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Novel Regulatory Properties of *Saccharomyces cerevisiae* Arp4

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***ARP4*, an essential gene of *Saccharomyces cerevisiae*, codes for a nuclear actin-related protein. Arp4 is a subunit of several chromatin-modifying complexes and is known to be involved in the transcriptional regulation in yeast. We used a mutant strain with a single amino acid substitution (G161D) in the conserved actin fold domain to investigate the influence of Arp4 on stress and nitrogen catabolite repression genes. The deficiency of functional Arp4 caused a highly increased sensitivity towards nitrogen starvation and to the macrolide antibiotic rapamycin. We show the changes of mRNA levels of selected genes under these conditions. The upregulation of stress genes as a consequence of treatment with rapamycin was largely Msn2p/Msn4p-dependent. The sensitivity towards rapamycin indicates a participation of Arp4 in the regulation of the TOR pathway. Consistently, a*rp4G161D* cells exhibited an affected cell cycle. Long-term cultivation, which leads to a G1 arrest in wild-type cells, provoked arrest in G2/M (more than 60%) in the mutant strain. The same effect was observed upon treatment with rapamycin, indicating an unexpected relationship of Arp4 to TOR-mediated cell cycle arrest.**

Key words: Arp4, cell cycle arrest, Msn2p/Msn4p, TOR, transcription regulation.

Abbreviations: NuA4, nuclear acetyltransferase complex with predominant specificity to acetylate histone H4; Ino80, a chromatin remodeling complex that contains the Ino80p ATPase, inositol requiring; SWR1, a chromatin remodeling complex that contains Swr1p, a Swi2/Snf2-related ATPase, sick with rat8 ts; Ty, transposon yeast.

Actin-related proteins (Arps) constitute a family of eukaryotic proteins which exhibit similarity to each other and to actin. The three-dimensional structure of rabbit skeletal muscle actin has been investigated earlier (*1*). Examination of the sequences of Arps suggests that they, like actin, most likely possess a common tertiary structure centered around a highly conserved ATP/ADP-binding pocket (*2*, *3*). Ten Arps are known in *Saccharomyces cerevisiae* and they are numbered according to their relatedness to conventional actin, with Arp1 being the most, and Arp10 the least similar (*4*). Arps 1–3 are found in the cytoplasm, Arps 4–9 in the nucleus, and Arp10 in both (*4*, *5*). Arps 1–3 play a role in association with actin (*6–8*). The nuclear Arps are suggested to be involved in the regulation of chromatin structure (*5*, *9*).

ARP4 is an essential gene in *S. cerevisiae*, coding for a protein of 489 amino acids (54, 9 kDa). This protein, Arp4, is known to be constituent of at least three chromatin-modifying complexes: NuA4, a histone acetyltransferase complex (*10*), and the chromatin-remodelling complexes Ino80 (*11*, *12*) and SWR1 (*13*). Arp4 is one of at least 12 components of NuA4 and was suggested to play a role in the integrity of this complex (*10*). The catalytic subunit of NuA4 is the histone acetyltransferase Esa1p, but virtually all constituents of this complex are essential (*10*, *14–16*). Recently, it was demonstrated that Arp4 plays an important role in double-strand break (DSB) repair. By interacting with phosphorylated histone H2A, Arp4 recruits NuA4 to sites of DSBs, where acetylation of histone H4 takes place (*17*).

In *S. cerevisiae*, inactivation of genes can be caused by insertion of a δ-element of Ty (a retrovirus-like transposable element) (*18–20*). Interference is caused by the strong δ-element-internal promoter, which competes with the transcription signals of the adjacent gene. Since mutations in *ARP4* can overcome δ-element inactivation of the *HIS4* gene (*his4–912*), *ARP4* belongs to the SPT (suppressor of Ty) genes. Products of SPT genes are often transcriptional regulators. Indeed, subsequent studies indicated that Arp4 is involved in transcriptional regulation (*21*). In our lab, we investigated the involvement of Arp4 in transcription regulation of stress genes in *S. cerevisiae*. For this purpose, a point mutation (*G161D*) in the highly conserved central region of Arp4 was created, resulting in replacement of glycine 161 with an aspartic acid. Using microarrays, 5,000 genes were analyzed in an *arp4G161D* mutant strain (*3*). Of these, 139 genes were found to be upregulated 2-fold or more, among them stress genes like *HSP12*, *HSP26*, *DDR2*, and *YGP1*. Only 16 genes were detected which were downregulated 2-fold or more. Remarkably, the products of many of these genes are involved in nitrogen and nucleotide metabolism: Cit2p, involved in citrate metabolism and glutamate biosynthesis; Mep2p, an ammonium permease, expressed under nitrogen catabolite repression (NCR) regulation; Ura1p, involved in the *de novo* biosynthesis of pyrimidine ribonucleotides; His4p, involved in the

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superpathway of histidine, purine, and pyrimidine biosynthesis; Gap1p, an amino acid permease regulated by nitrogen source; Glt1p, a glutamate synthase, also regulated by nitrogen source; Gln4p, a glutaminyl-tRNA synthetase; and Zrt1p, a high affinity zinc transport protein. Zinc is important for the nitrogen metabolism, since the four transcription factors Gln3p, Nil1p (Gat1p), Dal80p (Uga43p), and Nil2p share a characteristic zincfinger region, which recognizes the GATA sequences of nitrogen-regulated promoters (reviewed by Refs. *22* and *23*). Nitrogen metabolism is regulated by TOR (target of rapamycin), a central pathway controlling cell growth in response to nutrient availability. Inactivation of TOR kinases 1 and 2, caused by nitrogen starvation or the macrolide antibiotic rapamycin, results in the rapid translocation of Gln3p and Gat1p from the cytoplasm to the nucleus, where they activate transcription of nitrogen catabolite repression (NCR)–sensitive genes (*24*, *25*).

Here, we present a thorough examination of the influence of Arp4 on the transcription regulation of stress genes and NCR genes. We analyzed the impact of Arp4^{G161D} on transcription of a subset of genes comprising the three NCR genes *GLT1*, *MEP2*, and *GAP1*, together with *RPL9A*, which codes for a component of the large (60S) ribosomal subunit, and the stress genes *HSP12*, *HSP26*, and *YGP1*. We report the effects of the Arp4 mutation on the transcription levels of the selected genes following nitrogen starvation or rapamycin treatment. Additionally, we included an *esa1-414* mutant strain in our experiments to compare the effect of mutated Arp4 with that of altered histone acetyltransferase Esa1p. This *esa1-414* mutation, which causes temperature sensitivity, was created by Clarke *et al.* (*15*). Our results reveal the important role of Arp4 in the regulation of transcription and provide evidence for interference with the TOR pathway and cell cycle regulation.

