Mechanisms of Ageing and Development 177 (2019) 55–65

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Linker histones and chromatin remodelling complexes maintain genome stability and control cellular ageing

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ARTICLE INFO

Keywords:
Linker histones
Saccharomyces cerevisiae
Actin-related protein 4
Chronological ageing
Chromatin remodelling

ABSTRACT

Linker histones are major players in chromatin organization and *per se* are essential players in genome homeostasis. As the fifth class of histone proteins the linker histones not only interact with DNA and core histones but also with other chromatin proteins. These interactions prove to be essential for the higher levels of chromatin organization like chromatin loops, transcription factories and chromosome territories. Our recent results have proved that *Saccharomyces cerevisiae* linker histone – Hho1p, physically interacts with the actin-related protein 4 (Arp4) and that the abrogation of this interaction through the deletion of the gene for the linker histone in *arp4* mutant cells leads to global changes in chromatin compaction. Here, we show that the healthy interaction between the yeast linker histone and Arp4p is critical for maintaining genome stability and for controlling cellular sensitivity to different types of stress. The abolished interaction between the linker histone and Arp4p leads the mutant yeast cells to premature ageing phenotypes. Cells die young and are more sensitive to stress. These results unambiguously prove the role of linker histones and chromatin remodelling in ageing by their cooperation in pertaining higher-order chromatin compaction and thus maintaining genome stability.

1. Introduction

The molecule of DNA is compacted in the constrained nuclear compartment through binding with histone proteins which allows it to make nucleoprotein complex, known as chromatin. Chromatin is organized in nucleosomes which are composed of four pairs of the core histones – H2A, H2B, H3 and H4, around which 146 bp of DNA are wrapped (Luger et al., 1997; Richmond et al., 1984). Outside of the nucleosome though binds a fifth class of histone proteins, recognized also as the family of linker histones or H1 (Breneman et al., 1993; Kowalski and Pałyga, 2012). H1 are among the most intricate histone proteins whose functions have been studied for many years and yet some remain not well understood (Herghet and Schneider, 2015; Zhou et al., 2015). Histones H1 are less conserved than core histones. In metazoa they generally exist in multiple variants. For instance, deletion of one or two of the seven mouse somatic H1 variants have no detectable effects (Sirotkin et al., 1995). Unicellular eukaryotes such as *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Tetrahymena termophila* contain a single histone H1-like gene (Shen et al., 1995) and they are good models for studying the roles of linker histones in different processes and under various conditions. What is known until now about the role of linker histones in these model organisms is that they stabilize the nucleosome by transforming it in a larger unit called chromatosome (Ali and Thomas, 2004; Maier et al., 2008a). A more important function of H1 histones, both proven in yeast and higher eukaryotes, is their engagement in the building and maintenance of the higher-order chromatin structures like the putative 30 nm fibres and structures above it corresponding to differently sized chromatin loops (Georgieva et al., 2012a; McBryant et al., 2010; Robinson and Rhodes, 2006; Sarg et al., 2014).

Structural changes in the way the genome is organized at these higher levels of chromatin compaction direct and moderate gene expression and by this modulate cellular response to intrinsic and extrinsic signals. The dynamics in the upper levels of chromatin organization is governed by the linker histones through their dynamic interactions with remodelling complexes (Clausell et al., 2009; Corona et al., 2007; Georgieva et al., 2015b; Maier et al., 2008a; Ramachandran et al., 2003). This determines the way cellular programs

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https://doi.org/10.1016/j.mad.2018.07.002
Received 28 February 2018; Received in revised form 6 July 2018; Accepted 12 July 2018

are performed and also controls cellular normality (Burke and Stewart, 2014; Oberdoerffer and Sinclair, 2007). To fulfil these functions chromatin goes through various types of epigenetic modifications like DNA methylation, histone posttranslational modifications and non-coding RNA binding (Allis et al., 2007; Dhanasekaran et al., 2012; Georgieva et al., 2016). Many diseases and other pathologies like cancer, ageing, age-associated diseases and others have been linked with alterations in the structural organization of chromatin and its epigenetic make-up. Ageing, for example, is a multifactor process which has more epigenetic than genetic background. Recent studies from a number of model organisms including yeast have proven chromatin structure as a major contributory agent for ageing (Georgieva et al., 2016; Lazarus et al., 2013; Oberdoerffer and Sinclair, 2007). Despite the fact that changes to nuclear and chromatin structure by epigenetic modifications have been linked with aberrant yeast replicative ageing (*i.e.* cells in dividing state) (Dang et al., 2014; Kaeberlein et al., 2006), little yet is known about the involvement of chromatin in cellular adaptability to different types of stress during chronological lifespan (cells in non-dividing but yet alive state).

