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THE DELETION OF THE GENE FOR THE LINKER HISTONE IN *ARP 4* MUTANT YEAST CELLS IS NOT DELETERIOUS

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ABSTRACT
Certain evidence has been collected about the imperative role of chromatin remodeling complexes (CRCs) in the fine tuning of genome activity. One of the most abundant CRCs is INO80 which is evolutionary conserved from yeast to human. INO80 consists of several subunits, all of them playing important roles in its functioning. The actin-related protein -Arp4p, is one of these subunits. Although ARP4 gene is essential for the yeast cells certain arp4 mutants do exist, thus providing good opportunities for studying of INO80 roles in the higher-order building of chromatin. Using the advantages of S. cerevisiae we have traced the interaction between Arp4p and linker histones.

Keywords: *Saccharomyces cerevisiae*, chromatin, chromatin remodeling, actin-related proteins, linker histone - Hho1p

Abbreviations: arp: actin-related proten;
CRC: chromatin remodelling complexes;
PCR: polymerase chain reaction

Introduction
Eukaryotic genomes are organized in chromatin, a nucleoprotein complex, assuring both protection and at the same time proper functioning of DNA (14). All processes on DNA take part in chromatin environment. Therefore chromatin is the complex which by remodelling and specific modifications allows functional proteins to get access to DNA and to facilitate and further execute their functions. Modifications and remodelling of chromatin are done by specific factors and large complexes. Such complexes include ATP-dependent chromatin remodeling factors (1, 2). INO80 is the most conserved and abundant chromatin remodelling complex in the cells. It consists of more than ten subunits many of which essential for the cells (4, 6, 11). Ino80 is the largest subunit, while actin-related proteins - Arp4, Arp5 and Arp8 are part of the helicase domain, which is conserved in all chromatin remodelers. Arp8 and Arp5 are unique subunits of INO80, while the Arp4 subunit is also part of the SWR1 and NuA4 complexes (12). Our attention is caught by Arp4, a stoichiometric component of the NuA4, INO80 and SWR1 chromatin modulating complexes, whose role is recruiting these complexes onto chromatin. Single amino acid substitutions in Arp4 dramatically affect the functions of these complexes and lead to changes in chromatin organization and function (3).

In *arp4* mutant yeast cells we have knocked-out the gene for the linker histone, *HHO1*, thus creating a model for studying cells with impaired both chromatin organization and chromatin remodeling. Importantly, the deletion of the linker histone in *arp4* mutant genotype proved not to be deleterious for the cells. This offers opportunities detailed investigation of the fine interrelation between functional and structural components of chromatin.

Materials and Methods
Yeast strains
The genotype of the yeast strains used in this research is listed in **Table 1**.

TABLE 1
S. cerevisiae strains used and designed in the current study

DY2864 (*wild type*)	*MAT**a** his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3*	(5, 6)
Δhho1 deltahho1	*MAT**a** his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3 ypl 127C::K.L. URA3*	this work
DY4285 (*arp4* mutant)	*MAT**a** his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26*	(5, 6)
arp4 Δhho1 (double *arp4 delta hho1* mutant)	*MAT**a** his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26 ypl 127C::K.L. URA3*	this work

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TABLE 2
Oligomers used for *hho1Δ::KlURA3* cassette amplification and for validation of its right integration in the yeast genome

Oligomer name:	**Sequence:**
Forward primer: Ypl 127c up	GAT AAT GCT TGG CAG CGA GGG AAG CAA TTA TAA TAC AAC TAA AGC AAC GAG CGG CCG CTT GTT CCT TAC CAT TAA GTT GAT
Downstream primer: Ypl 127c dw	TGA TAG TAT TGC TAT CAC CAT TGA CAT TCT CGT TTG GAT ATT CAC TTT CGG GCC CGG AGA CAA TCA TAT GGG AGA AGC AAT TGG

Disruption of *HHO1* gene
The *HHO1* gene (GenBank Accession number: NC 001148) was disrupted in *Saccharomyces cerevisiae* progenitor strain DY2864, referred here as wild-type and in DY4285, designated as *arp4* mutant. The *HHO1* gene was disrupted by the well established gene disruption technique for *S. cerevisiae* (13). The successful disruption of *HHO1* gene was confirmed by PCR. The primers for cassette amplification and validation of its integration are given in **Table 2**.

Studying the cellular growth potential of the yeast cells
Cells were cultivated in rich media (1% Yeast extract, 2% peptone and 2% dextrose) to a logarithmic phase of growth for three hours. At every 20 min cellular aliquots were subjected to spectro-photometrical measurements of cellular growth at 600 nm wavelength.