MATERIALS AND METHODS

Saccharomyces cerevisiae Strains and Growth Conditions—The genotypes of all strains used in this study are listed in Table 1. Nitrogen starvation medium consists of 2% glucose and 0.17% yeast nitrogen base without ammonium sulphate and amino acids (Difco; Becton, Dickinson and Company).

Rapamycin medium was prepared as follows: sterile stock solution of rapamycin was added to autoclaved and precooled liquid complete medium (YPD; yeast extract 1%, peptone 2%, glucose 2%) at a final concentration of 100 ng/ml for flow cytometry, survival, 4′,6′-diamidino-2-phenylindole (DAPI) staining, or RNA preparation from liquid cultures, and 5 ng/ml for plates (YPD, 2% agarose), respectively.

Rapamycin treatment for flow cytometry analysis was carried out as follows: exponentially growing cells were incubated in rapamycin medium for 30 min, transferred to fresh YPD for 5 h, then harvested. For rapamycin survival assays, the cells were incubated for 3 h in rapamycin medium, then transferred to YPD for further cultivation. Due to the limited stability of the agent, rapamycin media were always prepared freshly.

Survival—For survival platings, cell cultures were diluted, cell numbers were determined with a hemocytometer (Reichert), and aliquots containing 100 to 250 cells were plated on YPD plates. The number of colonies was determined after two (wild-type strain) or three (*arp4G161D* strain) days at 30°C.

Table 1. **List of *Saccharomyces cerevisiae* strains used in this study.**

Strain	Relevant genotype	Source
K700	*MAT*α	K. Nasmyth
AK314	*MAT***a**/α *ARP4*/Δ*arp4::LEU2*	(*51*)
LK210	*MAT*α *esa1-414*	this study
LK100	*MAT*α *arp4G161D*	(*3*)
LK100A	*MAT*α *arp4G161D* [YCplac111ARP4]	this study
AK329	*MAT*α *arp4::LEU2* [YCplac33ARP4]	(*3*)
IG203	*MAT*α Δ*arp4::LEU2* [YCplac33arp4G161D]	(*3*)
W303 msn2msn4	*MAT*α Δ*msn2::HIS3* Δ*msn4::TRP1*	(*3*)
IG400	*MAT*α *arp4G161D* Δ*msn2::HIS3* Δ*msn4::TRP1*	(*3*)

All strains have the W303-1A genetic background: *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*.

Spot Assays—For spot experiments, strains were grown in liquid YPD, diluted, and plated at tenfold dilution steps onto solid media (20,000, 2,000, 200, and 20 cells, respectively). Pictures were taken after three to five days of incubation at 30°C.

RNA Preparation and Northern Blot Analysis—Cells were grown to a density of 0.6 to 1×10^7 cells/ml or for the indicated number of days in liquid YPD at 25°C. For the nitrogen starvation assay, exponentially growing cells were transferred to the nitrogen starvation medium and incubated for 30, 60 and 120 min before harvesting. Total RNA was extracted from frozen cell pellets using the hot acid phenol method as described by Ausubel *et al*. (*26*). Denatured RNA (15 μg) was electrophoresed in a 1% formaldehyde agarose gel and transferred to nylon membrane (Hybond^{TM}-N$^+$, Amersham). Blotted RNA was crosslinked by UV-irradiation (Stratalinker 1800, Stratagene) and hybridized with fluorescein-labelled probes according to the supplier's protocol (Gene Images CDP-Star detection module and Gene Images random prime labelling module, Amersham). The probes used for hybridization were PCR-amplified from selected regions of the particular genes using K700 chromosomal DNA as template. Following hybridization, the blots were exposed to Kodak Biomax Light film (Amersham). Quantification was performed using the computer programs Quantity One (BioRad) and MatLab (The MathWork, Inc.). All primers used for probes are listed in Table 2.

Flow Cytometry—Flow cytometry analysis was carried out with a FACScalibur flow cytometer (Becton Dickinson) equipped with an aircooled 15 mW argon ion laser system (excitation wavelength 488 nm). Yeast strains were grown in YPD for the time indicated and the following procedures were carried out prior to flow cytometry application: cells were harvested by centrifugation (4°C), washed with distilled water (4°C), and counted. Approximately 1×10^7 cells were suspended in ethanol (70%, –20°C) and incubated overnight at room temperature. The next day the cells were washed twice by centrifugation, resuspended in 1 ml of Na-Citrate (50 mM, pH 7.0), incubated for 1 h at

Table 2. **List of primers used in this study.**

Primer	Sequence
ACT1fw	TTCCCATCTATCGTCGGTAGACCA
ACT1rev	GAAGAAGATTGAGCAGCGGTTTGC
CTT1fw	CTCATCACCCATACGCTTCTCA
CTT1rev	TGAGTGAACAGCTTTGCCTGA
DDR2fw	TCTCACCCCTTATGGGACATCAA
DDR2rev	GGTATCATCATCGTGGCAGTAAGC
GAP1fw	TTGACGAAACAGGTTCAGGGTC
GAP1rev	GCAGAGACAATTTCCAATGGTAGC
GLT1fw	GCCTCTCTTCAAATAGGACTGGA
GLT1rev	GGAATGACACCTACCTCTGAAGCA
HSP12fw	ACAACAATGTCTGACGCAGGTAG
HSP12rev	ACTTCTTGGTTGGGTCTTCTTCAC
HSP26fw	TTAAGAGGCTACGCACCAAGACG
HSP26rev	GGTTCTTACCATCCTTCTGAGGCT
MEP2fw	ACAAACCACATTCCGTCACCTC
MEP2rev	CAATTTGAGCAGCGTCGGTA
RPL9Afw	CAGAAGGTGTCACTGTCAGCATC
RPL9Arev	CCTTGTGAGAAACGTAGATACCGT
YGP1fw	CCTGGAATGGGTCTAACTCTAGCA
YGP1rev	ACCAAACCTTGAACGGCAGAAC

37°C in RNAse A (final concentration: 200 μg/ml in Na-Citrate), then for 1 h at 50°C in proteinase K (final concentration: 1 mg/ml in Na-Citrate). Subsequently the cells were centrifuged, resuspended in 1 ml of Na-Citrate, vortexed for 30 s, and finally incubated overnight in 1 μM SYTOX Green nucleic acid stain (S-7020, Molecular Probes). Data were analyzed using the computer program Windows multiple document interface flow cytometry application (WinMDI; J. Trotter, Salk Institute for Biological Studies, San Diego, CA, USA).

DAPI Staining of Yeast—A 1-ml portion of each culture was harvested (microtube centrifuge, 3,000 rpm for 1 min at room temperature), the pellet was resuspended in 1 ml of H_2O bidest, again harvested, and the pellet resupended in 1 ml of ethanol (70%) and incubated for 20 min. The cells were again harvested, the pellet resuspended in 1 ml of H_2O bidest, then centrifuged at $10{,}000 \times g$ (1 min at room temperature), and the pellet resuspended in 1 ml of H_2O bidest. Approximately $1 \times 10^7 - 1 \times 10^8$ cells were incubated with 0.5 μg/ml DAPI (Roche) and used for fluorescence microscopy.