Considering the fact that linker histones and chromatin remodelling machineries are eminent players in chromatin dynamics (Maier et al., 2008b; Varga-Weisz et al., 1995) we therefore decided to search for new approaches to link their interactions in maintaining chromatin organization with the process of chronological ageing. Our previous results have shown that the yeast linker histone Hho1p and Arp4p (actin-related protein 4), which is a stoichiometric constituent of several chromatin modifying complexes in yeast and mammals, *i.e.* Ino80, NuA4, HAT, SWR1, hSWI/SNF (Galarneau et al., 2000; Gorzer et al., 2003; Harata et al., 2002), physically interact with each other and this interaction is crucial for chromatin structure organization and cellular morphology (Georgieva et al., 2015b). Here, we show our results which demonstrate that the healthy interaction between these two chromatin proteins is required for yeast chronological ageing. We have used double mutant yeast cells which simultaneously lack the gene for the linker histone (Hho1p) and bear a point mutation in Arp4p (Georgieva et al., 2008, 2012a; Georgieva et al., 2015b; Harata et al., 2000, 2002). By compromising the higher-order chromatin structure organization in these mutants we have studied the chronological ageing and the ability of cells to withstand different stress conditions. Remarkably, we have identified that the complex interplay between the two chromatin players turned out to be imperative for the normal process of chronological ageing and moreover for cellular adaptability to stress.

2. Materials and methods

2.1. Yeast strains

Saccharomyces cerevisiae strains with the following genotypes were used in the current study:

WT (DY2864, *ARP4* wild type) MATa his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3 (Harata et al., 2002)

arp4 (DY4285, *arp4* mutant) MATa his4-912δ -ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26 (Harata et al., 2002)

hho1delta (*HHO1* KO derivative of DY2864) *MAT***a** *his4-912δ -ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3 ypl127C::K.L.URA3* (Georgieva et al., 2015b)

arp4 hho1delta (double mutant derivative of DY4285) MATa his4-912δ -ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26 *ypl127C::K.L.URA3* (Georgieva et al., 2015b)

2.2. Culture media

Yeast strains were grown in rich YPD medium (1% Yeast extract, 2% Bacto peptone, 2% Dextrose) or in SDC media, containing 1.7% yeast nitrogen base w/o amino acids, 20 μg/ml of all required supplements according to the genotype of the strains and 2% Dextrose. Solid media contained 2% agar.

2.3. Chronological lifespan analysis

2.3.1. Chronological survival

Chronological lifespan was monitored according to (Fabrizio et al., 2004; Longo et al., 2012). All chronological lifespan studies were performed in cells grown in liquid synthetic complete media containing glucose (SDC) supplemented with standard amounts of amino acids and nucleotide bases. Overnight (ON) yeast cultures were diluted in fresh SDC media to an initial cellular density of 1×10^5 cells ml^{-1}). Cell cultivation continued for 19 days at optimal conditions (30 °C) in a water bath shaker. Cellular viability was determined at days: 3rd, 5th, 9th, 12th, 14th and 19th by CFU assay as described below. Cell culture aliquots were taken at the defined time points and after serial dilutions approximately 10^2 cells were plated on YPD agar petri dishes. Following cultivation at 30 °C for 48 h, the number of colonies was scored on the basis of viable cells' ability to propagate and form colonies. The experiments were done in three independent repetitions and the results were analysed by Microsoft Excel 2010 and GraphPad Prizm 3.0 software.

2.3.2. Analyses of cell cycle

100 μl aliquots from chronologically ageing yeast cells were taken from the culture and cells were fixed with 95% ice-cold ethanol at -20 °C, ON. Just prior the analysis, cells were harvested by centrifugation for 5 min at 1500 × *g* and resuspended in 1 ml of 50 mM sodium citrate, pH 5.6. After sonication for 20 s with output power of 40 W, the yeast cells were treated with RNase A (0.25 mg/ml) for 30 min at 37 °C and then washed with distilled water. At the next step cells were resuspended in S buffer (1 M Sorbitol; 25 mM NaH_2PO_4, pH 6.5) and treated with 0.25 mg/ml Zymolyase 20 T (Seikagako Corp.) for 15 min at 37 °C. Cells were then washed in 50 mM sodium citrate and further stained in 50 μg/ml propidium iodide for 20 min in dark.

FACS analyses were performed on BD FACSCalibur™ with excitation of 488 nm and emission of 564–606 nm settings filter (FL2), using Cell Quest software. Data acquisition was performed with FlowJo *V_10* software for single-cell flow cytometry analysis.

