptie activering tijdens de uitgang van quiescence door RNA Polymerase II; (IV) leidt tot het voorstel van een twee-staps model voor de inductie van quiescence; (V) identificeert genen en processen welke noodzakelijk zijn voor in- en uitgang van quiescence; (VI) karakteriseert een nieuwe genspecifieke transcriptie factor van de verhongering reactie en onthult zijn stroomafwaarts regulerend netwerk. Naast deze bevindingen werpt dit werk enkele nieuwe vragen op en verzorgt een kader voor verder onderzoek van cel rust.

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The results of my PhD studies are described in this thesis. It would take an additional one to describe how these last 4.5 years of living and working in The Netherlands influenced my life and personality. It has been an unforgettable journey, not without obstacles, but full of exciting discoveries and rewarding views. I would like to show gratitude to all people that have helped me along the way and made the journey more “gezellig”.

Frank, thank you for believing in me and giving me the opportunity to join your group. You taught me so much over these years, not only how to do good science, but also a few tricks about what a “real world” means and what do you need to survive in it. Thank you for your sensibility to understand who I am and kindness to respect it. I wish you all the best with “the next generation” of Genomics lab, and keep up with the top science!

My dear colleagues! Thank you so much for all your help and a nice working atmosphere. Theo, thanks for the best unofficial inburgering cursus in the world! I learned from you all those little unnecessary things that make Nederlanders Dutch. Nynke, you were the first to convince me that this lab is a great choice for my PhD and you have been there all along to share ups and downs of the lab life and the passion for yeasties. Good luck, whatever you decide to do. Dik, thanks for always being so helpful and kind to me. And for making me feel less lonely in the late lab hours… Patrick, my perfect opposite, yet with one common passion! I hope that we will once take that big rollercoaster ride together. Harm, thanks for all your help and that “I know what you mean” look throughout these years. I wish you and Dalila successful and happy life wherever you decide to settle. Nienke, thanks for arranging little things in the lab and for bringing up the girly spirit. Marian, dear and helpful, thanks for always being there for me. It has been a real pleasure working with you. Paul, your amazing attitude towards the life will certainly get you far. Good luck with your promotion and a further career path. Tony, I wish you all the best and take good care of the yeast lab. Arnaud, thanks for solving the little wars with my PC. Much of my work could not be done without you. Yumas, I’ll miss your “dobro jutro” and “zdravo” with a perfect pronunciation! Thanks for all help and especially for those exceptional 8GB. Erik, thanks for everything and good luck with your career. Philip, my paranimph, thanks for being extraordinary in an ordinary world! It has been great meeting someone with so many talents, not only in science but also in literature, music, outdoor activities, parenthood, …and this list is probably a misunderestimation! Thanks for accompanying me in solving the numerical puzzles during the last year! Your support meant a lot to me. I wish you good luck with your thesis and a soon promotion! Jean-Christophe, my lab-mate, office-mate, my neighbour, my dancing-partner, chill-out companion and simply my good friend, thanks for everything! Thanks for all discussions and tricks in the lab and out of it. I wish you success with your own group and ultimate happiness with your girl! Loes, Joris, Sake, our “next generation”, good luck with your projects! Enjoy your time in the Genomics lab and score some nice papers. Eveline, Alike, Esther, Geert, Maike, Maurice, Koen, it was great having you around! Good luck with your further studies and do not hurry with giving up partying… Elen, Thessa, Joop, thanks and good luck on your own paths! Mirjam, Lilian, Marjolein, Paul vd B., thanks for guiding me through the labyrinths of the administrative world. Peter van der Vliet, thank you for giving me the opportunity to become a member of the Physiological Chemistry. My work would not be possible without the help of many kind people from this great department. I would therefore like to thank all colleagues from Timmers, van der Vliet, Bos, Burgering, Wijmenga, Medema and Voest groups.

Monika and Marcin, thanks for aiding my first steps in the Netherlands, for the help with Belastingdienst forms, my first bike, and never letting me fall from the climbing wall (Marcin). I wish you good luck in the coming period and to be a very happy family soon! Lars, thanks for sharing

many great episodes with me (would take a chapter) and all your help with my lab “emergencies”. I wish you and Anna good luck with your PhDs and happiness with a three of you (kitten for a start)! Joost, I’ll miss you... Have a great trip, and then come back to tell all about it! Hetty, Chris was very lucky to have you in his team! It has been a pleasure chatting and working with you during all these years. Thanks for all your help! Chris, my soulmate in so many ways, thanks for all the parties. I wish you and Miranda all the best in NY. Klaas, it is amazing to look back and see us growing up (even me, as you said) throughout these years. Thanks for all funny and exciting discussions. I wish you good fortune with science and to become a great papa! Roland, thanks for always being there to give me a hand and for a plenty of nice chats in-between. Marcel (Winter), thanks for always cheering me up and good luck with your further career (and of course, girls). Marcel, I highly appreciate your patience and effort to clean all glassware from my huge yeast-growing experiments! Enjoy my absence! Marjolein, thanks for dealing with my countless emergency orders.

Vanesa, moja kumo i paranimfo (a to nije dugme), drago mi je sto su nam se igrom slucaja ukrstili putevi! Hvala za drustvo, razumevanje i podrsku tokom svih ovih godina. Zelim ti srecu sa svojim projektom i skoru najavu diplome.

Heel erg bedankt aan de familie van de Peppel and Frencken voor alle liefde, steun en gezellige momenten door de jaren heen.

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My beloved Jeroen, you gave the meaning to it all. Words are not enough to express my gratefulness for your endless patience, support and love that helped me to get this far. Beyond my imagination... My sweet Luna, only one look at you melts all my troubles away. Thank you both for being my family.

Curriculum vitae

Marijana Radonjić was born on the 24th of October 1976 in Belgrade, Yugoslavia (Serbia at present). From 1991 to 1995 she studied the natural sciences at The First Belgrade Gymnasium. Upon receiving the diploma in 1995, she started Molecular Biology and Physiology studies at the Faculty of Biology, University of Belgrade, Serbia. The second part of the university studies included courses from Applied Biochemistry program and a research project at the Laboratory for Molecular Genetics of Industrial Microorganisms, Institute for Molecular Genetics and Genetic Engineering, Belgrade, under the supervision of Prof. Dr. Ljubisa Topisirović. As the result of the university studies, she recieved the equivalent of Bachelors and Masters degree in Molecular Biology and Physiology in February 2001. Upon the graduation, she stayed at the laboratory of Prof. Dr. Ljubisa Topisirović as the junior researcher. In December 2001, she started her Ph.D. studies at the Department of Physiological Chemistry, Utrecht University, The Netherlands, under the supervision of Prof. Dr. Frank Holstege. The results of this research, conducted during the period from 2001 to 2006 in the Genomics Laboratory are described in this thesis.

