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The Nuclear Actin-Related Protein Act3p/Arp4 Influences Yeast Cell Shape and Bulk Chromatin Organization

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Abstract *ACT3/ARP4* is an essential gene, coding for the actin-related protein Act3p/Arp4 of *Saccharomyces cerevisiae* located within the nucleus. Act3p/Arp4 is a stoichiometric component of the NuA4, INO80, and SWR1 chromatin modulating complexes, and recruits these complexes onto chromatin for their proper chromatin functions. Mutated Act3p/Arp4 leads to impairment of the functions of these complexes and affects transcription of specific genes. Our results revealed significant disorder in the cell size and shape of *act3/arp4* mutant cells, when grown at permissive temperature. *act3/arp4* mutants have also demonstrated an increase in their nuclear diameters, thus suggesting that Act3p/Arp4 is a key regulator in the maintenance of cellular shape and nuclear organization. Furthermore, the use of Chromatin Yeast Comet Assay (ChYCA) for assessment of single-cell bulk chromatin organization in *act3/arp4* mutant cells allowed us to detect an elevated sensitivity toward nuclease action, denoting differences in higher-order chromatin structure of the mutants. J. Cell. Biochem. 104: 59–67, 2008. © 2007 Wiley-Liss, Inc.

Key words: *Saccharomyces cerevisiae*; *ACT3/ARP4*; chromatin; FACS; yeast comet assay

In eukaryotic organisms DNA is packed into chromatin together with histones and various non-histone proteins. The resulting chromatin structure affects transcription, DNA-replication, repair and recombination. These processes are governed and controlled by two dominant concepts—the "histone code" [Turner, 1993; Sun and Allis, 2002] and the chromatin remodeling [Imbalzano and Xiao, 2004]. The generality and diversity of the histone modifications as well as the ubiquity of chromatin remodeling are carried out by specific protein complexes.

Actin-related proteins (Arps) are regularly discovered in chromatin remodeling and histone acetyltransferase (HAT) complexes from yeast to mammals [Sunada et al., 2005]. In the budding yeast *Saccharomyces cerevisiae* ten Arps were identified which were numbered according to their similarity to actin. Increasing numbers show decrease in relatedness with actin [Poch and Winsor, 1997]. Arps 1p-3p are located in the cytoplasm, while the other six, Act3p/Arp4, Arp5p, Arp6p, Arp7p, Arp8p, and Arp9p are nuclear proteins [Weber et al., 1995; Harata et al., 2000].

Act3p/Arp4 is an essential protein for the budding yeast [Harata et al., 1994]. Genetic studies revealed that this protein was involved in transcriptional regulation of *HIS3* and *LYS2* and in the activity of the *his4-912δ* promoter [Jiang and Stillman, 1996]. Later on Act3p/Arp4 was found in NuA4 HAT complex and in SWR1 and INO80 chromatin remodeling complexes as an essential subunit [Galarneau et al., 2000; Shen et al., 2000; Mizuguchi et al., 2004]. Act3p/Arp4 binds to core histones, and is involved in the recruitment of remodeling complexes to chromatin [Harata et al., 1999, 2002; Downs et al., 2004]. In addition, it is suggested that the ATP-binding pocket of Act3p/Arp4 regulates the dynamics of these

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complexes [Sunada et al., 2005]. Based on these observations, Act3p/Arp4 is expected to be one of the key component of chromatin organization. However, because of its necessity for cell life, the phenotypes of Act3p/Arp4 mutated cells have not been analyzed enough.

By taking advantage of the FACS analysis we determined the cell size and surface of *act3/arp4* mutants. Surprisingly, our FACS results showed that *act3/arp4* mutant cells, even when grown at permissive temperature, are characterized by diversity in the cell size and intracellular complexity. Moreover, by measuring the nuclear diameters of these mutants we detected a substantial increase in the nuclear size when compared to the wild-type.