2.3.3. Vacuole morphology examination

Yeast vacuoles were stained with Neutral red as described in other authors' works (Kuchitsu et al., 1987) with slight modifications. In brief, aliquots from the chronologically ageing yeast cultures were centrifuged and cell pellets were washed with sterile distilled water. Cells were then incubated in 50 mM HEPES-KOH buffer (pH 7.6) containing 6.9 mM neutral red (CAS-No553-24-2) for 5–10 min. Stained yeast vacuoles were visualized under a light microscope Lietz (Orthoplan, VARIO ORTHOMAT 2) and were documented using Olympus μ800 camera.

For quantitative assessment of vacuolar morphology we have assumed a classification according to the previously reported studies (Abudugupur et al., 2002; Li and Kane, 2009). To this end, at least 50 cells were examined per each time point. Based on the number, the size and the shape of their vacuoles cells were classified into three well distinguishable groups: Type 1 represented cells with one central vacuole; Type 2 – cells with one or several smaller vacuoles; and Type 3 – cells with one swollen, big and highly wrinkled vacuole.

2.4. Analysing the ability of yeast mutants to survive different types of stress

2.4.1. Induction of replicative stress

Yeast cells from mid logarithmic phase (10^6 cells ml^{-1}) were treated with 200 mM HU for two hours at 30 °C. Cells were then washed and inoculated in YPD in order to allow recovery from the stress. 10^2 cells were plated on solid YPD media and were cultivated for three days at 30 °C. The CFUs of the studied mutants were counted and compared to the CFUs of wild type. FACS analyses were performed prior and after HU treatment. Recovery after replicative stress was followed by studying the transition of HU treated cells through the phases of the cell cycle for a period of 18 h.

2.4.2. Cold temperature stress

Mid-logarithmic yeast cells (10^6 cells ml^{-1}) were incubated at 10 °C for 14 h and subsequently 10^2 cells were plated on YPD solid media and were cultivated for 3 days at 30 °C. CFUs were counted for each strain and were compared to the CFUs of controls. Cold stress tolerance was further assayed by FACS analyses for a period of 5 h after the stress. Control cells were cultivated at optimal conditions and aliquots were also analysed by FACS at same periods of time as for the treated samples.

2.4.3. Oxidative stress

Cellular viability after induction of oxidative stress was assessed after treatment of logarithmically growing cells (day 1st) and cells that have entered post-diauxic phase (day 3rd) with hydrogen peroxide. Exponentially growing and stationary phase yeast cell cultures were treated with 100 mM H_2O_2 for 30 min at 20 °C. Six 10-fold dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were prepared for each test strain. 50 µl from dilutions ranging between 10^{-3} - 10^{-6} were dropped on YPD agar plates and the growth phenotype of treated (wild type and mutant) cells and untreated controls was examined after incubation at 30 °C for 48 h.

2.5. Gene expression analyses

2.5.1. RNA isolation

Yeast cells from the four studied strains were cultivated at optimal and under stress conditions (as described in 2.4). Aliquots were taken and total RNA was isolated according to (Collart and Oliviero, 2001). Briefly, cells were washed with 1 ml of ice-cold RNase-free water and collected by centrifugation at 1500*g* for 10 min. Cell pellet was resuspended in 400 µl of TES solution (10 mM Tris, pH 7.5, 10 mM EDTA, pH 7.5 and 0.5% SDS) followed by addition of 400 µl of pre-warmed at 65 °C acid phenol and incubation for 60 min at 65 °C with 10 s vortexing at every 15 min. After centrifugation for 15 min at 10 000*g* the purification of nucleic acids continued with three subsequent extractions with 400 µl of hot acid phenol each and a final extraction with 400 µl chloroform. Nucleic acids were precipitated at -20 °C ON with three volumes of cold 100% ethanol and 1/10 volumes of 3 M sodium acetate, pH 5.2. Total RNA was then washed with 70% ethanol, dried and dissolved in RNase-free water. The concentration and purity of the isolated total RNA was determined using NanoDrop® ND-1000 spectrophotometer.

2.5.2. First strand cDNA synthesis and quantitative PCR (RT-qPCR)

The level of the *ScACT1*, *ScCDC28* and *ScRAD9* mRNAs was analysed by real-time reverse transcriptase PCR (RT-qPCR). 1 µg of DNase I-treated total RNA was reverse transcribed into cDNA using oligo-dT$_{18}$ primer and RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions.