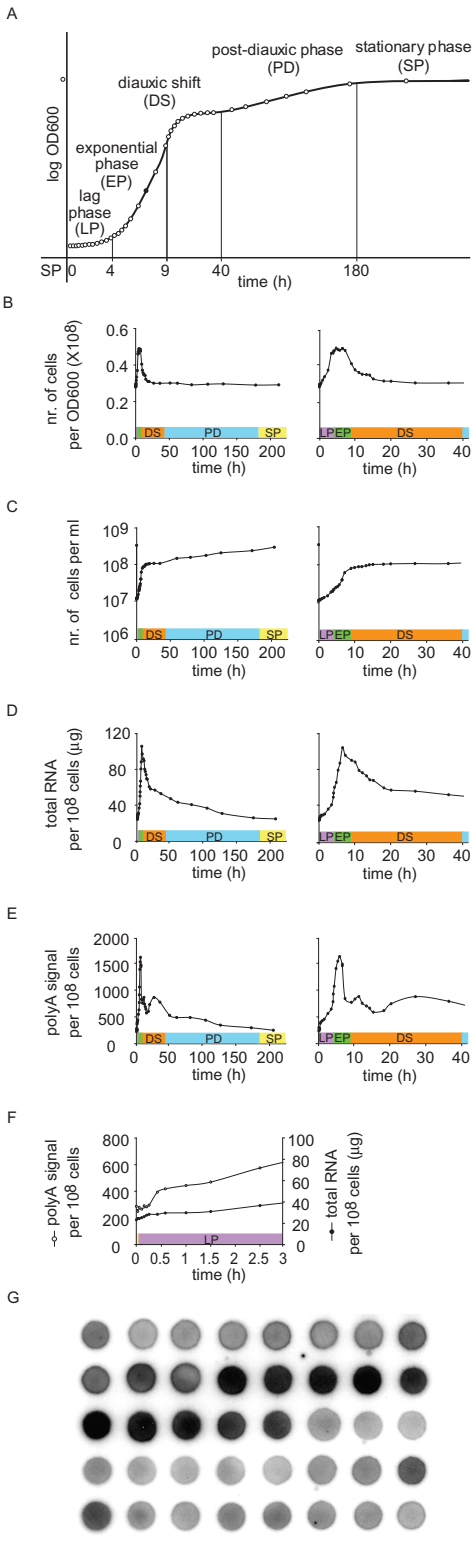
List of publications

M. Radonjic, P. Lijnzaad, H. van Bakel, M. Groot Koerkamp, D. van Leenen and F.C.P. Holstege
Integrative genomic analysis characterizes a novel regulator of the *S. cerevisiae* starvation response
Submitted

M. Radonjic, J.C. Andrau, P. Lijnzaad, P. Kemmeren, T.T.J.P. Kockelkorn, D. van Leenen, N.L. van Berkum, F.C.P. Holstege
Genome-wide analyses reveal RNA polymerase II located upstream of genes poised for rapid response upon *S. cerevisiae* stationary phase exit
Molecular Cell. 2005 Apr 15;18(2):171-83

van de Peppel J, Kemmeren P, van Bakel H, Radonjic M, van Leenen D, Holstege FC.
Monitoring global messenger RNA changes in externally controlled microarray experiments.
EMBO Rep. 2003 Apr;4(4):387-93

Colour figures



Chapter 2, Figure 1. Global changes during quiescence exit and entry

(A) Schematic representation of a glucose starvation experiment, with circles representing the 39 samples analyzed. The single filled circle in the rapid exponential growth phase represents the common reference sample used in the microarray experiments. The open circle, left of the horizontal axis, represents the aliquot of the SP culture (day 9) that was used to start the experiment. See Experimental Procedures for a complete list of time points.

(B) Cell count per OD600 throughout the culture (left) and for the first 40 hours (right). The colored bar represents the various culture periods, with abbreviations according to A.

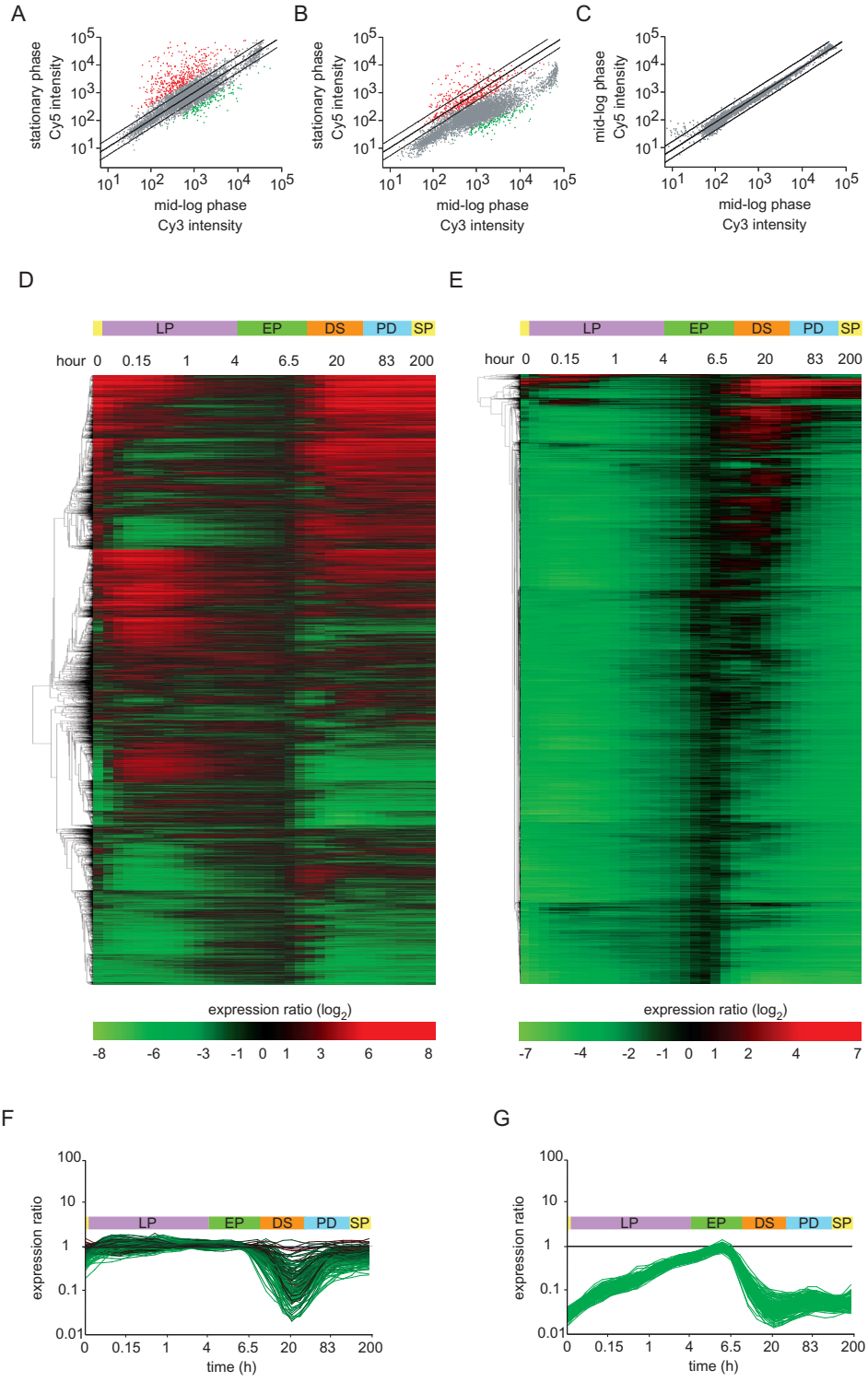
(C) Number of cells/ml throughout the culture (left) and for the first 40 hours (right).