Distinct single amino acid substitutions in Act3p/Arp4 have led to temperature sensitive phenotype [Harata et al., 2002; Steinboeck et al., 2006]. It is known that mutations within the actin fold affect the stability of Act3p/Arp4 and cause the formation of irregular Act3p/Arp4 complexes, thus affecting its association with chromatin [Harata et al., 2002].

Recently, it was shown that in mutant *act3/arp4* cells changes in chromatin structure appear in the his4-912δ chromatin region [Harata et al., 2002]. In order to shed more light on the nature of the mutant phenotype we used mutants (ts12 and ts26) which contain amino acid substitutions in the actin fold domain of Act3p/Arp4. For our experiments we have combined the method yeast comet assay [Miloshev et al., 2002] with treatment of chromatin in the cells with nucleases: micrococcal nuclease and deoxyribonuclease I. The resulting method—Chromatin Yeast Comet Assay (ChYCA) allowed us to observe overall differences in the higher-order structuring of chromatin. Using this method we have looked for the in vivo bulk chromatin organization of *act3/arp4* mutants and revealed that Act3p/Arp4 plays a more general role in this organization.

MATERIALS AND METHODS

All chemicals and reagents used in this study were purchased from Sigma unless stated otherwise.

Yeast Strains

Yeast strains DY2864 (MAT**a** *his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3*), referred in this study as a wild-type (WT), DY4519 (MAT**a** *his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts12*), designated as ts12 and DY4285 (MAT**a** *his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26*), assigned as ts26 were used in this study. The *act3-ts12* and *act3-ts26* alleles have a single amino acid substitution of G455S and G187R respectively [Harata et al., 2002].

YPD growth medium was used as standard medium for the culturing of yeast cells.

Survival Assay

Cell cultures were grown at permissive temperature 30°C for one night. After 50-fold dilution of the culture the cell number was determined with a hemocytometer (Neubauer). Aliquots containing ~300 cells were plated on YPD plates. Colony-forming units (CFUs) were determined for each of the strains after 3 days growth at 30°C and 5 days growth at 20 and 37°C. Percentage of viable cells was calculated as a part of the number of viable wild-type cells, which was assumed as 100%.

Microscopic Analyses

Yeast cells were grown at 30°C to a logarithmic phase to an approximately 1.8×10^7 cells/ml.

Aliquots of cells from the wild-type and *act3/arp4* mutants were taken and were observed under a light microscope. Pictures were taken with an Olympus digital camera with maximal resolution of 8 Mpx.

Additional aliquots of wild-type and *act3/arp4* mutant cells were stained with SYBR green I (Molecular Probes) and were visualized under fluorescent microscope Leitz (Orthoplan, VARIO ORTHOMAT 2) using 450–490 nm bandpass filter. Quantification of the diameter of 20 nuclei from each of the analyzed yeast strains was done by a built-in microscopic eyepiece.

FACS Analysis

Yeast cells from strains wt, ts12 and ts26 grown at 30°C to logarithmic phase (1.8×10^7 cells/ml) were spinned down in a microfuge tube for 1 min. The pellet was washed with 1 ml of 70% ethanol and cells were fixed for 1 day at −20°C. After that cells were pelleted for 1 min and resuspended in 1 ml of buffer 50 mM Na citrate pH 7 and sonicated for 15 s. After subsequent pelleting and resuspending of the cells in 1 ml of the same buffer cells were treated with RNase A (0.1 mg/ml) and incubated at 37°C

for 1 h. After pelleting and washing of the cells, they were resuspended in 1 ml of Na citrate buffer and were stained with propidium iodide (PI) 50 μg/ml final concentration for 20 min. Afterward FACS analysis proceeded at a BD FACS Canto apparatus, according to the manufacturer's recommendations.

FACS Data Quantification

Data acquisition and analysis were performed using the computer program Windows Multiple Document Interface flow cytometry application (WinMDI Version 2.8).