Assessment of cellular morphology
Light microscopy
Yeast cells from the wild type, the mutants *arp4* and *deltahho1*, and the double mutant *arp4 deltahho1* were cultivated in rich media - YPD (1% yeast extract, 2% peptone and 2% dextrose) to a logarithmic phase of cell growth $1x10^6$ cells/ml. Aliquots were taken from the four strains and were prepared for light microscopy observation of cellular size and morphology. Pictures were taken with a built-in camera at 8 mpx resolution. Images were elaborated with Adobe Photoshop CS5.1.

FACS analyses
Wild type, *arp4*, *deltahho1* and *arp4 deltahho1* cells grown to logarithmic phase were collected by centrifugation for 1 min at 10 000 g. The pellet was washed with 1 ml of 70% ethanol and cells were fixed for one day at -20°C. After pelleting, the cells were resuspended in 1 ml of 50 mM Na citrate, pH 7, sonicated for 15 sec, treated with RNase A (0.1 mg/ml) for 1 hour at 37°C and then washed. Finally, the cells were resuspended in 1 ml of Na citrate buffer and were stained with 50 µg/ml propidium iodide for 20 min. The FACS analysis was performed by BD FACS Canto apparatus. Data acquisition and analysis were performed using the WinMDI Version 2.8 software 2000.

Results and Discussion

Knocking-out the gene for the linker histone in *arp4* mutant yeast cells
In order to study the interactions among chromatin remodelling complexes and the linker histone as a structural builder of the higher-order chromatin structure we set off a series of experiments on *S. cerevisiae* cells which have mutations in the actin-related protein - Arp4p. These mutants have a point mutation in *ARP4* (*YJL081C*) gene which leads to a single amino acid substitution (G187R) located in one of the ATP-binding domains of Arp4p. The latter led to non-functional chromatin modifying complexes. We have demonstrated that as a consequence of chromatin remodelling dysfunction *arp4* mutant cells display severe defects in cellular and nuclear morphology (3). Several studies have shown that the linker histone Hho1p is not essential for the yeast cells (8, 9) in contrast to Arp4p (4). Therefore we have decided to knock out the gene for the linker histone in *arp4* mutants. The disruption of *HHO1* (*YPL127C*) was performed by the commonly used methodology, described by Wach et al. (13). Shortly, the technique includes three subsequent steps- design of the disruption cassette, transformation in the yeast cells and then verification of the proper integration in the genome.

The disruption cassette for the yeast linker histone gene- *HHO1*, referred here as *hho1Δ::KlURA3*, was bearing the *URA3* gene from *Kluyveromyces lactis* flanked by two 48 base pair long sequences homologous to the outer ends of the chromosomal copy of *HHO1* (**Fig. 1**). The cassette was successfully transformed in wild type and *arp4* mutant yeast cells and the verification of the proper cassette integration at *HHO1* locus was done by PCR. The primers for the verification of the correct cassette integration flank the cassette with the outlying 5' and 3' regions of the *HHO1* locus. A representative gel with the PCR products with the expected size is shown on **Fig. 2**. This confirmed that the obtained yeast cells did not have the gene for the linker histone. Thus at the end we had four yeast strains which were further subjected to investigation: wild type, *deltahho1*, *arp4* and double *arp4 Δhho1* mutants (**Table 1**). Importantly, the double mutants were viable which allowed us to continue with characterization of the impact of the *HHO1* knock-out in *arp4* mutant genotype.