RESULTS AND DISCUSSION

Transcription Regulation—The present data imply that Arp4 functions in both activation and repression of gene transcription. As a component of the histone acetyltransferase complex NuA4, it is involved in the stimulation of transcription via the acetylation of the N-terminal tails of histone H2A and H4 (*10*, *14*, *15*). Previous experiments revealed a decreased basal transcription of some genes in an *arp4* ts mutant (*10*, *21*). Recently, we created a mutant strain expressing $Arp4^{G161D}$, where a single amino acid (glycine 161) was changed to aspartic acid. This residue is highly conserved and is situated in the core region of proteins of the actin family and related proteins. The *G161D* mutation resulted in a temperature-dependent decay of Arp4, probably due to incorrect folding of the protein. At the permissive temperature of 30°C, only a moderate reduction of the Arp4 pool was observed, still enabling maintainance of viability. Microarray gene expression analysis of this mutant strain revealed that 139 of the 5,000 genes studied were upregulated 2-fold or more, among them many stress genes, and only 16 genes were downregulated 2-fold or more, several of them involved in the nitrogen metabolism (*3*).

To gain more detailed data on the effect of Arp4 on gene regulation, we extracted RNA of three haploid yeast strains [K700 (wild-type), LK100 (*arp4G161D*), LK210 (*esa1-414*)], grown in liquid YPD medium, after a shift to nitrogen starvation medium or after treatment with rapamycin (100 ng/ml). We compared the mRNA levels of three nitrogen-regulated genes (*GLT1, GAP1, MEP2*), two stress genes (*HSP12, YGP1*), and *RPL9A*, coding for a protein of the large (60S) ribosomal subunit. We obtained similar results from experiments with cells undergoing nitrogen starvation or rapamycin treatment, respectively. Fig. 1 (A and B) shows the results prior to, and after 30, 60, and 120 min of nitrogen starvation. In wild-type cells, the transcription of *GLT1*, coding for a glutamate synthase, was downregulated after 30 min of nitrogen starvation, returning to the value of non-stressed cells after 120 min. *GAP1*, coding for a general amino acid permease, *MEP2*, coding for an ammonium permease, and the stress genes *HSP12* and *YGP1* were upregulated after 30 min of nitrogen starvation. Further incubation led to a decrease of *GAP1, HSP12*, and *YGP1*, whereas the expression level of *MEP2* remained high. The ribosomal protein *RPL9A* was dramatically downregulated to a very low level already after 30 min of nitrogen starvation.

Mutation of *ESA1* did not cause significant changes in the pattern of mRNA levels of the genes investigated. The results obtained from *esa1-414* mutant cells were roughly similar to those from wild-type cells, the stress genes (*HSP12, YGP1*) being slightly elevated.

Mutation of *ARP4* on the other hand, triggered substantial changes in the transcription levels of some genes. The most striking differences were observed for the stress genes. In agreement with previous observations (*3*), transcription of *HSP12* and *YGP1* in *arp4G161D* mutant cells was highly induced already under normal growth conditions. Transcription of *RPL9A* and *GLT1* was downregulated in these cells, the latter one even to approximately 50% compared with the wild-type strain. The amount of *GAP1* and *MEP2* mRNA was slightly elevated compared to the wild-type strain. Upon nitrogen starvation, transcription of the stress genes *HSP12* and *YGP1* was additionally increased in the *arp4G161D* mutant strain. *GAP1* and *MEP2* were also upregulated, *GAP1* similarly to the wild-type strain, *MEP2* to a clearly lesser extent. The level of *GLT1* remained unchanged at the decreased level, even after 120 min of nitrogen starvation. *RPL9A* was downregulated comparably to the wild-type cells.

Gorzer *et al.* (*3*) have described that the elevated transcription of certain stress genes (among them *YGP1* and *HSP12*) in *arp4G161D* cells is not just part of an unspecific stress response. Our data, obtained from Northern blot experiments, evidence that Arp4 is involved in transcription regulation of NCR and stress genes. The analyzed stress genes were extensively upregulated as a consequence of the $Arp4^{G161D}$ mutation. The NCR genes were