(D) Total RNA per 10^8 cells.

(E) Average poly(A) dot blot ^{32}P radioactive signal per 108 cells as determined by analysis of two dot-blot experiments, one of which is depicted in G.

(F) poly(A) signal per 10^8 cells (open circles) and total RNA per 10^8 cells (filled circles) for the first 3 hours.

(G) poly(A) dot blot for determination of mRNA levels throughout the experiment. The first dot (left upper corner) represents the nine day SP culture from which the experiment was started. The 20th and 21st spot (counting from left to right) are duplicates of the 6.5 hour time point.


A
B
C
stationary phase
Cy5 intensity
mid-log phase
Cy3 intensity
D
E
LP
EP
DS
PD
SP
hour 0 0.15 1 4 6.5 20 83 200
expression ratio (log2)
-8 -6 -3 -1 0 1 3 6 8
-7 -4 -2 -1 0 1 2 4 7
F
G
expression ratio
time (h)
0 0.15 1 4 6.5 20 83 200

Chapter 2, Figure 2. Genome-wide changes in transcript levels during quiescence exit and entry

(A) Standard, all genes normalized scatter-plot of average background subtracted microarray gene spot intensities, comparing ML samples with SP samples (9 days). The three lines represent two-fold up, no change and two-fold down boundaries, from top to bottom respectively. Colored in red and green are genes that were determined to show statistically significant changes in mRNA expression in the analysis of the eight SP versus ML replicate measurements.

(B) External control normalized result of the experiment shown in A, with the same genes colored red and green as in A.

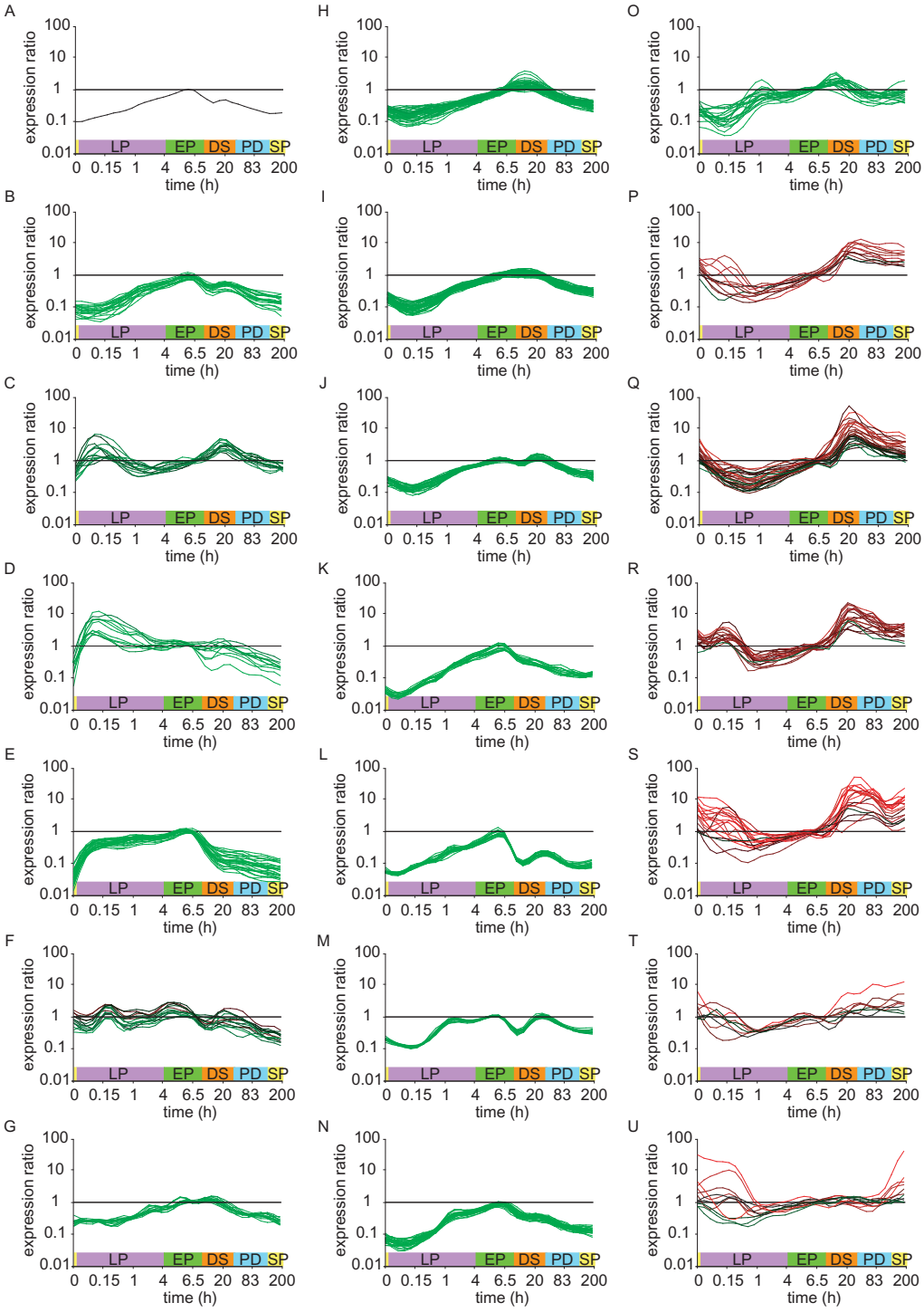
(C) External control normalized result of a same versus same, ML replicate microarray hybridization.

(D) Standard, all genes normalized, hierarchical clustering diagram for the entire time-course (left to right), of all 6357 yeast genes represented on the microarrays. The colored bar on top represents the various culture periods, with abbreviations according to Figure 1A. Clustering was by average linkage analysis (UPGMA) with a standard (cosine) correlation. Supplemental table S1 contains a fully annotated version of the cluster diagram.

(E) Hierarchical clustering diagram of the external control normalized microarray data.