Chromatin Yeast Comet Assay

Treatment of cells with nucleases. Cells were resuspended in S buffer (1 M Sorbitol; 25 mM NaH_2PO_4, pH 6.5). Aliquots of yeast cells (4×10^5 cells/ml) were mixed with 0.7% final concentration of low-gelling agarose (type VII) and 5 μg/ml of Zymolyase (Seikagaku Corp.). The agarose-cell suspension was quickly layered on precoated with normal agarose (type II-A) glass slides and covered with coverslips to allow the gel to spread. Incubation at 37°C for 14 min was performed in order to spheroplast the yeast cells. A drop, containing 100 units/ml of micrococcal nuclease (MNase; MBI Fermentas) or 100 units/ml of DNase I, was applied in situ on the gels, covered with coverslips and incubated at 37°C for 1 min. The enzyme reaction was stopped by immersing the slides into lysis solution.

Yeast comet assay. After preparing the gels and treatment of the cells the slides were immediately placed in a cold lysis solution (1 M NaCl, 50 mM EDTA, pH 8, 30 mM NaOH, 0.1 % *N*-lauroylsarcozine; pH 10) for 1 h. DNA was denatured by incubating the slides for 3×20 min in a denaturing solution (30 mM NaOH, 10 mM EDTA; pH 12.6). Electrophoresis was conducted at 10°C for 15 min at 0.45 V/cm in an electrophoresis buffer (30 mM NaOH, 10 mM EDTA; pH 12.6). The glass slides were then washed with 0.5 M Tris–HCl, pH 7.5 for 5 min to neutralize the alkali and were dehydrated by sequential washes in 75% and 95% of ethanol for 5 min each. The comets in the gel were stained with SYBR green I (Molecular Probes) and visualized under fluorescent microscope Leitz (Orthoplan, VARIO ORTHOMAT 2) using 450–490 nm bandpass filter. Pictures were taken with a built-in microscope photo camera.

Quantification of Yeast Comet Assay

A total number of 100 comets for each of the nucleases were analyzed by calculating the parameters comet length (C_{length}) and tail moment (TM; Fig. 3).

Comet length (C_{length}). The parameter C_{length} was defined by measuring the distance between the beginning of the comet head and the end of the comet tail according to Olive [Olive et al., 1990].

Tail moment (TM). TM was calculated by multiplying the percentage of DNA in the tail by the comet length [Olive and Durand, 2005]. The quantity of DNA in the comet was measured by the software Gel Pro Analyzer Version 3.0—Media Cybernetics (MD). DNA percentage in the tail was calculated as a part of the whole DNA content in the comet, which was assumed as 100%.

Statistical Analysis

Statistical analysis was done by means of the GraphPad Prism software, (GraphPad Software Inc., 2000) by using one-way ANOVA with Newman–Keuls comparison test.

RESULTS

act3/arp4 Mutant Cells Growth Features

ACT3/*ARP4* is a *S. cerevisiae* gene coding for a polypeptide of 489 amino acids with a calculated mass of 54.8 kDa [Harata et al., 1994]. We used *act3*/*arp4* mutant yeast cells: ts12 and ts26, each with a single amino acid substitution of G455S and G187R, respectively [Harata et al., 2002] and the isogenic wild-type strain. When grown for 3 days at permissive temperature 30°C on YPD plates the *act3*/*arp4* mutant cells showed no obvious growth defects in comparison with the wild-type cells. The number of CFUs of *act3*/*arp4* mutants was indistinguishable from the CFUs of the wild-type. However at 20 and 37°C the G455S and G187R mutations led to growth inhibition of *act3*/*arp4* cells. The percentage of viable cells of ts12 and ts26 mutants, grown at 20°C, when compared to the wild-type cells, was $18\% \pm 0.2$ and $91\% \pm 0.5$, respectively. At 37°C *act3*/*arp4* cells did not grow at all, thus showing major temperature-dependency of these cells.

act3/arp4 Mutant Cells Exhibit Differences in the Cell Size and Nuclear Diameter

To assess whether mutated Act3p/Arp4 causes major cellular rearrangements at permissive temperature of growth we have checked cellular size and shape. Yeast cells were grown at permissive temperature 30°C to a logarithmic phase of growth and were prepared for light microscopy.