 DOI: 10.5504/50YRTIMB.2011.0025

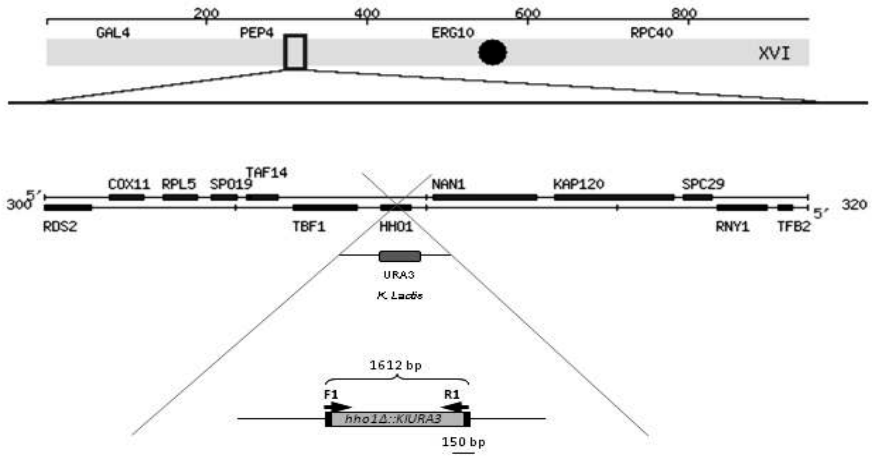

Fig. 1. Design of the disruption cassette for the knock out of the gene for the yeast linker histone, *HHO1*
The gene for the linker histone of *S. cerevisiae* is located on chromosome XVI. The disruption cassette was designed to bear the genomic copy of the gene *URA3* from *K. lactis*, flanked by two 48 bp long ends homologous to the 5' and 3' ends of *HHO1* locus. The disruption cassette was designated as ***hho1Δ::KlURA3***

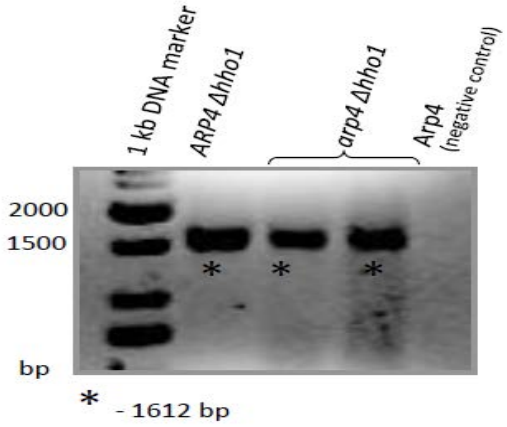

Fig. 2. PCR verification of *HHO1* knock-out in wild type (Arp4) and *arp4* mutant cells
Wild type and *arp4* mutant cells were transformed with the disruption cassette for *HHO1*. After transformation cells were cultured on selective media for picking up only cells which had the cassette integrated in their genome while further verification of the proper cassette integration was done by PCR. Genomic DNA was isolated from the selected mutants and was used for cassette amplification with the designed primers. The size of the disruption cassette is 1612 bp. The starts are written on the picture with the agarose gel representing the amplified disruption cassette in the wild type- *Arp4 Δhho1*, and in two *arp4* mutants- *arp4 Δhho1*. As a negative control genomic DNA from cells in which no transformation was performed, was also used for PCR with the same primers, thus assuring their selectiveness and specificity- Arp4 (negative control)

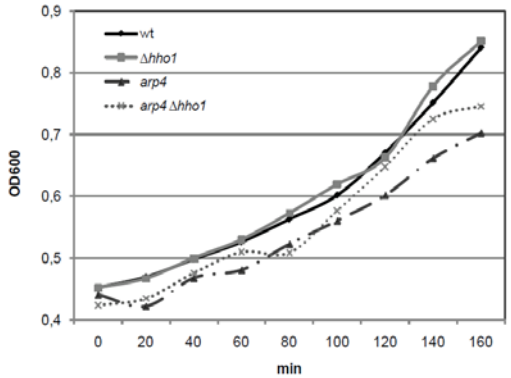

Fig. 3. Growth curves of wild type, *deltahho1*, *arp4* and *arp4 deltahho1* yeast cells
Yeast cells were cultured in rich media for three hours. At every 20 min aliquots were spectrophotometrically measured and the obtained values for OD_{600} were plotted on a graph thus allowing comparison among the four strains

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Characterization of wild type, *deltahho1*, *arp4* and *arp4 deltahho1* mutants

In order to study what would be the impact of the knock-out of the gene for the linker histone in *arp4* genetic background we started characterization of the cellular growth together with probing of the cellular morphology of the yeast cells.

Assessing cellular growth

Cells were cultivated in rich media for three hours in order to enter exponential phase. At every twenty minutes the optical density at 600 nm wavelength (OD_{600}) was spectrophotometrically analyzed. Results representing the growth potential of the cells are shown on **Fig. 3**. As was expected and in good relevance with other authors' data (8, 9) wild type and *deltahho1* cells did not exhibit significant difference in the growth potential when cultured in rich media. In contrast, cells which have mutated *arp4* and the double mutants - *arp4 deltahho1*, i.e. having mutated *arp4* and at the same time lacking the gene for the linker histone demonstrated different growth. *Arp4* mutant cells were growing slower than the wild type. As for the double mutant it was growing slower when compared to the wild type, but when compared to the *arp4* mutant strain it demonstrated faster growth. This could be explained with a partial compensation of the *arp4* mutant phenotype by the knock-out of the gene for the linker histone. Taken together all these results prompt interesting interplay between linker histones and chromatin remodelling complexes, namely the Arp4p subunit which is essential part of their ATPase and helicase domains.