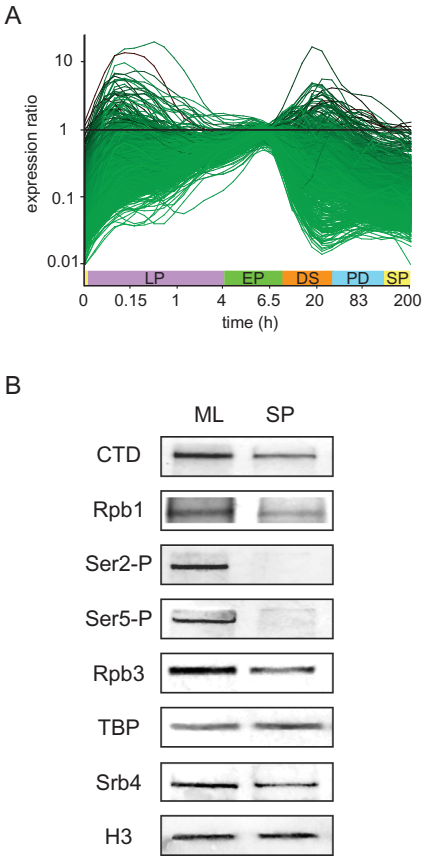
(F) Expression ratios of 123 ribosomal protein genes throughout the time-course when the data is normalized by the standard, all genes approach. The collective expression ratio of all genes is artificially set at one (no change) in each individual time-point by this approach. The RPGs appear only to fluctuate once during the time-course, at 20 hours. This actually represents the time-point when these transcripts deviate most from the behavior of all the genes, with which the data is normalized here.

(G) Expression ratios of 123 ribosomal protein genes when data is normalized with external controls. This results in ratios for each individual transcript, relative to the level of that transcript in the reference sample. The reference sample was ML, which is why all transcripts show a ratio of one at this time-point.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
expression ratio
100
10
1
0.1
0.01
LP
EP
DS
PD
SP
0
0.15
1
4
6.5
20
83
200
time (h)

Chapter 2, Figure 3. Many distinct patterns of gene expression during quiescence exit and entry

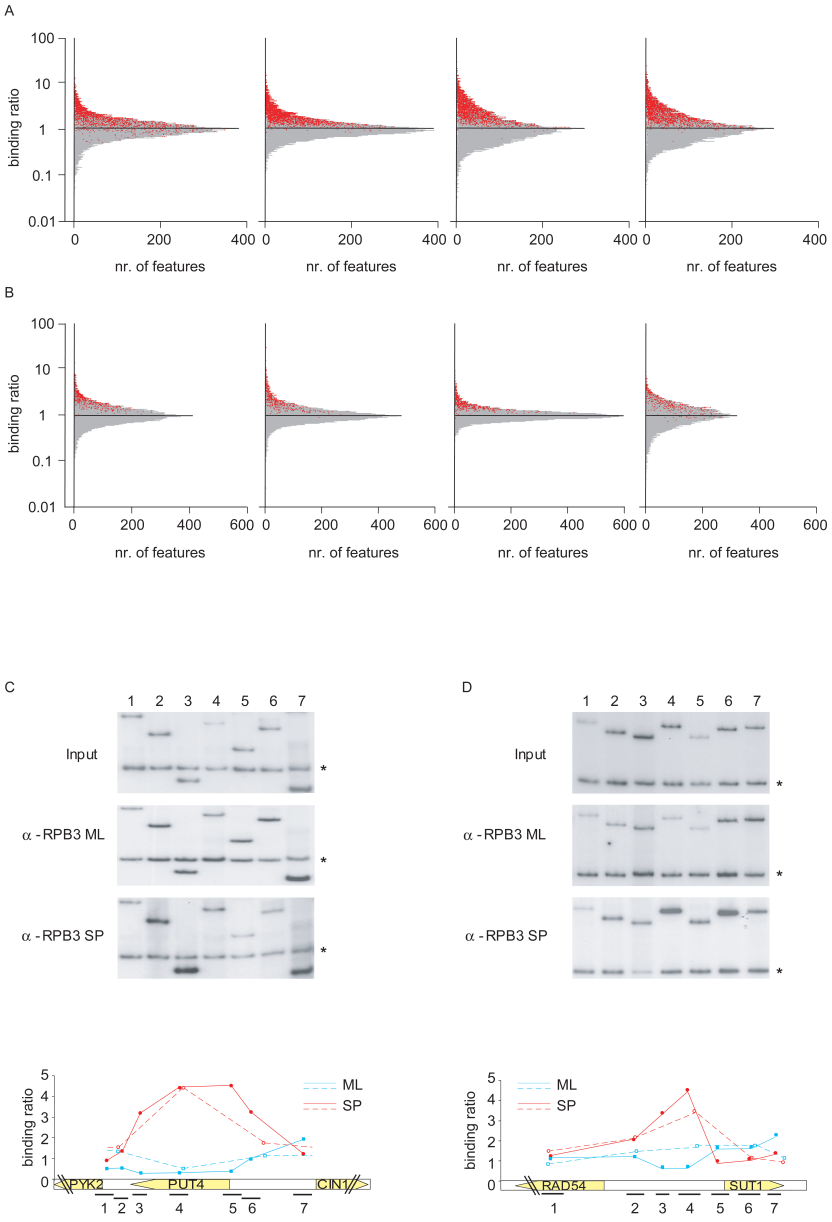
(A) The average expression ratio of all genes across the time-course.
(B) Genes that most closely follow the average transcript behavior.
(C) Rapid SP exit genes, transiently induced prior to both periods of proliferation.
(D) Rapid SP exit genes, transiently induced only prior to proliferation on glucose.
(E) Rapid SP exit genes, induced prior to proliferation and maintained at high levels during exponential growth.
(F) Transcripts that fluctuate several times during the time-course. These are mainly genes with uncharacterized molecular function and/or biological process.
(G) A group of uncharacterized/hypothetical ORFs induced at the end of lag phase.
(H) Genes that escape the general DS repression and which are induced very early in DS, at 10 to 11 hours.
(I) Similar to H, but with less prominent induction.
(J) Transcripts that peak at the end of rapid exponential growth and late in the DS.
(K) Transposon TyB Gag-Pol genes. The tight co-regulation is likely due to their overall identity.
(L) Transposon TyA Gag genes.
(M) Subtelomeric Y' helicases and homologs.
(N) A cluster of various functionally related genes induced late in lag-phase and including negative regulators of the Swe1p kinase, SPT6 and SPT16, genes involved in bud site selection and replication.
(O) Transcripts that show two transient peaks, late in lag phase and very early during DS. Half of these genes are involved in mating.
(P) SP enriched transcripts, induced late in DS and remaining high thereafter. This includes many of the stress and aerobic respiratory genes enriched in SP.
(Q) SP enriched transcripts, more transiently induced late in DS, but still remaining elevated in SP compared to ML. This also includes many of the stress and aerobic respiratory genes enriched in SP.
(R) SP enriched transcripts, as Q, but with an additional induction early in lag phase, although not immediately upon exit. This suggests that these genes may be required for transitions to any new carbon source.
(S) SP enriched transcripts as Q and R, but that show a second induction upon SP entry, after the post-diauxic growth phase.
(T) SP enriched transcripts that start to rise late in lag phase and continue to do so into SP. This group includes previously characterized SP genes such as SNO1, SNZ1 and SNZ2.
(U) SP enriched transcripts showing induction upon the transition between slow post-diauxic growth and SP. Groups B-O were obtained by clustering across the entire time-course. Groups P-U were clustered based on their behavior only in the latter part of the time-course. The genes depicted in each cluster are listed in Supplemental Table S2. More genes with similar behavior can be found in Supplemental Table S1.