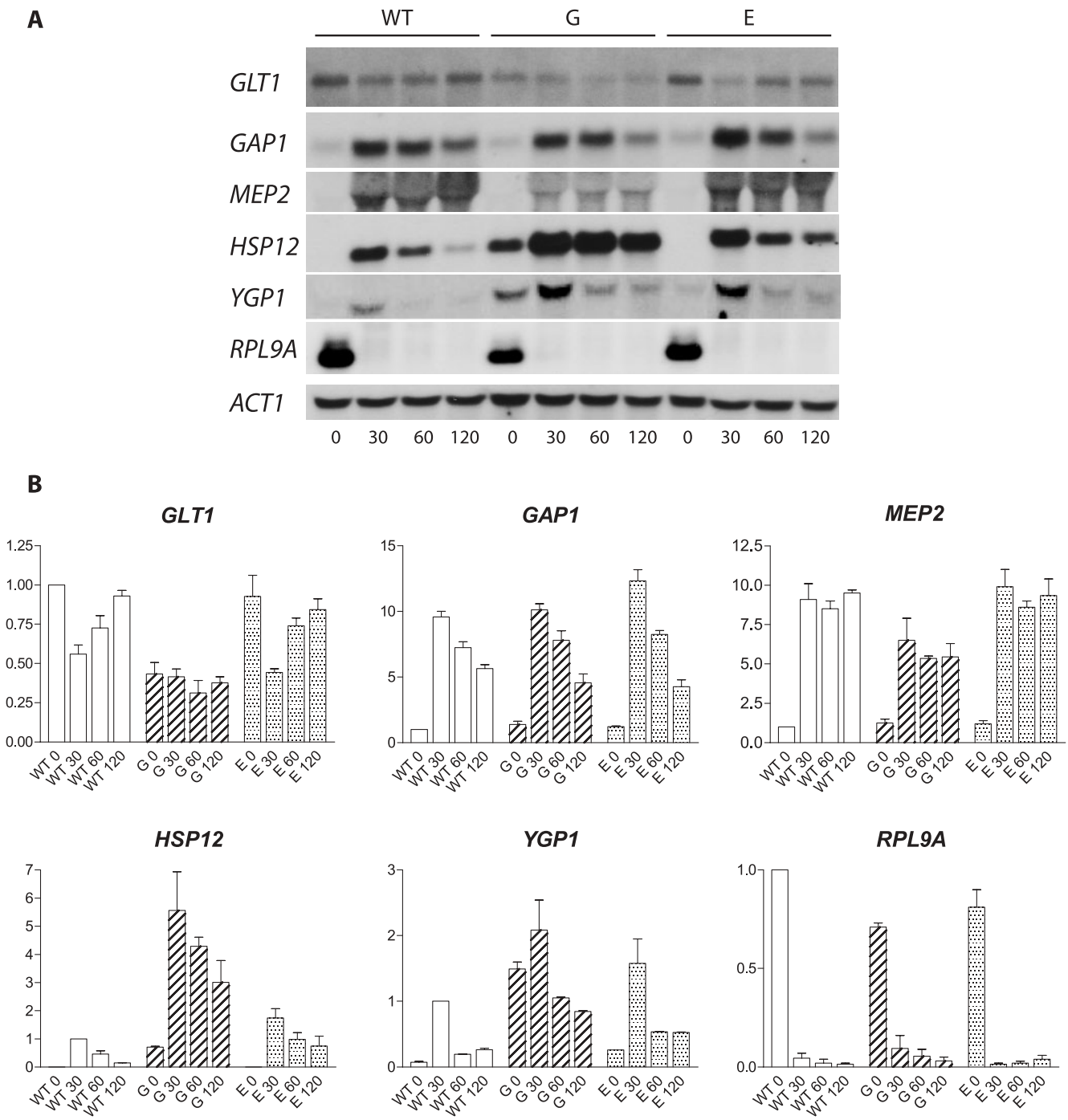

Fig. 1. **mRNA levels of selected genes in the wild-type (WT), and *arp4G161D*(G) and *esa1-414*(E) mutant strains**. (A) Northern blots (15 μg of RNA per lane) were probed with DNA fragments of the genes indicated. RNA was prepared from cells of the three strains under normal growth conditions (0) and after 30, 60 , and 120 min of nitrogen starvation (30, 60, and 120, respectively). Actin (*ACT1*) was used as an internal control. (B) Quantification of the Northern blots. At least three independent experiments were used for each probe to relatively quantify the mRNA levels. For the nitrogen-dependent genes (*GLT1, GAP1, MEP2*) and RPL9A, the mRNA levels of untreated cells of the wild-type strain were set to 1. For the stress genes (*HSP12, YGP1*), the mRNA levels of wild-type cells treated for 30 min with rapamycin were set to 1 (due to the lack of expression of *HSP12* in untreated cells).

affected in different ways: downregulated (*GLT1*), more or less unaffected (*GAP1*), or less upregulated upon nitrogen starvation (*MEP2*). However, unlike the stress genes, no upregulation of NCR genes was evident in *G161D* cells in comparison to wild type cells under the same conditions. We propose that the impact of native Arp4 on the regulation of transcription occurs in at least two different ways: a more general mechanism for the regulation of stress genes, and a rather individual regulation of several other genes, among them some NCR genes.

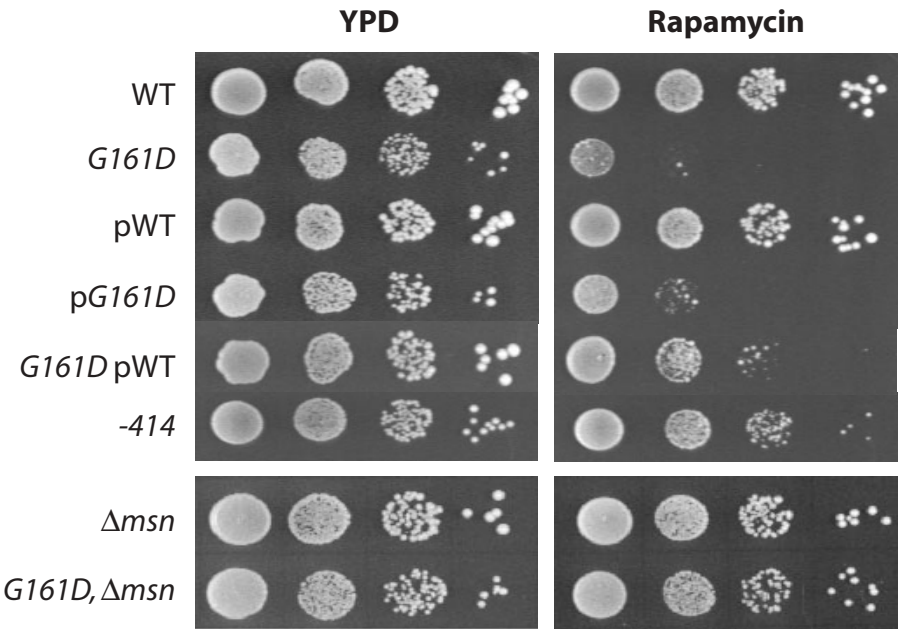

Fig. 2. **Phenotypic analysis of *ARP4* and *ESA1* mutant alleles.** Cultures were tenfold serially diluted on YPD and rapamycin (5 ng/ml) plates, respectively, and subsequently incubated at 25°C for three days. (WT) wild-type strain K700, (*G161D*) *arp4G161D*, (pWT) *arp4::LEU2* with *ARP4* on plasmid YCplac33, (pG161D) *arp4::LEU2* with *arp4G161D* on plasmid YCplac33, (*G161D* pWT) *arp4G161D* with *ARP4* on plasmid YCplac111, (*–414*) *esa1–414*, (Δ*msn*) W303 Δ*msn2::HIS3* Δ*msn4::TRP1*, (*G161D*, Δ*msn*) W303 *arp4G161D* Δ*msn2::HIS3* Δ*msn4::TRP1*; the complete genotypes of the strains are listed in Table 1. No effect of this rapamycin concentration was evident for (WT) and (Δmsn), and only a slight growth effect was visible for (*–414*). Note the severe growth inhibition caused by rapamycin of strains that exclusively possess mutated Arp4 (*G161D* and p*G161D*). The wild-type phenotype of the Arp4^{G161D} strain could be restored by knockout of Msn2p/Msn4p (*G161D*, Δ*msn*) or partially by means of plasmid-encoded Arp4 (*G161D* pWT).

The arp4G161D Mutation Causes Hypersensitivity to Rapamycin—Considering the role of Arp4 in the regulation of NCR and stress gene transcription, we were interested to learn if the sensitivity to rapamycin was changed by the mutation Arp4^{G161D}. Rapamycin, first derived from *Streptomyces hygroscopicus* isolated on Easter Island (Rapa Nui), is able to form a complex with a small 12-kDa peptidyl-prolyl isomerase, FKBP/Rbp1. This complex is a potent inhibitor of the activity of TOR kinases 1 and 2 (*27*). Treatment with rapamycin, as well as a shift to a medium with proline as a source of nitrogen, results in the dephosphorylation of Gln3p phosphate and its translocation to the nucleus.