Gene expression analyses were performed with Maxima® SYBR Green qPCR Master Mix (Fermentas) on Rotor Gene 6000 (Corbett LifeScience) following manufacturers' protocols. All primer pairs were designed through SGD Web Primer design tool. qPCR was performed in duplicate, using 1/40th volume (*i.e.* 25 ng total RNA) of the cDNA template and 0.2 µM each of the forward and the reverse gene-specific primer (Table 1) in a 20 µL reaction mixture. Cycling conditions used for all primer sets consisted of an initial denaturation at 95 °C for 10 min and 45 cycles of: 95 °C for 15 s, 60 °C for 60 s. The specificity of the resulting amplicons was confirmed by melting curve analysis and agarose gel electrophoresis.

The expression of each gene was normalized to the reference gene *ScACT1*. Relative quantification expression levels were calculated by comparative C$_T$ method (Schmittgen and Livak, 2008) *via* Rotor-Gene Q Series Software 1.7.

Table 1
Primers for qPCR.

Oligomer name	Sequence 5′–3′	Amplicon length, bp
*ScACT1*_For	CGGTAACATCGTTATGTCCGGTG	93
*ScACT1*_Rev	ATGGAAGATGGAGCCAAAGCG	
*ScCDC28*_For	AGGAAACCAATCTTCAGTGGCGA	91
*ScCDC28*_Rev	CTGGCCATATAGCTTCATTCGGC	
*ScRAD9*_For	GCATGTTTGAGCGCAGGTAG	102
*ScRAD9*_Rev	TCTGGGTACTAAAGAATCTAAGGCA	

2.6. Reproducibility of the results and statistical analysis

All experiments were repeated twice and the data reported for CFUs counts represent the Mean ± SD. Results were statistically elaborated by using GraphPad Prizm 3 Software (La Jolla, USA) by One-way Anova analysis of variance. Difference in values was considered significant when $p < 0.05$.

3. Results

Saccharomyces cerevisiae cells are a brilliant model organism for studying ageing. With their high genetic tractability and the possibility to investigate both replicative (RLS) and chronological lifespan (CLS) they offer opportunities for addressing major questions regarding ageing in higher eukaryotes (Kaeberlein, 2010; Mazzoni et al., 2013). Some of these questions take up serious matters like the relationship between the non-dividing state of chronological ageing, and the dividing state during which cells age replicatively. In point of fact no one other organism allows addressing of this issue. Our goal was to establish approaches that target better understanding on how chromatin higher-order organization and its dynamics are involved in the process of chronological ageing when cells are alive but do not divide.

3.1. Hho1p interaction with Arp4p is crucial for yeast chronological lifespan

In order to study the influence of chromatin organization on the process of chronological lifespan we have used yeast chromatin mutants which we have previously demonstrated to have abnormal higher-order chromatin structure organization due to the lack of the gene for the linker histone and because of a point mutation in the gene for Arp4p (Georgieva et al., 2015b; Uzunova et al., 2013). The way these cells were chronologically ageing was further examined following standard protocols as described in (Georgieva et al., 2015a; Longo and Fabrizio, 2012; Uzunova et al., 2013).

Investigation of yeast chronological lifespan (CLS) started by diluting the overnight cultures of the four studied *Saccharomyces cerevisiae* strains – the wild type (wt) and the mutants *hho1delta*, *arp4,* and *hho1delta arp4* to an initial density of 1×10^5 cells ml^{-1} in 50 ml of synthetic complete medium, containing 2% glucose (SDC). The yeast cultures were then incubated at 30 °C for a period of 19 days. After approximately 10 h of exponential growth, the yeast switched from a fermentation to a respiration-based metabolism. According to other studies after this 'diauxic shift', the yeast cells obtain most of the energy from mitochondrial oxidative phosphorylation (Fabrizio et al., 2004). The diauxic shift is then followed by a post-diauxic phase, in which culture growth continues slowly until approximately the 48th hour, and then stops. According to standard protocols for yeast chronological lifespan studies (Breitenbach et al., 2012; Longo and Fabrizio, 2012) in the post diauxic phase the metabolic rates in the yeast cells remain high until day 5–6. Then cells get into stationary phase. Therefore, in our experiments we have measured yeast cell cultures' optical densities in