As shown in Figure 1A *act3/arp4* mutant yeast cells exhibit very pronounced and characteristic features. ts12 cells are larger and with more prolonged form than WT cells. A closer inspection of the pictures reveals that ts26 cell population is mixed, consisting of large, medium-, and small-sized cells.

To corroborate these observations we performed a more precise measurement of the size, granularity, and complexity of cells, grown at permissive temperature, by FACS analysis. Figure 1B represents comparison between light-scattering properties of wt, ts12 and ts26 yeast cells.

Forward (FSC) and side scattering (SSC) properties are measured in arbitrary units on a linear scale and are determined by cell complexity and size, respectively. The intensity of forward scatter and the side scatter are proportional to the size, shape, and optical homogeneity of the cells. A representative result of one from three independent experiments is shown. Each dot stands for a single cell. In order to calculate the percentage of cells with different size and complexity we conditionally divided the dot-plots into four different groups according to the forward and side scattering characteristics of the cells. Group I combines cells with normal size and complexity, group II—cells, which are normal in size, but with higher granularity and complexity, group III joins the cells with larger size and normal complexity and group IV merges the cells which are with the largest size and with the highest complexity. Interestingly, calculation of percentage of cells in each group (Table I) revealed that ts12 mutant was represented by the highest percentage of cells in groups III and IV,