For our experiments we used haploid *S. cerevisiae* strains bearing *arp4G161D* or *esa1-414* on the chromosome, replacing the respective wild-type gene. Spotted onto solid YPD, all strains exhibited similar growth after three days at 25°C (Fig. 2). However, if the medium contained 5 ng/ml rapamycin, strains expressing Arp4^{G161D} clearly showed inhibited growth, suggesting that Arp4 plays a role in the TOR signalling pathway. In contrast, cells expressing Esa1p^{-414} exhibited only a slight growth impairment, and the wild-type strain was not affected by this concentration of rapamycin. *arp4G161D* cells, transformed with *ARP4* on a centromeric plasmid (*G161D* pWT), displayed an intermediate growth phenotype between wild-type (WT) and *arp4G161D* (G161D) cells. Surprisingly, these data suggest that the wild-type version of Arp4 is not fully dominant. In contrast, Δ*arp4* cells, as well transformed with *ARP4* on a centromeric plasmid (pWT), were resistant to 5 ng/ml rapamycin, restoring the wild-type phenotype. The lack of dominance of the episomal *ARP4* wild-type allele is hardly consistent with the interpretation of Gorzer *et al.* (2003), that the G161D mutation does not have an effect *"per se"* but rather leads to a deprivation of functional Arp4, and requires further clarification.

The importance of gene dosage was marginal. Cells bearing *arp4G161D* on a centromeric plasmid, which is present in one to three copies per cell, in a Δ*arp4* genetic background, grew only slightly better than those carrying the chromosomal version (Fig. 2).

Due to the effect of Arp4^{G161D} on the transcription of stress genes, we presumed that the increased rapamycin sensitivity could be Msn2p/Msn4p-dependent. We used strain IG 400, a haploid strain that expresses Arp4^{G161D}, but lacks Msn2p and Msn4p, to test our hypothesis. Growth of this strain, just like the *ARP4* control strain W303msn2msn4 (lacking both Msn proteins, but expressing Arp4), remained unaffected by 5 ng/ml rapamycin (Fig. 2). Thus, the increased rapamycin-sensitivity of *arp4G161D* cells is indeed Msn2p/Msn4p dependent. To analyze this correlation in more detail, we compared the expression of selected genes between the Msn2p/Msn4p-proficient and -deficient *arp4G161D* strains. Although the transcription of stress genes in *arp4G161D* cells was already highly increased under non-stress conditions, a further enhancement occurred upon nitrogen starvation (Fig. 1, A and B) or rapamycin treatment (Fig. 3). In the *msn2*Δ/*msn4*Δ genetic background, the induction of stress genes (*CTT1, DDR2, HSP12, HSP26, YGP1*) after treatment with rapamycin was abolished or dramatically reduced (Fig. 3). Thus, similar to the effect of osmotic stress (*3*), the upregulation of stress genes following rapamycin treatment was Msn2p/Msn4p-dependent. These transcription factors are involved in the activation of stress-inducible genes (*28, 29*) and regulated by the TOR and the Ras cAMP pathways (*30*). However, the transcription of stress genes in the mutant cells was not exclusively dependent on Msn2p/Msn4p. As is apparent from *HSP12*

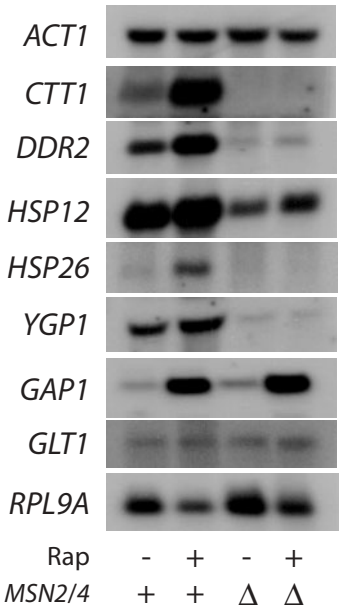

Fig. 3. **Stress gene transcription activation is Msn2p/Msn4p-dependent.** mRNA levels of selected genes in *arp4G161D* cells expressing or lacking Msn2p and Msn4p are shown. We compared cells treated with rapamycin (Rap +), or untreated (Rap –). Total RNA of the cells (15 μg per lane) was analyzed with probes of the genes indicated. Basal as well as rapamycin-induced transcription of stress genes was dependent of the availability of Msn2p and Msn4p.

(Fig. 3), other transcription factors may also be involved, which act in the absence of Msn2p/Msn4p, for example, Hsfp1 (*31, 32*).

The dramatically enhanced sensitivity of *arp4G161D* cells towards rapamycin indicates that Arp4 exerts influence on the TOR pathway. As is evident from Fig. 3, the mRNA levels of the nitrogen-regulated genes (*GAP1, GLT1*), as well as the ribosomal gene *RPL9A*, and *ACT1*, which was used as a control, were not affected by the absence of Msn2p/Msn4p. Thus, the impact of Arp4 on the transcription of NCR genes seems not to be mediated by Msn2p/Msn4p. Arp4 might be recruited directly to the respective promoter regions. Besides its interaction with histone H2A (*33*), Arp4 has been shown to bind to the his4–912delta promoter region (*34*). The ability of Arp4 to bind to the promoter was altered by single amino acid substitutions G187R and G455S, both located within the actin fold, just as is G161D (*3, 4*).

Affected Entry into Stationary Phase—During exponential growth on rich media (log phase), *S. cerevisiae* grows by fermentation of the available glucose. When glucose becomes limiting, the cells switch to a respiratory mode of energy production ("diauxic shift"). During the subsequent post-diauxic growth period, the cells grow slowly on the ethanol that was produced during the previous period of fermentation. When this ethanol is finally exhausted, the cells enter the stationary phase (reviewed in Ref. *35*). Although no specific molecular markers for the stationary phase exist, it is known that, while the overall level of mRNA is decreased, some genes are temporarily transcriptionally induced upon diauxic shift or in early stationary phase. Two of them are the genes coding for the stress-responsive heat shock proteins Hsp26 and Hsp12 (*36–38*). We analyzed the mRNA levels of several genes (*HSP12, HSP26, DDR2, YGP1, RPL9A, GAP1, GLT1, ACT1*) in the wild-type and the *arp4G161D* mutant strain during protracted cultivation over a period of seven days (Fig. 4, A and B). In wild-type cells, transcription of the stress-related genes (*HSP12, HSP26, DDR2, YGP1*) reached a maximum at day 2, subsequently declining rapidly, whereas transcription of *GAP1, GLT1, RPL9A* and *ACT1* was largely confined to the logarithmic phase.

In *arp4G161D* cells, however, the situation was more diverse. Except for the wild-type–like mRNA levels of *RPL9A* and *GLT1*, transcription of all other genes studied was remarkably different. The amount of *ACT1* mRNA was initially equal in logarithmic mutant and wild-type cells. During further incubation, *ACT1* was downregulated only very slowly in *arp4G161D* cells, in contrast to wild-type cells, where the transcription level was almost zero at day 2. The same was true for *GAP1*, where the level of mRNA in the mutant strain was already elevated roughly twice in logarithmic cells, and increased to a peak at day 2. Similarly, transcription of all stress genes examined was elevated throughout the days following logarithmic phase, although in a varying manner for the different genes (Fig. 4, A and B). In summary, these results show that upregulation of some genes upon diauxic shift and early stationary phase occurs in both strains. Only in *arp4G161D* mutant cells were the mRNA levels of the stress genes and *GAP1* dramatically elevated, and the expected relatively prompt downregulation (*39*) was not observed.