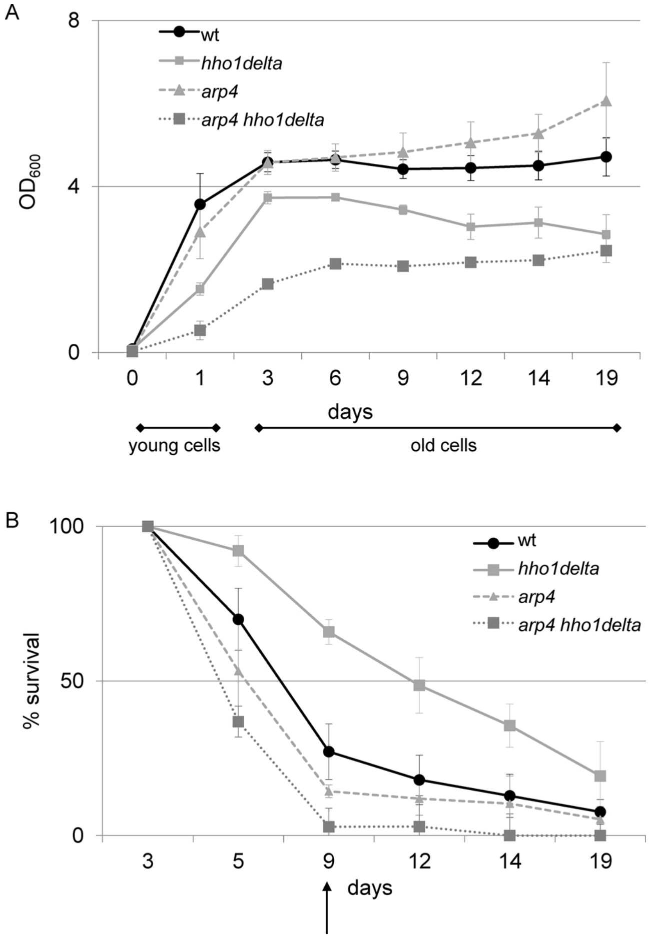

Fig. 1. *arp4 hho1delta* double mutant cells are short-lived.

Yeast cellular growth and chronological lifespan were estimated by measuring the optical density of the yeast cultures (OD_{600}) at 600 nm at eight time points (day 1, 3, 6, 9, 12, 14 and 19) and by calculating the percentage viability of the chronologically ageing cells.

A: Growth of yeast cultures in the timeline of their chronological lifespan.

B: Percentage viable cells.

The percentage of viable cells was calculated from the results of CFU (colony - forming units) experiments as is described in 2.3. Values represent MEAN ± STDV.

minimal media by spectrophotometric measurements at 600 nm wavelength (OD_{600}) at certain days and the results are plotted as growth curves (Fig. 1A). The three chromatin mutants exhibited changed cell culture growth. In comparison to the wild type which we assume as the control in all our experiments, the chromatin mutants lacking the gene for the linker histone *hho1delta* and the double mutant *arp4 hho1delta* had slower growth. Considering the overall growth rate, *i.e.* the average of the values for OD_{600} at all studied time points we have noticed that in comparison to the wt the double mutant cells had in total 50% slower cell growth, while *hho1delta* cells had around 20% slower growth. Notably, the difference in the growth rate between the mutants lacking the gene for the linker histone (*hho1delta*) and the double mutants (*arp4 hho1delta*) started to appear from the first day with the double mutant OD_{600} value being 6 times reduced than the wt OD_{600} at the same day. The results speak of strong synergism between the two mutations (the deletion of the gene for the linker histone combined with the point mutation in *ARP4*) in relation to cellular growth during CLS. In the time course of CLS the double mutant cells have the severest cell growth phenotype which proves our hypothesis that cells with aberrant chromatin structure that lack the gene for the linker histone – *hho1delta* and *arp4 hho1delta* (Georgieva et al., 2012a,b) could experience cellular growth stress. Surprisingly, *arp4* mutant exhibited slightly faster cellular growth after day 6th in comparison to all other strains but following the measurements we consider this increase as insignificant especially for the time points between days 6th and 14th.

In a standard post-diauxic experiment we have monitored the survival of the four studied strains by measuring the ability of individual yeast cells to form colony-forming units (CFUs) on YPD plates starting from the 3rd day and taking samples for cellular viability studies at the 5th, 9th, 12th, 14th and 19th day of cultivation. CFUs were counted and percentage survival was assessed for each strain independently, with the number of CFUs at day 3 considered to be the initial survival (100% survival). Surprisingly, *hho1delta* mutants exhibited better survival rates than all other strains (Fig. 1B). In contrast, the double mutant cells exhibited the poorest cellular survival which again proved the strong synergistic cellular growth phenotype of the double mutants. These cells died at day 9th, while the other mutant cells preserved their survival rate until day 19th though at a highly reduced rate. 100% of dead cells at early time points in a chronologically ageing yeast population is considered as a hallmark of premature ageing phenotype and as an indication of abnormal cellular ageing of the cell population. The result for the double mutant cells, which even at day 5th had 50% reduced cellular viability in comparison to the wt and at day 6th had almost 100% dead cells, proved that the double mutant cells have abnormal chronologic ageing. And as this is a double chromatin mutant in which the higher-order chromatin organization is abolished (Georgieva et al., 2015b) the result suggests that chromatin thus indeed serves as a platform for the normal process of cellular ageing (Georgieva et al., 2016).