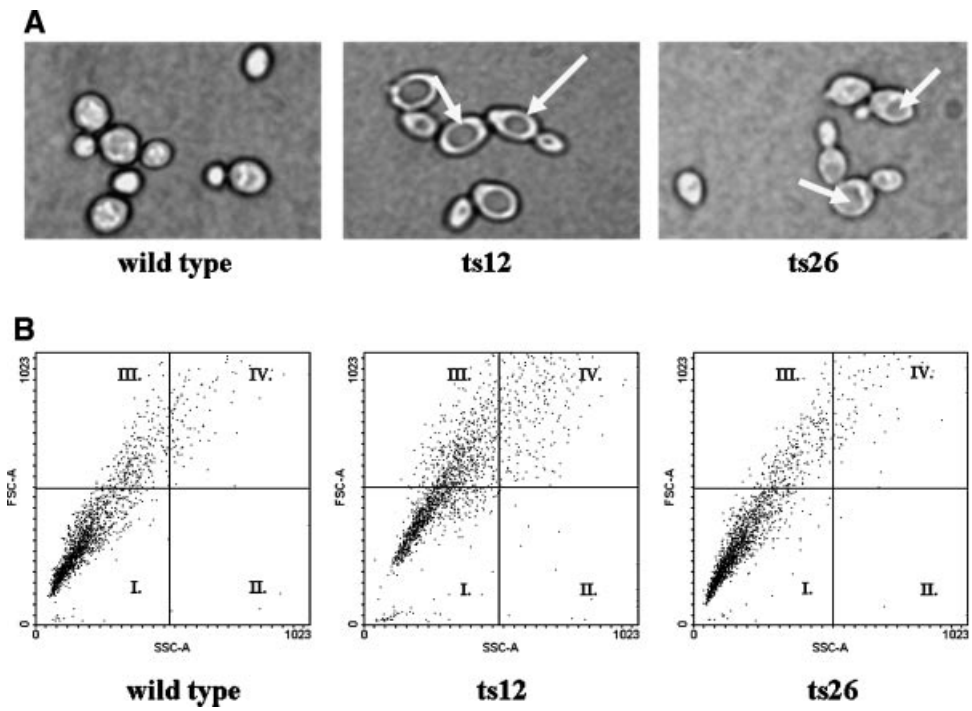


Fig. 1. Comparison between cell size and complexity of wild-type and *act3/arp4* mutant yeast cells. **A**: Light microscopy of wild-type, ts12 and ts26 yeast cells (magnification 600×). Arrows indicate cells larger in size in the cell population of *act3/arp4* mutants. **B**: FACS analysis. Comparison of light-scattering properties of wt, ts12 and ts26 yeast cells. Relative SSC-A and FSC-A are measured in arbitrary units on a linear scale and are determined by cell complexity and size, respectively. Each dot-plot is conditionally divided into four different groups representing cells with different size and complexity. Groups I and II—characterize yeast cells with normal size, III stands for yeast cells larger in size and IV—corresponds to cells with higher complexity as well as larger in size.

TABLE I. A Distribution of Cells With Different Size and Complexity

	wt (%)	ts12 (%)	ts26 (%)
Group I	83.78	45	81
Group II	0.5	0.6	0.2
Group III	15.12	30.4	11.8
Group IV	0.6	24	7

Percentage of cells in the groups (I, II, III, and IV) for each strain was calculated as a part of the whole cell population, which was considered as 100%

respectively ~30% and ~24% in comparison with the wt and ts26 cells. Thus ts12 mutant displayed a higher percentage of cells larger in size and very complex in cell surface. As was expected from the observation by light microscopy, ts26 mutant was represented by a highly mixed population of cells with characteristics resembling both wt and ts12. For example, the distribution of cells in group I for ts26 was ~81%, similar to the wild-type cells (~83%) while group IV was represented by ~7% in contrast to the wild-type which exhibited insignificant percentage 0.6%. What should be highlighted is that all the observed differences between ts12, ts26 and wt cells were manifested at permissive temperature, attesting that all cells were viable. Statistical analysis of the results proved their significance ($P < 0.01$).

act3/arp4 Mutant Cells Possess Larger Nuclei

Wild-type, ts12 and ts26 cells were grown at permissive temperature to a logarithmic phase and were prepared for fluorescent microscopy. Determination of the nuclear diameter of 20 nuclei for each of the three strains was performed by a microscopic eyepiece and the results are summarized in Figure 2. The diameters of ts12 nuclei (1.67 μm ± 0.31) and ts26 cells (1.33 μm ± 0.27) were respectively 1.6 and 1.3 times lager in size than the nuclear diameter of the wild-type cells (1.03 μm ± 0.25). The observed differences are significant and represent an interesting relationship between *act3/arp4* mutations and increasing of the nuclear size.

The Chromatin of *act3/arp4* Mutant Cells Demonstrates Higher General Sensitivity Toward Nuclease Action as Revealed by Chromatin Yeast Comet Assay (ChYCA)

In order to elucidate whether Act3p/Arp4 has more general role in the chromatin organization, we have utilized the ChYCA.

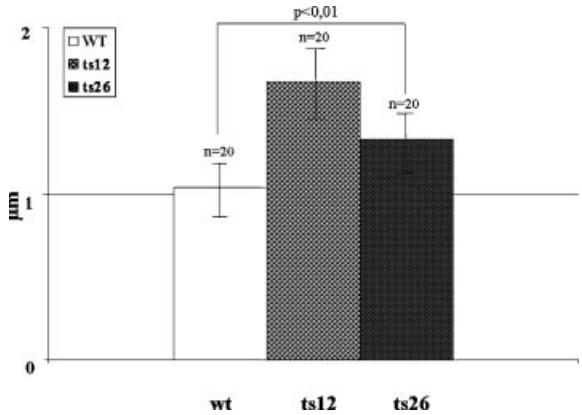


Fig. 2. Quantification of nuclear diameter of wt and *act3/arp4* yeast cells. Cells were stained with SYBR green and visualized under fluorescent microscopy. Cell nuclei diameter was determined by microscopic eyepiece. Bars ± STDV.