Assessing cellular morphology

Previous data (3) have shown that *arp4* mutants possess abnormal cellular and nuclear morphology- large cells with highly articulated cellular granularity. Hence, it was of interest to follow the cellular morphology of the double mutants.

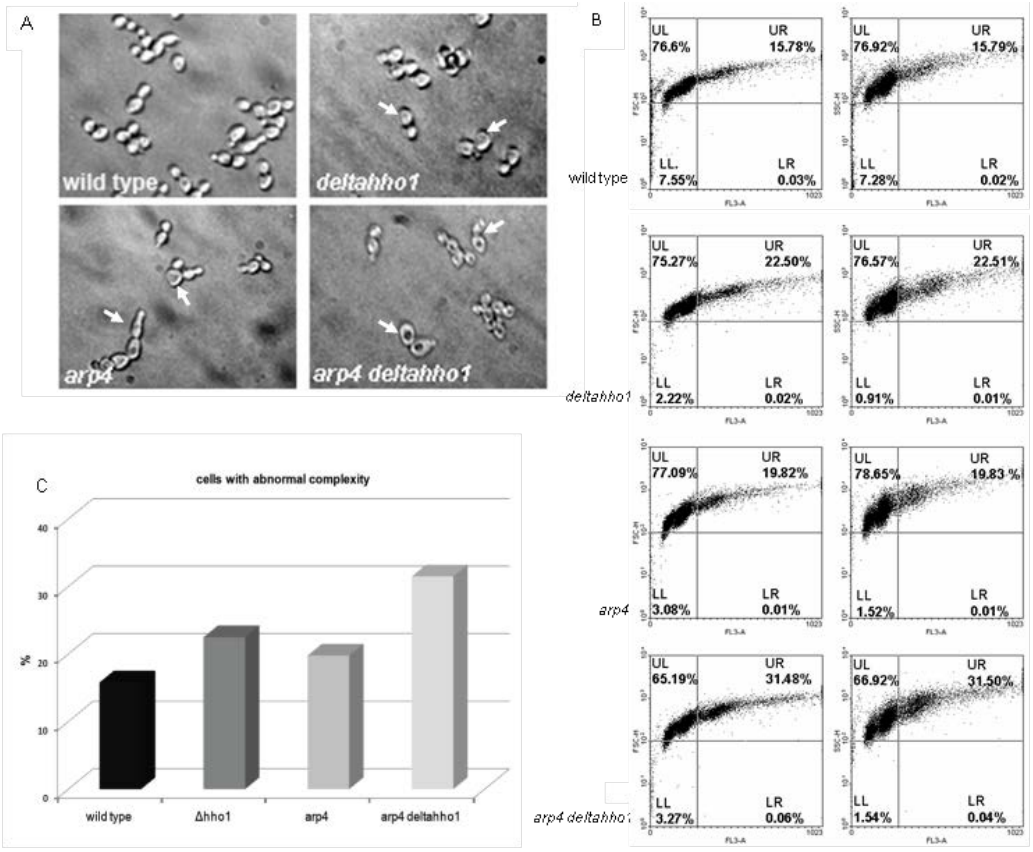

Fig. 4. Cellular morphology of wild type, *deltahho1*, *arp4* and *arp4 deltahho1* yeast cells

A: Light microscopic images of logarithmically growing yeast cells from the four studied strains. Cells with atypical and abnormal morphology are pointed with arrows

B: FACS analysis of cellular morphology. Dot plots representing the distribution of cells with different size and intracellular granularity are given for each of the four yeast strains. The dot plots are divided into four sections each standing for the distribution of cells with given size and granularity. Results are statisticallelaborated by the FACS apparatus and are given as % in each of the four subdivisions of the dot plots

C: Percentage of cells with abnormal cellular morphology. Cells with abnormal morphology were determined after elaboration of the results obtained by the FACS analysis

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Light microscopic analysis

In order to better characterize the four studied strains we have done a set of light microscopic observations. Results are shown in **Fig. 4A**. A closer inspection of the light microscopic micrographs of the studied yeast strains revealed that the cells with obvious heterogeneity, i.e. larger cells with abnormal cellular shapes, were from the *deltahho1*, *arp4* and *arp4 deltahho1*mutants (arrows point out this cellular heterogeneity in the three mutants, **Fig. 4A**).