3.2. Premature ageing phenotypes are detected at early stages of the chronological lifespan of yeast cells with mutations in HHO1 and APR4

The yeast vacuoles, often described as the counterpart of mammalian lysosomes, are dynamically regulated, both in number and size in response to environmental conditions, including those linked to replicative lifespan (Li and Kane, 2009). For instance, nutrient restriction, which extends lifespan causes yeast cells to undergo vacuolar fusion events, leading to one large vacuole in place of several normal in size vacuoles. Moreover, recent results show that vacuolar membranes become disordered in replicatively old cells (Gebre et al., 2012; Tang et al., 2008). In order to precisely characterise the way the studied chromatin mutants which had abolished normal chromatin organization age chronologically and to look for any changes in the vacuolar morphology during this process we have performed light microscopy vacuolar morphology examinations at certain time points of the CLS.

3.2.1. Chromatin mutants had altered vacuolar morphology at early stages of their lifespan

Yeast vacuolar morphology was studied by staining the cells gathered at four time points (days 1st, 6th, 12th and 20th) with Neutral red. The observation of stained vacuoles was carried out under a light microscope. For quantitative assessment of vacuolar morphology cells were classified into three groups according to (Li and Kane, 2009). Cells with one central vacuole was designated as Type 1; Type 2 presented cells with one or several smaller vacuoles; and cells with one swollen wrinkled vacuole were classified as Type 3 (Fig. 2A). The last type of vacuoles are accepted as vacuoles indicative for already aged yeast cells (Georgieva et al., 2015a; Li and Kane, 2009). We have followed the dynamics of the accumulation of cells with the three types of vacuoles during the chronological lifespan in the studied chromatin mutants and in the control wt (Fig. 2). Notably, in all cultures the percentage of cells with the first type of vacuoles gradually decreased with time whereas the percentage of cells with the third type increased. The differences in the way this interchange happens is indicative for the way by which cells age and change their metabolism. The intersection point of bars representing the first and the third vacuolar morphology types was on