From these results, we suspected that at least a fraction of the *arp4G161D* mutant cells failed to arrest and did not enter stationary phase. Wild-type cells under conditions of lacking nutrients should not proceed to START, the key regulatory transition point in the late G1 phase, but instead exit the mitotic cycle in early G1 phase and enter a stationary G0 phase (*40*), reviewed in (*41*). If our hypothesis was correct, the *arp4G161D* mutant cells should be clearly distinguishable from the wild-type cells in this respect. We examined both types of cells, from logarithmic phase and cultures up to seven days old, using flow cytometry, as well as light and fluorescence microscopy (Figs. 5 and 6). In addition, we determined the cell viability of both strains, untreated and pretreated with rapamycin, respectively (Fig. 7).

In fact, morphological differences were visible. *arp4G161D* mutant cells were more frequently irregularly shaped, damaged, or unusually small or large (Figs. 5 and 6). Analysis of the DNA content by flow cytometry showed the expected result in the wild-type strain: cells in all phases of the cell cycle during log phase, followed by G1 arrest of cells from two, five, and seven days old cultures. In *arp4G161D* mutant cells from logarithmic phase cultures, the relative amount of cells in G2/M phase was elevated compared to the wild-type cells. However, within the next seven days the portion of cells in G2/M phase further increased, indicating that at least some of these cells were not arrested in the G1/G0 phase (Fig. 5). This belief was corroborated by calculating the relative percentages of cells in G1, G2/M, and S phase (Table 3). In the observed period following the logarithmic phase, the relative amount of G2/M cells was more or less zero in the wild-type

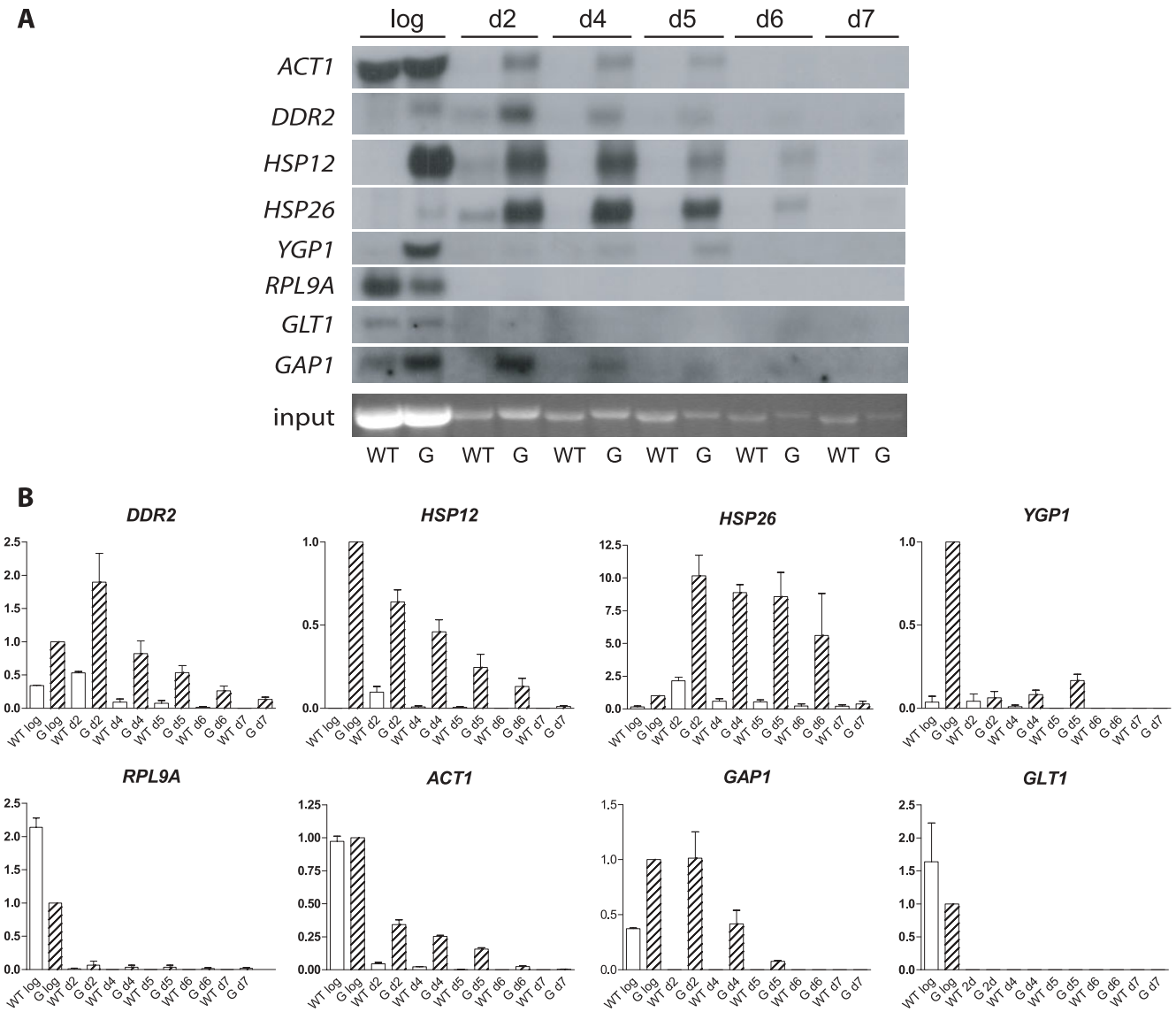

Fig. 4. **Time-course of the expression of *RPL9A,* NCR (*GLT1, GAP1*) and stress (*DDR2, HSP12, HSP26, YGP1*) genes in wild-type (WT) and *arp4G161D* mutant cells (G) during long-term cultivation.** (A) Northern blots (15 μg of total RNA per lane) were probed with DNA fragments of the genes indicated. Actin (*ACT1*) was used as an internal control. RNA of both strains was prepared from cells in the logarithmic phase (log) and cultures grown for up to seven days (d2, d4, d5, d6, d7). Control: 26S rRNA (input); (B) Quantification of the northern blots; at least three independent experiments were used for each probe to relatively quantify the mRNA levels. For all genes the mRNA levels of the cells of the *arp4G161D* mutant strain from logarithmic phase were set to 1.

strain, but around 60% in the mutant strain. We also tried to arrest cells of both strains with rapamycin (100 ng/ml). As expected (*27*), the wild-type cells were arrested in G1 phase. On the other hand, approximately 50% of the *arp4G161D* cells were in G1, and more than 40% in G2/M phase (Table 3). The high ratio of mutant cells in G2/M phase might be explained by a certain portion of cells arresting in G2 phase or mitosis, instead of G1. Theoretically, the apparent amount of cells in G2/M phase could be artificially increased by cells containing two nuclei or by cells with nonseparated daughter cells, a phenotype which occurred as a result of rapamycin treatment, as described below. However, as a result of this, only minor deviations could arise, since the number of cells with two nuclei was not high enough, and cells with several buds—as an effect of rapamycin treatment—similarly occurred in the wild-type strain, where the amount of cells in G2/M phase was low in logarithmic cultures and evanescent after long term cultivation.