FACS analysis

An elegant experiment for assessing cellular morphology is the FACS (fluorescence activated cell sorting) analysis. It allows scattering of cells with different sizes and cellular granularity and thus allows easy and fast assessment of cellular morphology and thus permits comparison between different yeast species. Aliquots from logarithmically growing yeast cells were gathered by centrifugation and processed for FACS analysis. Cells were spread out by the forward side scattering (FSC-H), which reveals differences in the cellular size (left panels on **Fig. 4B**) and by the side scattering (SSC-H), which denotes cellular granularity (right panels on **Fig. 4B**). The dot plots according to FSC-H and SSC-H parameters showed specific distribution of *deltahho1*, *arp4* and *arp4 deltahho1* cells, revealing incidence of cells with bigger sizes and higher heterogeneity in comparison to the wild type cells. Statistical elaboration of the obtained FACS results has been performed (**Fig. 4C**). Interestingly, *arp4* mutants had fewer cells with bigger sizes than the other two mutants. Moreover, when the gene for the linker histone has been knocked out in these mutants the percentage of cells with abnormal morphology went 20% higher and pretty much resembled the *deltahho1* mutants.

These data unambiguously show that the yeast linker histone- Hho1p together with Arp4p are involved in the maintenance of the cellular morphology. These data provoke plans for future investigations of this complex interplay between structural chromatin proteins and functional complexes in the cells.

Conclusions

Sketching the interplay among functional and structural chromatin complexes

The way chromatin is organized in the eukaryotic nucleus is decisive for the way genes are expressed. Stable and at the same time quite dynamic chromatin structure allows flexible and all together precise regulation of genetic information. Light is being progressively shed on this with the advance of Epigenetics, which deals with the differences in gene expression among closely related cells and organisms, even among genetically identical ones. Epigenetics studies alterations in chromatin structure and organization leading to variations in gene expression pattern without any nucleotide changes in the sequence of interest (7). The mechanisms by which chromatin is executing these sophisticated functions have long been a challenge for Molecular biologists. *S. cerevisiae* is a brilliant model for the purpose. Studying the complex interactions between chromatin structure and chromatin modifying complexes in the yeast cells is easy, fast and very well correlates with higher eukaryotic organisms (10).

We have successfully knocked out the gene for the linker histone in *arp4* mutant yeast cells and thus were able to obtain a model for studying cells with impaired both chromatin structure and chromatin remodeling. This offers wonderful opportunities for searching for the fine interplay among the builders of chromatin structure, i.e. the linker histones, and the functional complexes that remodel chromatin structure. The initial characterization of the double *arp4 deltahho1* mutants together with the comparison with the wild type and the single *arp4* and *deltahho1* mutants revealed that the *HHO1* knock-out in *arp4* mutants suppresses *arp4* mutation when cellular growth in rich media was assessed. The double mutants explicitly exhibited highly distorted cellular morphology regarding cellular size and intracellular granularity. Obviously, the yeast cells with non-functional chromatin remodelling complexes and without the linker histone had very heterogeneous cellular populations, consisting of cells with larger size and higher granularity.

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REFERENCES

1. **Becker P.B. and Horz W.** (2002) Annu. Rev. Biochem., **71**, 247-273.
2. **Eberharter A. and Becker P.B.** (2004) J. Cell Sci., **117**, 3707-3711.
3. **Georgieva M., Harata M., Miloshev G.** (2008) J. Cell. Biochem., **104**, 59-67.
4. **Harata M., Karwan A., Wintersberger U.** (1994) Proc. Natl. Acad. Sci. USA, **91**, 8258-8262.
5. **Harata M., Oma Y., Tabuchi T., Zhang Y., Stillman D.J., Mizuno S.** (2002) J. Biochem., **128**, 665-671.
6. **Harata M., Zhang Y., Stillman D., Matsui D., Oma Y., Nishimori K., Mochizuki R.** (2002) NAR, **30**, 1743-1750.
7. **Jenuwein T. and Allis C.D.** (2001) Science,

 DOI: 10.5504/50YRTIMB.2011.0025

293, 1074-1080.
8. **Landsman D.** (1996) Trends Biochem. Sci., **21**, 287-288.
9. **Patterton H., Landel C.C., Landsman D., Peterson C.L., Simpson R.T.** (1998) J. Biol. Chem., **273**, 7268-7276.
10. **Pina B.** (2000) Food Technology and Biotechnology, **38**, 253-262.
11. **Shen X., Mizuguchi G., Hamiche A., Carl W.** (2000) Nature, **406**, 541-544.
12. **Steinboeck F., Bogusch A., Kaufmann A., Heidenreich E.** (2007) J. Biochem., **141**, 661-668.
13. **Wach A.** (1996) Yeast, **12**, 259-265.
14. **Wolffe A.** (1995) Chromatin Structure and Function, Academic Press, San Diego.