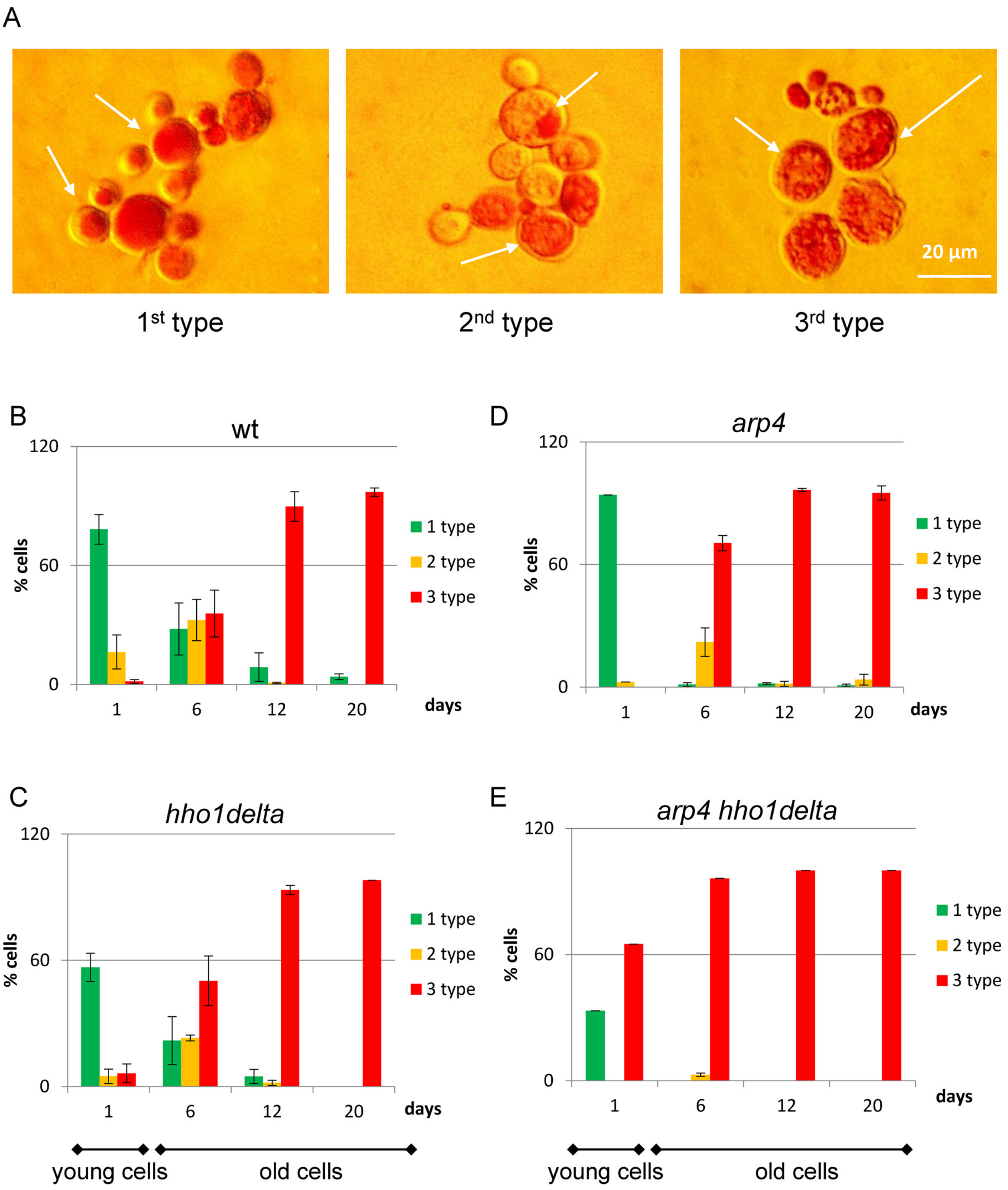

Fig. 2. Vacuole morphology is disturbed earlier in the mutants.
Yeast vacuolar morphology and acidity was studied by staining of cells gathered at four time points (day 1, 6, 12 and 20) with Neutral red. The observation of stained vacuoles was carried out with a light microscope Lietz (Orthoplan, VARIO ORTHOMAT 2). For quantitative assessment of vacuolar morphology cells were classified into three groups according to the type of their vacuoles: Type 1 represented cells with one central vacuole; Type 2 – cells with one or several smaller vacuoles; and Type 3 – cells with one swollen wrinkled vacuole.
A: Classification of cells depending on the vacuolar morphology observed during chronological ageing.
B: Distribution of the three types of vacuoles during the chronological lifespan of the control wild type.
C: Distribution of the three types of vacuoles during the chronological lifespan of *hho1delta* mutant.
D: Distribution of the three types of vacuoles during the chronological lifespan of *arp4*.
E: Distribution of the three types of vacuoles during the chronological lifespan of *arp4 hho1delta* double mutant cells.

the 6th day for the wild type (Fig. 2B), whereas for the *hho1delta* and *arp4* mutants this point was earlier (Fig. 2C and D). For *hho1delta* cells the 3rd type of vacuoles already prevailed at day 3rd. While on day 6th almost 50% of all *hho1delta* cells had one big central and highly wrinkled vacuole (Fig. 2C). For *arp4* mutants at day 6th almost 70% of the vacuoles were classified in the 3rd group (Fig. 2D). A well pronounced contrast demonstrated the double mutant (Fig. 2E). It had highly changed distribution of the three types of vacuoles when compared not only to the wt but also to the other two single mutants. At day 1st almost 70% of cells were with the 3rd type of vacuoles which was an indication of accelerated ageing and presence of metabolic stress in these cells. This trend continued until the end of the chronological