Chapter 2, Figure 4. The general transcription machinery is maintained during quiescence

(A) Rapid and broad transcription activation upon exit from stationary phase (SP). The expression ratios throughout the time-course for the 769 genes induced more than two-fold within the first three minutes of SP exit are depicted. The genes are listed in Supplemental table S3.

(B) Western blot analysis of mid-log (ML) and stationary phase (SP) protein extracts for RNA polymerase II subunits Rpb1p, its C-terminal domain (CTD) and Rpb3p. Ser2-P and Ser5-P refer to specific phosphorylation sites within the heptapeptide repeat of the CTD. TBP refers to TATA-binding protein and Srb4p is a subunit of the Mediator complex. Extracts from equivalent amounts of cells were loaded and this was verified using an antibody specific for the invariant part of Histone H3 (H3).

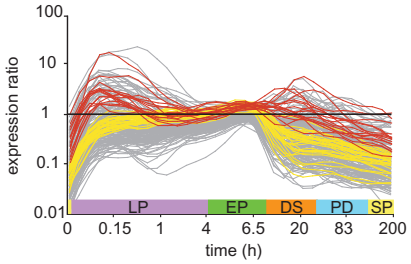


Chapter 2, Figure 5. Genome-wide location analysis of RNA polymerase II

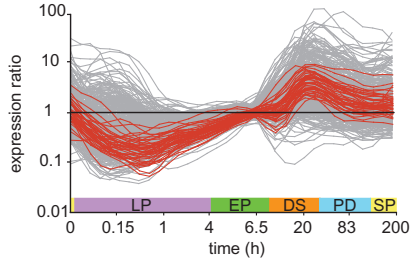
Histograms of Pol II binding ratios of all genomic sequences for ML (A) and SP (B). Each histogram is the result of a single microarray hybridization. The vertical axis represents the normalized binding ratios. In red are genomic features selected as significantly enriched in each collection of four hybridizations.

Conventional ChIP analysis of *PUT4* (C) and *SUT1* (D), analyzed by PCR in the linear range of amplification, loaded on a 7% polyacrylamide gel and quantified using a phospho-imager as described under Experimental Procedures. Relative positions of the PCR products are indicated below the graphs. The asterisk represents the position of the internal normalization control for PCR. The binding ratios depicted on the graphs are from the microarray (dashed lines) and conventional ChIP (solid lines), for ML (blue) and SP (red), respectively.

A



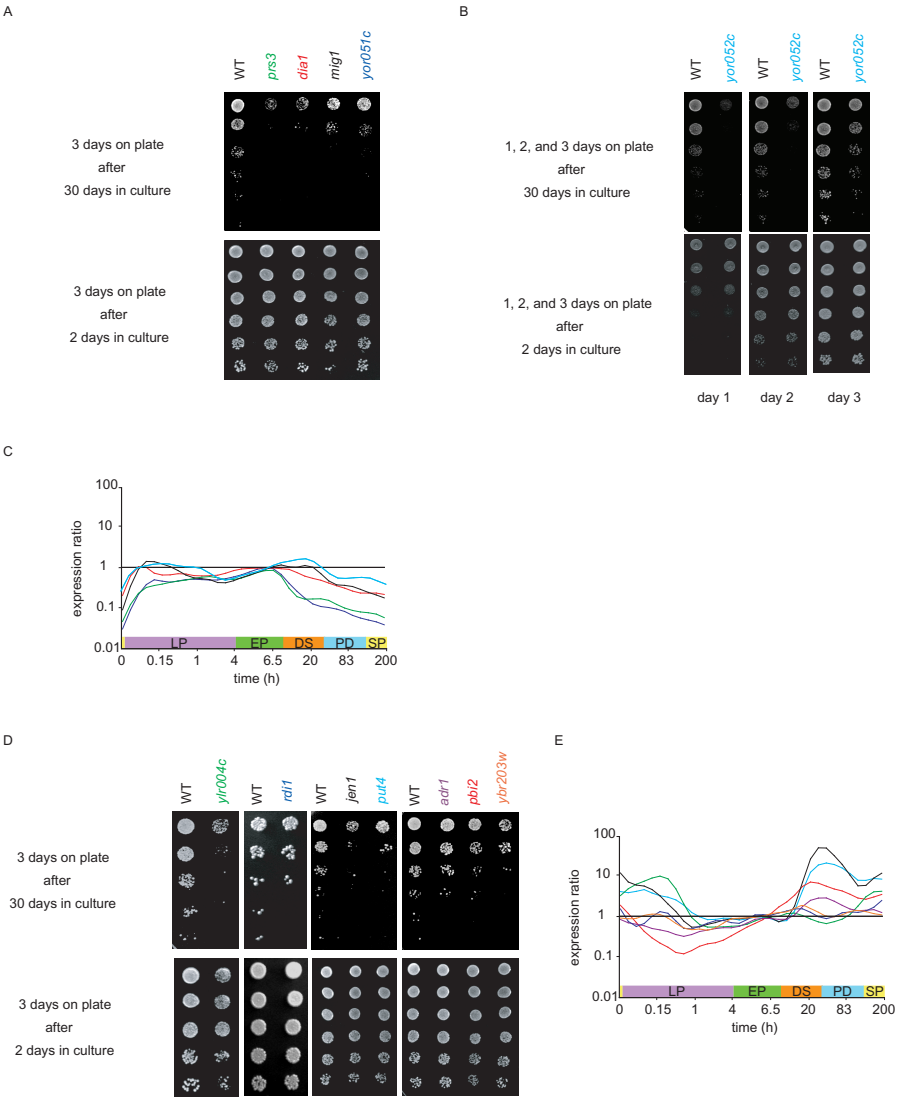
B



Chapter 3, Figure 1. mRNA expression profiles of genes selected for functional analysis

(A) Genes induced more then 4 fold in the three minute time-point after stationary phase (SP) exit compared to the SP starting sample (Radonjic et al., 2005). Representatives of the functional categories “ribosome biogenesis and assembly” and “DNA dependent transcription” are shown in yellow and red, respectively. The remainder of SP exit genes is shown in gray. The coloured bar at the bottom of the graphs represents the various culture periods: LP, lag phase; EP, exponential phase; DS, diauxic shift; PD, post-diauxic phase; SP, stationary phase.

(B) Genes induced in stationary phase (Radonjic et al., 2005). Representatives of the functional category “respiration” are shown in red and the rest of the SP induced genes are shown in gray. All genes shown in (B) are induced during SP at least three fold more than in exponential phase, relative to expression of all genes in the genome.