One of the distinct properties of cells entering G0 is a 1n DNA content. Our results indicate that *arp4G161D* cells do not enter cell cycle arrest comparably to the wild-type cells. In this respect it is interesting that Minoda *et al.* (*42*) reported that a single amino acid substitution (S402N) in the conserved C-terminal domain of Alp5, the fission yeast homolog of Arp4, caused mitotic arrest. We observed that in the mutant strain the DNA content, determined by flow cytometry, did not shift towards 1n (Fig. 5). In contrast

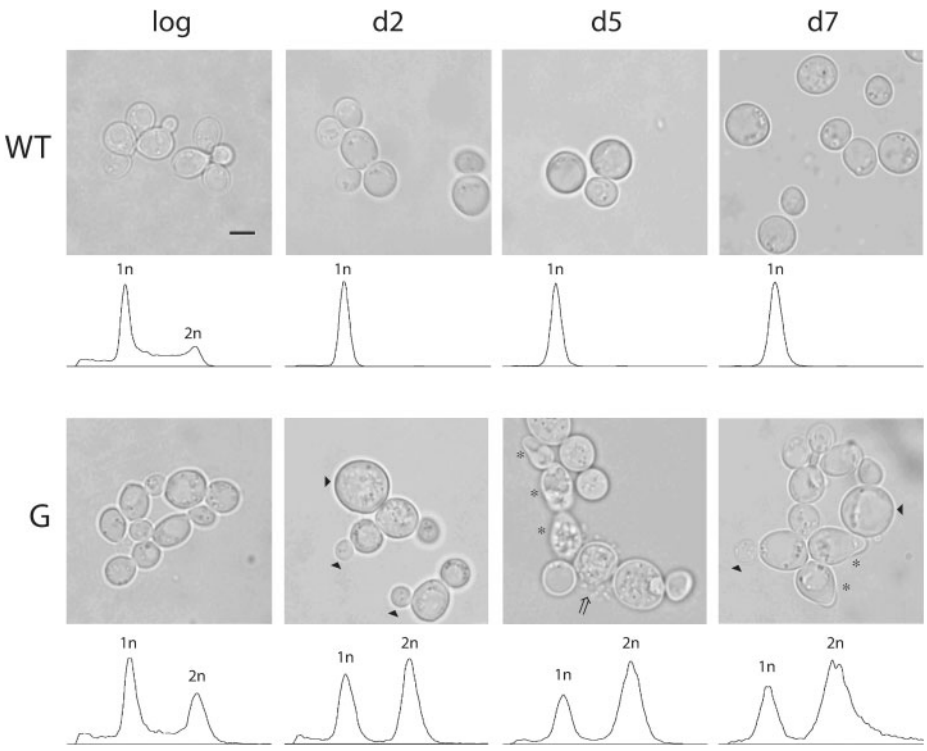

Fig. 5. **Microscopic images of wild-type (WT) and *arp4G161D* mutant cells (G) during logarithmic phase (log) or after long-term cultivation for two (2d), five (5d), or seven (7d) days.** Mutant cells are more often irregularly shaped (asterisks), damaged (arrows), or of unusual size (arrowheads). The corresponding flow cytometry histograms (FL1-A) are shown below. 1n and 2n refer to DNA content. Bar: 5 μm.

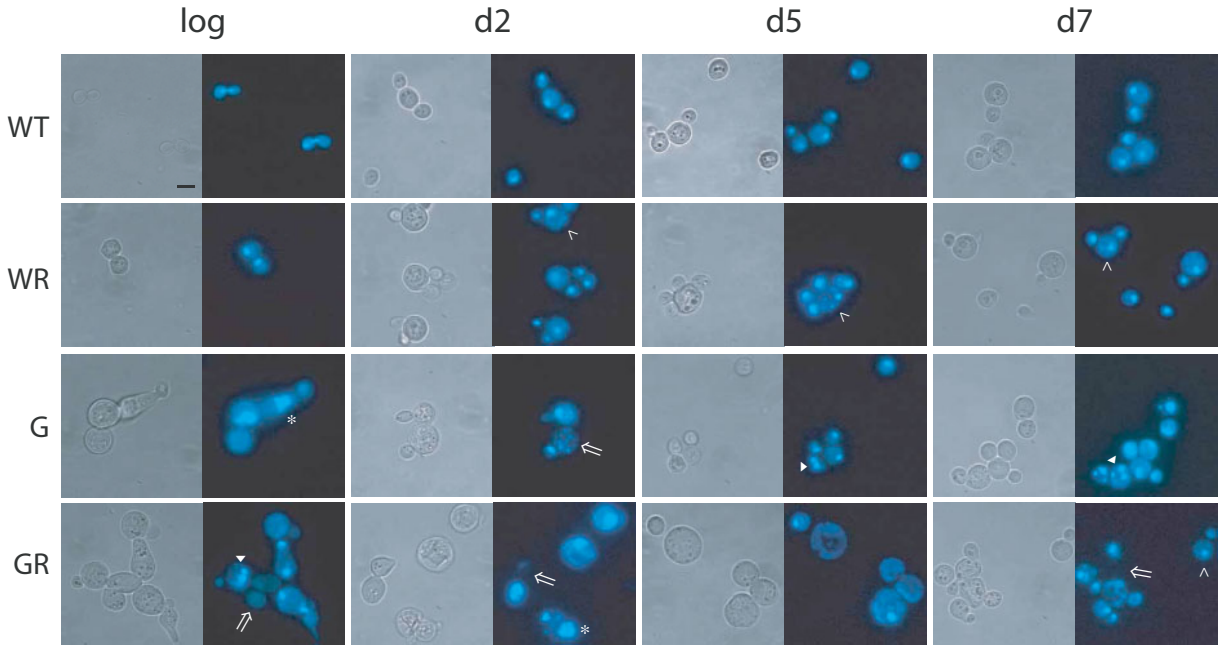

Fig. 6. **Wild-type and *arp4G161D* mutant cells, untreated (WT and G, respectively) or pretreated with rapamycin (WR and GR, respectively), during logarithmic phase (log) or after long-term cultivation for two (2d), five (5d), or seven (7d) days.** The microscopic appearance of the cells is shown in the left images, and DAPI DNA-staining of the same fields of cells is shown in the right images. Rapamycin pretreatment (WR, GR) causes a phenotype of cells with one, two, or more relatively large buds, which obviously can not be separated from the mother cell (angles). Several *arp4G161D* cells (G, GR) exhibit misarrangement of the nucleic DNA: extensive irregular nuclei (asterisks), cells without nuclei (arrows), or with two nuclei (arrowheads). Bar: 5 μm.