Figure 3 represents a general depiction of ChYCA. The method provides detection of damages in the molecule of DNA at a single-cell level. After embedding of the cells in agarose

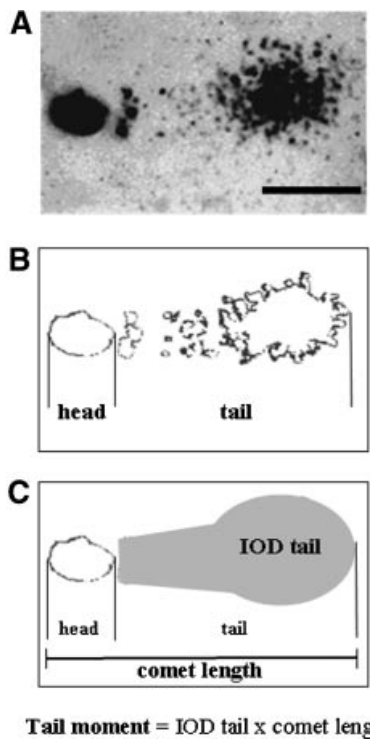


Fig. 3. Chromatin Yeast Comet Assay of wt and *act3/arp4* mutant yeast cells. **A**: A typical yeast comet. Negative shown. Bar = 100 μm. **B**: An outline of the yeast comet, represented in (A). Outlining of the comet was done by means of the software programs Photoshop 6.0 and RasVector. Yeast comet characteristics are illustrated: comet head and comet tail. **C**: Main parameters for yeast comet assay quantification of results.

and cutting of their DNA by MNase or DNase I the chromatin loops are released. The electric field allows the migration of the relaxed chromatin loops away from the nucleus and the formation of a comet-like shape. We have chosen MNase and DNase I as two enzymes that are commonly used for probing of chromatin structure, but cut DNA in the chromatin differently. MNase makes double-stranded DNA breaks [Telford and Stewart, 1989], while DNase I is a single-stranded DNA cutting enzyme with a preference to the active chromatin domains [Wolffe, 1995]. We used 100 units/ml of both nucleases a concentration which best revealed the differences in the studied chromatins.

Figure 3A shows a typical comet from yeast obtained by the method of ChYCA. It represents a typical fluorescent image of a yeast comet, obtained by treatment of the yeast cells with 100 units/ml of MNase. For a more detailed and descriptive explanation of the method we have presented Figure 3B, which is an outline of the yeast comet, demonstrated in Figure 3A. Each yeast comet is composed of a head, which consists of the undamaged DNA, and a tail, representing the chromatin loops (Fig. 3B). Quantification of the results from yeast comet assay could be done by measurement of several parameters [Tice et al., 2000; Hartmann et al., 2003]. We have chosen the most often used parameters, comet length (C_{length}) and TM Figure 3C. These parameters represent DNA liberated from the head of the comet and are proportional to the degree of chromatin relaxation [Tice et al., 2000; Chandna, 2004]. Both parameters are representative and in the case of nuclease treatment definitely revealed changes in the chromatin organization of the eukaryotic nucleus.

Comet length (C_{length}). Comet length was defined by measuring the distance between the beginning of the comet head and the end of the comet tail in μm. Comet length of 100 comets was measured for any of the DNA cutting enzymes and the results are presented on Figure 4A. We observed that *act3/arp4* mutants treated with MNase provided longer comets when subjected to ChYCA in comparison with the wild-type comets. The differences were ~10% longer comets for ts12 cells and ~20% longer for ts26 cells. Both mutants yielded comets with different length, suggesting differences in their chromatin loop organization.

According to our results comets obtained from *act3/arp4* mutant cells by the other DNA cutting enzyme DNase I displayed statistically insignificant differences in comet lengths when compared to the wild-type comets.