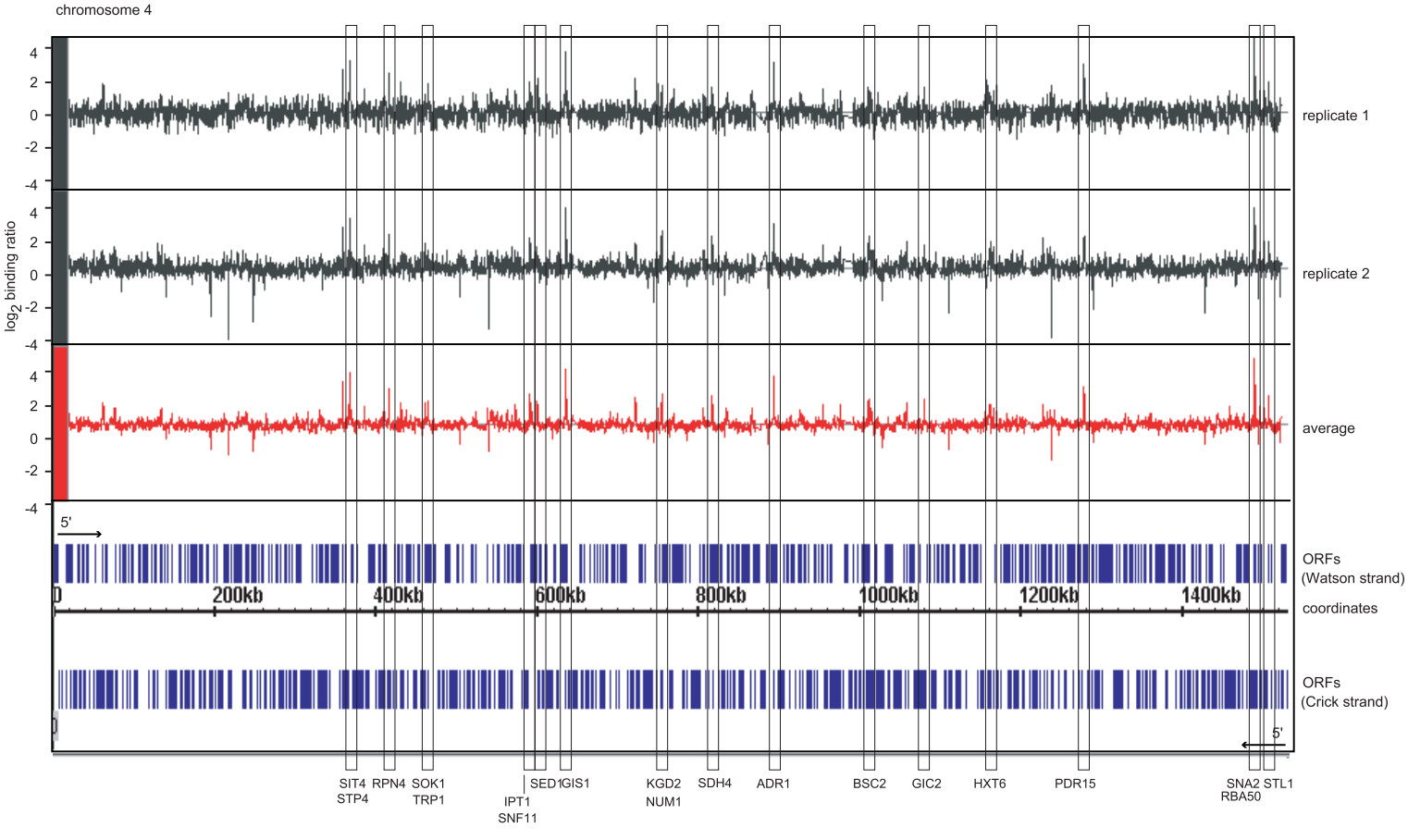
Chapter 3, Figure 3. Stationary phase phenotypes

(A, B) Examples of phenotypes for mutants bearing deletion in stationary phase (SP) exit genes. Wild-type and mutant strains were grown for 30 days in YPD culture at 30°C. Cells from 30-day (top panels) or two-day (bottom panels) cultures were spotted in five-fold serial dilutions on YPD plates and incubated for 3 days at 30°C. Loss of viability in stationary phase is estimated from the number of colonies formed on the third day upon replating (A). Slow recovery from stationary phase is deduced from the size of colonies formed during first, second and third day upon replating (B).

(C) mRNA expression profiles of genes with phenotypes shown in (A) and (B). The colour of mRNA expression graphs corresponds to the colour of gene names in (A) and (B). The bar at the bottom of mRNA expression graphs represents the various culture periods, with abbreviations according to Figure 1.

(D) Examples of phenotypes for mutants bearing deletions in SP induced genes, revealed by an identical analysis as in (A) and (B).

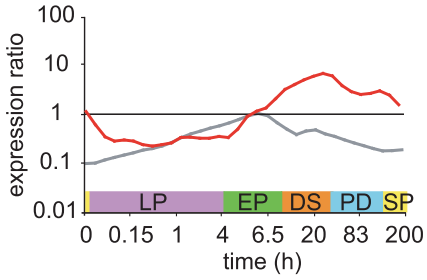
(E) mRNA expression profiles of genes with phenotypes shown in (D). The colour of mRNA expression graphs corresponds to the colour of gene names in (D).


chromosome 4
log2 binding ratio
4
2
0
-2
-4
replicate 1
replicate 2
average
5'
ORFs
(Watson strand)
0
200kb
400kb
600kb
800kb
1000kb
1200kb
1400kb
coordinates
ORFs
(Crick strand)
5'
SIT4
STP4
RPN4
SOK1
TRP1
IPT1
SNF11
SED1
GIS1
KGD2
NUM1
SDH4
ADR1
BSC2
GIC2
HXT6
PDR15
SNA2
RBA50
STL1

◀ Chapter 4, Figure 2. Pattern of Yjl103c binding to chromosome 4

An example of Yjl103c binding to an entire chromosome, visualized using the Integrated Genome Browser (IGB) (http://www.affymetrix.com/). Three binding tracks are shown, representing $\log_2$ binding ratios of Yjl103c for all chromosomal features. The two top graphs (black) are derived from the two replicate ChIP on chip experiments and the bottom graph (red) shows the average values of these replicates. The horizontal axis illustrates the chromosomal position of the array features along *S. cerevisiae* chromosome 4, and the blue blocks represent the array features themselves on Watson and Crick DNA strands. Framed peaks of binding ratios and their corresponding array features are selected as significantly enriched after applying statistical analyses and "distance from ORF" criterion (Experimental procedures). The open reading frames (ORFs) located downstream of these enriched features are shown. ORF pairs *SIT4* and *STP4*, *SOK1* and *TRP1*, *IPT1* and *SNF11*, *KGD2* and *NUM1*, and *SNA2* and *RBA50* have common enriched features, as these are positioned within sufficient proximity upstream of both ORFs.