to the wild type cells, a certain amount of *arp4G161D* mutant cells still passes through S phase after 2 and 5 days of cultivation (around 14% and 4%, respectively), indicating that at least these cells are not arrested in G1/G0 (Table 3). Throughout protracted cultivation, the portion of cells in G1 remained constant, whereas the amount of cells in G2/M increased commensurately with the decrease of cells in S phase. This suggests that *arp4G161D* mutant

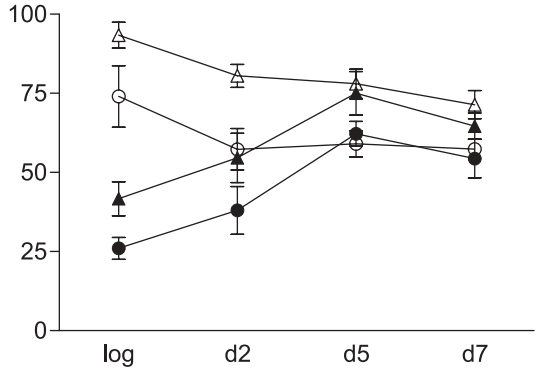

Fig. 7. **Viability assay of wild-type (triangles) and *arp4G161D* mutant cells (circles) sampled from logarithmic cultures (log), or after long-term cultivation for 2, 5, or 7 days (2d, 5d, and 7d, respectively).** Cells were grown in YPD at 30°C, either untreated (empty symbols) or pretreated with rapamycin (full symbols). The survival of the mutant strain is clearly decreased compared with the wild-type strain. Pretreatment with rapamycin (3 h, 100 ng/ml, for details see 'MATERIAL AND METHODS') causes significantly lower survival rates for logarithmic cells of both strains. The survival rates are increased at day 2, and after 5 days the cells have overcome the effect of rapamycin and the survival is back to the level of untreated cells. Data represent the mean ± SEM of six experiments.

Table 3. **Relative amounts of G1-, S-, and G2/M-phase cells (%).** Flow cytometry of wild-type (WT) and arp4G161D mutant cells (G) from logarithmic phase, untreated (log), or treated with rapamycin (log+rap), and from cultures grown for up to seven days (d2, d5, d7).

	log	log+rap	d2	d5	d7
WT					
G1	44.0	96.3	99.2	99.0	98.7
S	47.0	1.9	0.4	1.0	1.3
G2/M	9.0	1.8	0.3	0	0
G					
G1	31.9	50.8	31.3	31.5	32.1
S	35.3	5.9	13.7	4.2	1.0
G2/M	32.8	43.3	55.2	64.3	66.9

cells either arrest in G1/G0 phase or in G2/M phase. Arrest in G2 or mitosis is probably at least partly retarded, most likely after a temporary arrest in the S phase.

DNA staining with DAPI revealed that the *arp4G161D* mutant strain has a problem with the regular distribution of the nuclear DNA. Beginning in the logarithmic phase, and increasing in older cultures, a fraction of the mutant cells exhibited somehow tattered chromosomal DNA instead of a homogenous round nucleus. Moreover, occasionally cells occurred that had no nucleus, whereas others had two (Fig. 6). These phenomena were independent of rapamycin treatment, although the frequency was slightly elevated in treated cells. However, the phenotypic effect of rapamycin, visible in both strains, was a frequently observed phenotype of large round cells with one, two, or more relatively large buds, obviously not able to be abscised (Fig. 6).

We also determined the cell viability for both the wild-type and the *arp4G161D* mutant strain. Not unexpectedly, considering the problems with the distribution of the nuclear DNA, the mutant strain displayed decreased viability (Fig. 7). More than 90% of the logarithmic wild-type cells were viable, and around 70% were still viable after seven days. The survival of mutant cells was approximately 75% for logarithmic cultures, decreasing to roughly 55% for cultures grown for up to seven days. Rapamycin-pretreated cells of both the wild-type and the mutant strain showed an initially significantly decreased viability (about 40% and 25%, respectively). In the course of further cultivation, cells exited rapamycin-induced quiescence and the survival rates increased to levels similar to those of untreated cells at days 5 and 7 (Fig. 7). The mechanisms that control the transition from quiescence back to proliferation are poorly understood and have received surprisingly little attention thus far. Only few publications, Gray *et al.* (*43*) and Dubouloz *et al.* (*44*), have dealt with this subject. Nevertheless, the re-adjustment of rapamycin-pretreated cells was obviously not affected by the *arp4G161D* mutation. Although on a lower level, the increase of the survival rate back to the level of untreated cells occurred correspondingly to the wild-type strain.

The cell cycle can be arrested at multiple points. From our results, we assume that the cells which do not exit the cell cycle in G1, arrest in G2 or mitosis, at least some of them following an afore arrested S phase. The mode of action of *arp4G161D* remains unclear. We can not rule out a yet unknown direct interaction of Arp4 with a cell cycle–regulating protein. Nevertheless, we consider it more likely that the observed effect of mutated Arp4 takes place via influence on the TOR pathway. It is known that cells lacking only the TOR2-unique function are defective in actin cytoskeleton organization and arrest in the G2/M phase of the cell cycle (*45*). Furthermore, it is known that the TOR and the Ras/cAMP signalling pathways regulate cell growth. An increasing amount of evidence suggests that these two major pathways in yeast are linked. Schmelzle *et al.* (*30*) present a model in which TOR signalling bifurcates into two separate effector pathways (TAP42/SIT4 and Ras/cAMP, PKA). The yeast TOR1 and TOR2 proteins control translation and cell cycle progression in response to nutrients (*46–50*). The irregular size and shape of the *arp4G161D* mutant cells we observed also supports a model in which Arp4 is connected with the TOR pathway.

Together, our results support the view of Arp4 as a multifunctional protein. *Via* its role in the chromatin-modifying complexes, it unspecifically mediates the transcription of many genes. Beyond it, Arp4 has an impact on the TOR pathway, thereby regulating—mainly Msn2p/Msn4p-dependently—the transcription of stress genes. Furthermore, the impact of $Arp4^{G161D}$ on the TOR pathway probably has more consequences: irregular growth of the cells and influence on the cell cycle, resulting in a G2/M instead of a G1/G0 arrest upon starvation or rapamycin treatment. The mode of interaction of Arp4 with the TOR pathway is not yet clarified. Currently we do not know at which step of the TOR pathway Arp4 takes effect. The illustration of this and the identification of interacting promoter regions will be rewarding tasks for further investigations.

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