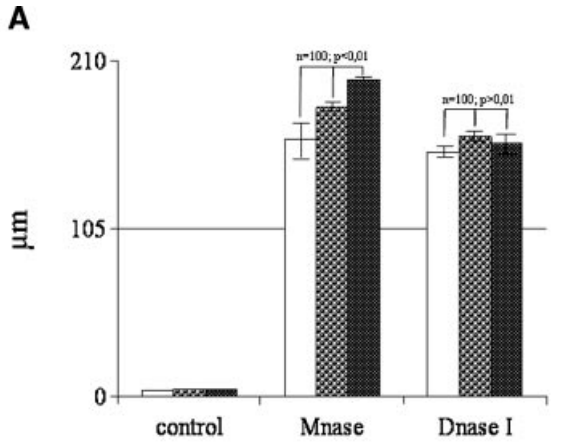
Tail moment (TM). TM is a more complex parameter for Comet assay quantification. It combines several parameters of the analyzed comets, thus providing a more precise estimation of DNA damage (Fig. 3C) [Olive and Banath, 2006]. By quantifying the TM of wt, ts12 and ts26 yeast cells we were able to detect more profoundly the differences in chromatin structure. Treatment of *act3/arp4* mutant cells in comparison with the wild-type cells showed higher sensitivity to both nucleases. As can be observed from Figure 4B the TMs of ts26 and ts12 MNase comets are with ~25% and ~13% respectively higher than that of wt comets. Interestingly, DNase I comets of both *act3/arp4* mutant strains displayed ~20% higher TMs when compared to the TMs of wild-type comets. Data presented by TM include intensity of the DNA in the tail. Thus the higher TM, when the length of tail remains unchanged as in the case of digestion with DNase I, means more frequent cuttings in DNA. Basically both mutants portray more accessible, that is, more relaxed, chromatin structure.

DISCUSSION

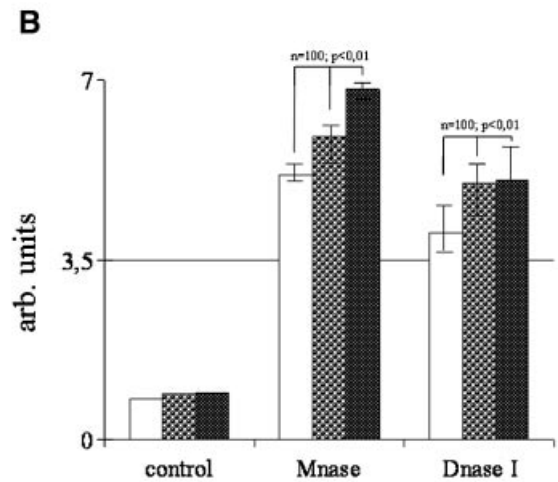
Why *act3/arp4* Mutant Yeast Cells Exhibit Different Cell Size and Surface?

The observed growth defects at restrictive temperature in *act3/arp4* mutant cells suggest that if we intend to scrutinize the overall role of Act3p/Arp4 in nuclear and chromatin maintenance, we should cultivate cells at a permissive temperature.

Fluorescent-activated cell sorting (FACS) demonstrated that *act3/arp4* mutant yeast cells were characterized by a higher side scatter (a measure for intracellular complexity) and higher forward scatter (a measure for the cell size) in comparison with the wild-type. How could be explained the relationship between *act3/arp4* mutations in the cells and their size and shape? Recently, it was shown that *act3/arp4* mutant cells at permissive temperature 30°C display high levels of expression of several stress genes, including *HSP12* and *HSP26* genes, which exhibit an increased expression in comparison with the wild-type cells [Görzer



	control	Mnase	Dnase I
□ wt	3,44	161,3	153,1
▨ ts12	3,9	181,6	162,9
■ ts26	4,3	198,13	158,24



	control	Mnase	Dnase I
□ wt	0,8	5,17	4,04
▨ ts12	0,87	5,902	5
■ ts26	0,9	6,839	5,06