A



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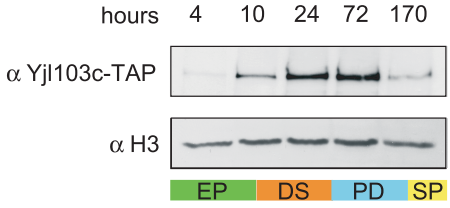
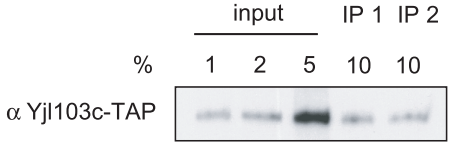


C

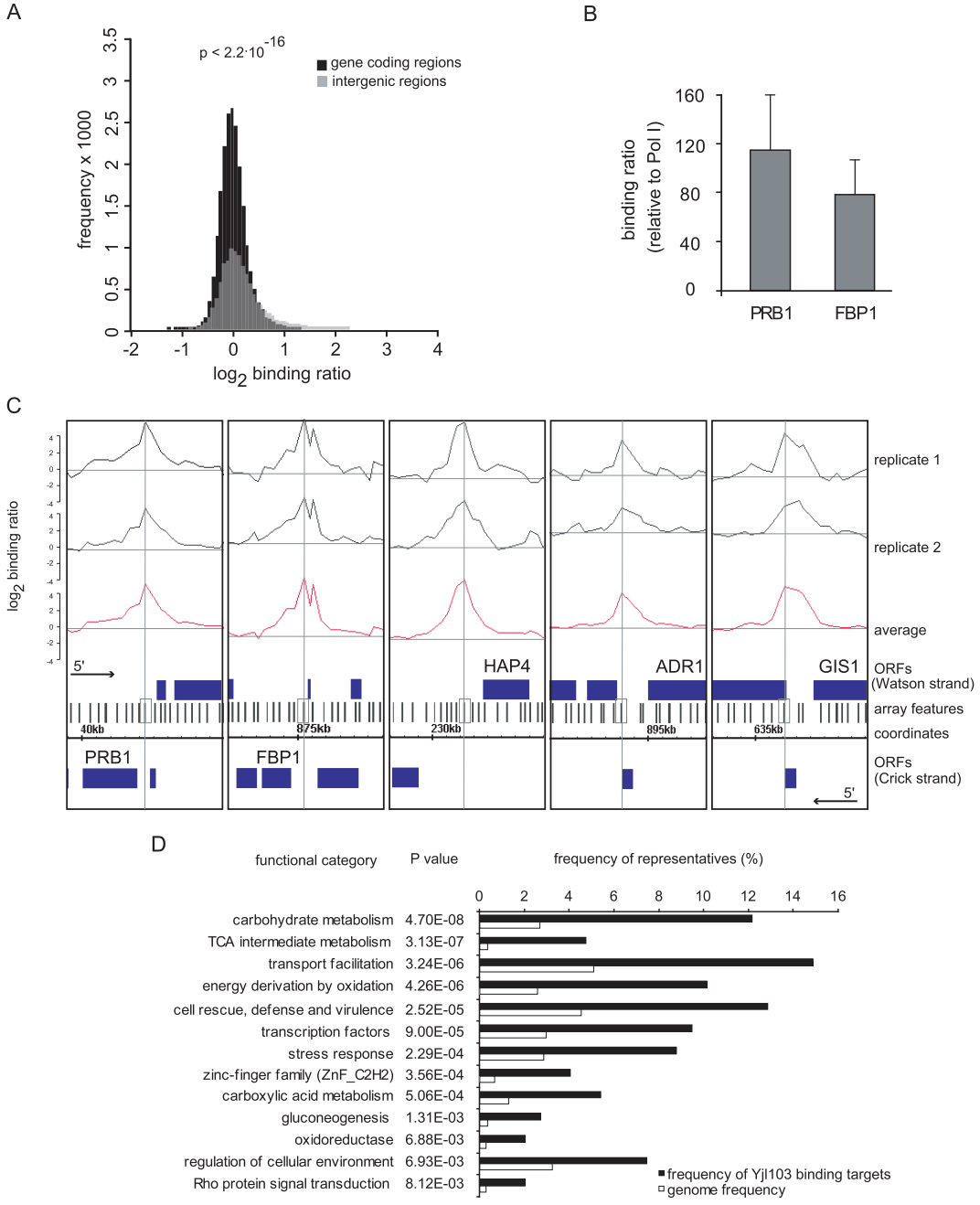


Chapter 4, Figure 1. Yjl103c is expressed upon glucose depletion

(A) mRNA expression profile of *YJL103C* (red) during the entire *S. cerevisiae* growth cycle, including exit from stationary phase (SP), entry into SP and intervening phases. The gray line represents the average expression of all genes. Data are normalized through external controls (Radonjic et al., 2005). The coloured bar at the bottom of graphs illustrates the various culture periods: LP, lag phase; EP, exponential phase; DS, diauxic shift; PD, post-diauxic phase; SP, stationary phase.

(B) Yjl103c protein expression during the exponential and post-exponential growth phases. Western-blot analysis of chromatin enriched protein extracts was performed using an antibody against TAP-tagged Yjl103c. Extracts from equivalent amounts of cells were loaded and this was verified using an antibody specific for the invariant part of Histone H3 (H3). The coloured bar at the bottom of the graphs corresponds to the growth phases described in (A).