Fig. 4. Chromatin Yeast Comet Assay of wt and *act3/arp4* mutant yeast cells. Wt, ts12 and ts26 cells were subjected to Yeast Chromatin Comet assay. **A**: Comet length C_{length} was calculated by measuring the distance between the beginning of the comet head and the end of the comet tail. **B**: TM was calculated by multiplying the distance between the beginning of the residual nucleus and the end of the comet tail (in arbitrary units) by the intensity of DNA in the comet tail (IOD tail). Data are the mean of three independent experiments; bars ± STDV. Statistical analysis revealed that the existing differences in chromatin sensitivity of act3/arp4 mutant yeast cells when compared to wt cells are significant ($P < 0.01$).

et al., 2003]. On the other hand, data show that some of the upregulated stress genes in *act3/arp4* mutants are responsible for the cell wall integrity and flexibility [Fernandes et al., 2001, 2004]. Later on a genome-wide analysis in *S. cerevisiae* unquestionably proved the role of chromatin modifiers in transcription [Steinfeld et al., 2007]. The newly developed high-throughput methodology allowed the authors to assess the dependence of transcription factor

function on chromatin modifiers. Over 60 potential chromatin modifiers and their role on gene transcription were analyzed. Our own analysis on their results revealed that the modifiers INO80 and SWR1, in which Act3p/Arp4 is a constituent, influence the transcription of more than 200 transcription factor cohorts, with a single cohort including between 40 and 200 different genes. Therefore, it is quite possible the mutations in Act3p/Arp4, leading to impairment of the functions of INO80 and SWR1 chromatin modifying complexes, to generate changes in the expression of many genes which could generally lead to overall changes in the cell size and surface of *act3/arp4* mutant cells.

Recently another *act3/arp4* mutant with a single amino acid substitution G161D in the conserved actin fold domain of Act3p/Arp4 was studied [Steinboeck et al., 2006]. Interestingly, these authors have also detected morphological differences between their *act3/arp4* mutant and the wild-type. Mutant cells were irregularly shaped or unusually large or small. The results of Steinboeck et al. and ours unambiguously prove that a single amino acid substitution in Act3p/Arp4 could lead to serious changes in the cellular morphology.

What About the Chromatin Structure of *act3/arp4* Mutant Cells?

In 1999, Harata et al. showed that Act3p/Arp4 binds to each of the core histones and that conditional *act3* mutation affects chromatin structure of an episomal DNA molecule, indicating that the Act3p in complex with other proteins may be involved in the establishment, remodeling, or maintenance of chromatin structure [Harata et al., 1999]. Later it was shown that Act3p/Arp4 is a component of the NuA4 HAT complex and Ino80 chromatin remodeling complex [Shen et al., 2000] and it was suggested that it functions to recruit components of these complexes onto chromatin [Harata et al., 2002; Sunada et al., 2005]. However, none of these articles present analysis of the influence of Act3p/Arp4 in the global chromatin maintenance and organization.

By utilizing well-established chromatin nucleases in Comet assay we designed a method in order to look at the loop organization of the nucleus at single cell level. The method, which we called ChYCA is sensitive enough to differentiate changes in the higher order chromatin structures. Analyzing by this method the chromatin structure of the wild-type and the two *act3/arp4* mutant cells we observed specific features of their chromatin. Results disclosed general higher sensitivity of *act3/arp4* mutant chromatin toward the action of nucleases in comparison with the chromatin of wild-type cells. Although unexpected, the observed difference in the compaction of chromatin of yeast *act3/arp4* mutant could explain the increased nuclear diameter of these mutants. Thus our results reveal an unforeseen effect of Act3p/Arp4 in the overall chromatin structuring in the yeast nucleus.

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