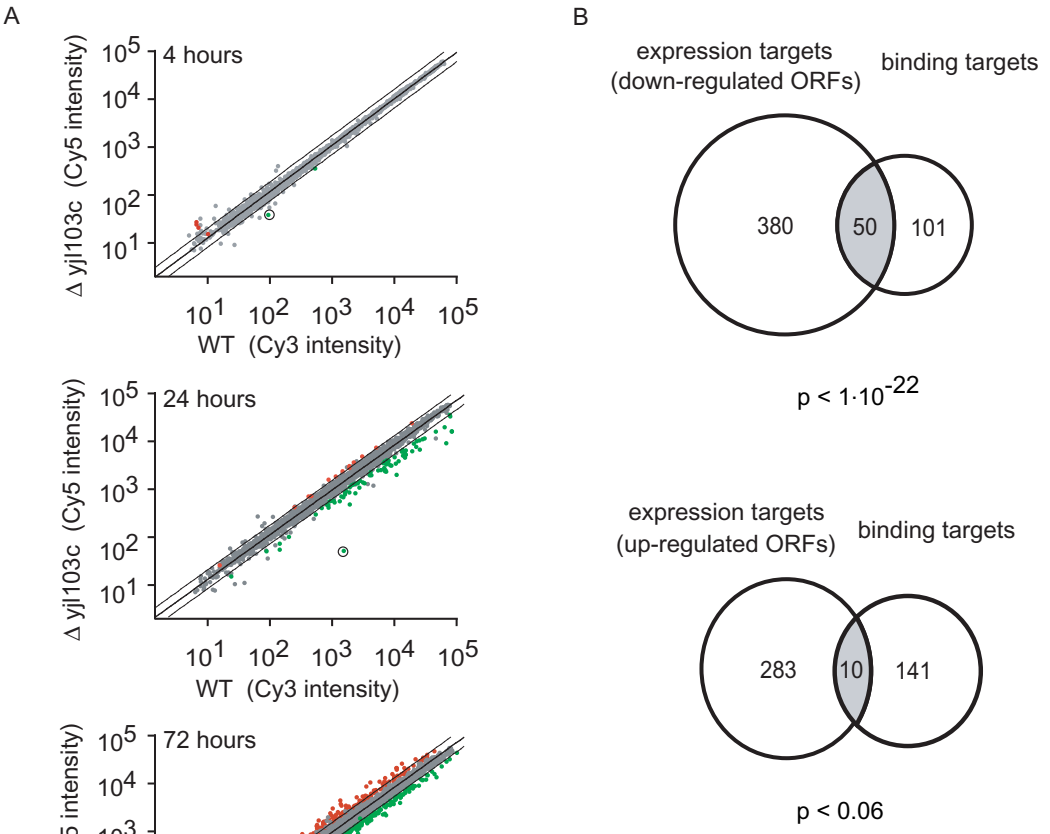
(C) Immunoprecipitation (IP) efficiency of Yjl103c from the chromatin extracts (CE) at the 24 hours time-point (see B). IP efficiency was monitored by western blot analysis comparing 1, 2 and 5% of input (CE) to 10% of IP extracts, and estimated to be approximately 20% of the input.



Chapter 4, Figure 3. Genome-wide location analysis of Yjl103c

(A) Distribution of $\log_2$ binding ratios (BRs) for gene coding regions (GCR) and intergenic regions (IGRs). The shift of IGR binding ratios towards higher values implies preferential binding of Yjl103c to IGRs. The p-value is determined by a t-test that assessed the probability of binding ratios for GCRs and IGRs being equal. After applying the significance cutoff, 257 features are selected as significantly enriched and 192 of those are annotated as intergenic regions.

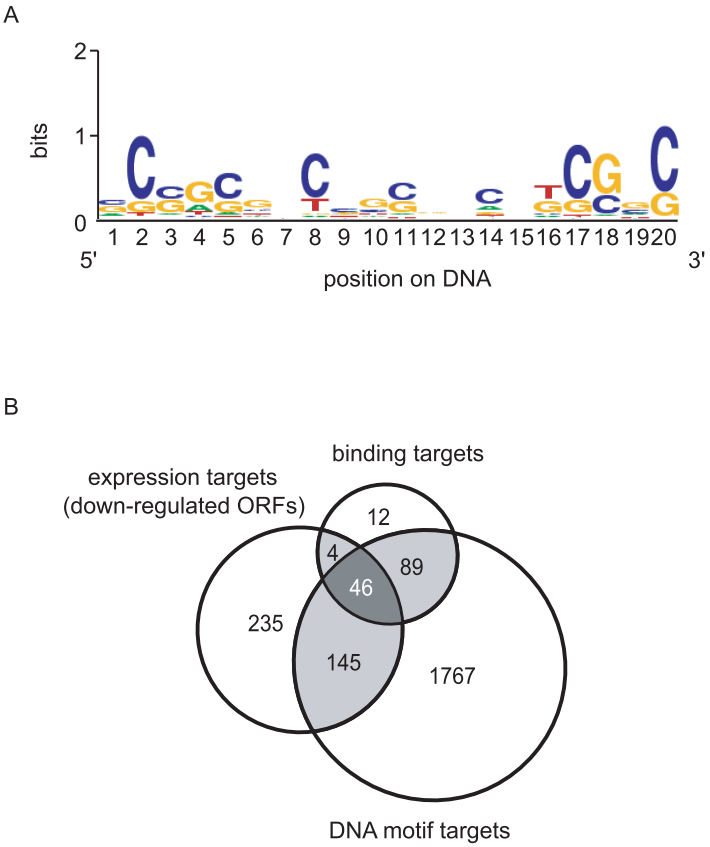
(B) Confirmation of the ChIP on chip results by quantitative real-time PCR (qPCR). Binding ratios (IP / IP Pol I) for ChIP experiments were generated using real time PCR with specific primer sets corresponding to two Yjl103c bound regions (as determined by ChIP on chip), located upstream of *PRB1* and *FBP1*. A *POL1* reaction was performed concomitantly and later used as a relative ratio reference. The values shown are an average of two biological replicate experiments and each of these included 3 replicate PCR reactions performed in parallel and averaged. The standard deviation of values derived from the two biological replicate experiments is shown.
(C) Examples of Yjl103c binding. The binding of Yjl103c is represented equivalently as in Figure 2, with the focus on the 1-kilobase (kb) wide surroundings of the peak of Yjl103c enrichment. The vertical line is drawn to mark the array features with maximal binding ratios. Their corresponding target ORFs are labeled with gene names. The numbers on the horizontal axis represent the location of array features on their corresponding chromosomes.
(D) Functional categories enriched among Yjl103c binding targets. ORFs located downstream of Yjl103c binding sites were analysed for overrepresentation of functional categories derived from MIPS and GO databases. The functional category is shown on the left, followed by the p-value as determined by a hypergeometric test. The black bar indicates how many of the queried genes are assigned to a category and the white bar, how many genes in the genome belong to that category.



Chapter 4, Figure 4. Genome-wide expression changes in the yjl103c deletion mutant

(A) Scatter-plots of average microarray gene spot intensities, comparing *yjl103c* deletion mutant samples (Cy5) with the wild type samples (Cy3) in four time points (4 hours, exponential phase; 24 hours, diauxic shift; 72 hours, post-diauxic phase; 144h, entry into stationary phase). The three lines represent 1.6-fold up, no change and 1.6-fold down boundaries, from top to bottom respectively. Colored in red and green are genes that were determined to show statistically significant mRNA expression changes in the analysis of the Δ*yjl103c* versus wild type replicate measurements per each time-point. The deleted gene, *YJL103c*, is marked with a circle.

(B) Overlap between Yjl103c targets identified by location and expression analyses. Venn diagrams show overlap between ORFs with altered mRNA expression in Δ*yjl103c* in any of the time points and ORFs located downstream of Yjl103c binding sites. The p-values for comparisons of overlapping sets of genes are based on a hypergeometric test with multiple testing correction.



Chapter 4, Figure 6. Yjl103c DNA binding motif

(A) Sequence logo of the DNA motif identified as the most specific among Yjl103c binding sequences. The motif was identified using MEME (Bailey and Elkan, 1994). The relative size of each letter represents the frequency of its appearance on the corresponding DNA position multiplied by the total bits of information.

(B) The overlap of ORFs that contain the motif shown in (A) in their upstream sequences with Yjl103c binding targets and genes downregulated in Δ*yjl103c*.