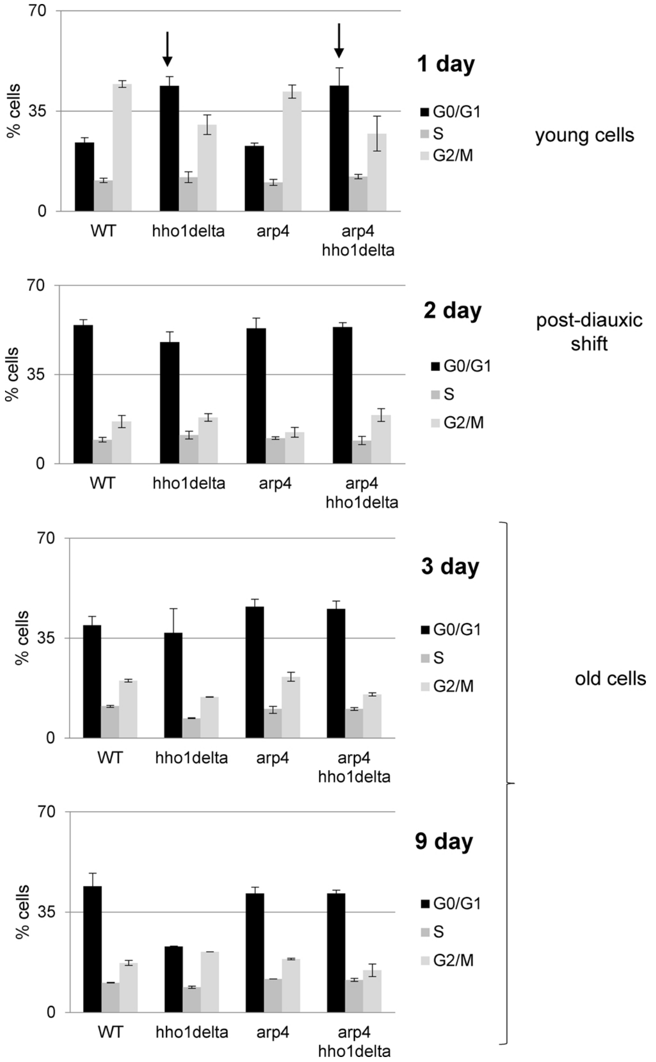

Fig. 3. *hho1delta* cells and the double mutant cells *arp4 hho1delta* predominantly accumulate cells in G0-G1 phases of the cell cycle even at the early (logarithmic) stage of their chronological lifespan.
The proliferative potential of the yeast mutant cells was assessed by FACS analysis and was compared to the control wild type cells. The aliquots were taken at the four time points: day 1, day 2, day 3 and day 9. Percentage of cells in different phases of the cell cycle has been calculated by means of specialized software FlowJo V10.

lifespan studies with the 3rd type of vacuoles being monotype in the studied double mutant cells (Fig. 2E).

3.2.2. Linker histoneless chromatin mutants had reduced proliferation capacity

In order to see how the proliferation activity is affected in the chronologically aging yeast mutants we have performed FACS analysis on cell aliquots taken at days 1st, 2nd, 3rd and 9th. This allowed us to compare the progression of the mutant cells through the phases of the cell cycle with that of the wild type (Fig. 3). As can be seen from Fig. 3 the mutants that lack the gene for the linker histone, *i.e* the single mutant *hho1delta* cells and the double mutant *arp4 hho1delta* demonstrated abnormal cell cycle in comparison to the control wt at day 1st (Fig. 1, day 1, arrows). The strength of this result comes from the fact that day 1st designates a population of young cells. At the beginning of the chronological lifespan the mutants that lack the gene for the linker histone exhibited predominant accumulation of cells in G0/G1 phase of the cell cycle which was not the case for the other two strains (Fig. 3, day 1st). Around 50% of *hho1delta* and *arp4 hho1delta* cells were in G0/G1 at day 1st. The wt and *arp4* cells demonstrated normal distribution through the cell cycle phases with more cells in G2/M phase which is a characteristic of logarithmically growing yeast cell cultures (Zhang and Siede, 2004) at day 1st of the chronological lifespan (Breitenbach et al., 2012; Kaeberlein, 2010). Probably, the lack of the proper chromatin compaction of the ageing chromatin mutants without the gene for the linker histone (*hho1delta* and *arp4 hho1delta*) impaired cellular proliferation capacity at early stages of the chronological lifespan (Fig. 3, 1st day) and thus affected the later stages of cellular ageing (Figs. 1A and B and 2B and D).

3.3. The deletion of the gene for the yeast liker histone in arp4 mutant influences S. cerevisiae cells' potential to cope with different stress conditions

During time cellular stress response and repair pathways gradually start to decline which defines the process of ageing as the outcome of an impaired balance between damage and repair (Haigis and Yankner, 2010). Oxidative and replicative stress occur throughout the whole lifespan and when repair pathways are impaired this leads to premature ageing phenotypes. In order to study the way cells with disordered chromatin organization response to different stress conditions we performed a set of experiments in which we studied cellular viability of the studied chromatin mutants after three types of stress (replicative, cold temperature and oxidative stress) and moreover followed the change in expression rate of a set of genes which are known to have a role in cellular damage response.

Having in mind the importance of chromatin remodelling complexes in DNA damage repair we expected that the mutant strains will exhibit low resilience to stress in comparison with the wild type.

3.3.1. Replicative stress affects chromatin mutants severely

Cultivation of cells on media supplemented with hydroxyurea (HU) was performed for assaying the ability of the studied mutants to cope with and restart hydroxyurea-stalled replication forks. To this end, logarithmically growing cells from the four studied strains were treated with 200 mM HU for two hours, then plated on YPD agar and cultivated for three days at 30 °C. The CFUs of both treated and non-treated cells were counted. The number of colonies from HU treated cells was presented as a percentage of the number of colonies from the non-treated cells (Fig. 4A). The single *arp4* mutant exhibited severe growth defects after treatment with HU - only 17% of the cells remained viable. As for the *hho1delta* and the double *arp4 hho1delta* mutants around 40% and 42% of the cells, respectively, survived. The double mutant exhibited strong positive epistatic phenotype in cellular survival after replicative stress in which cells had weaker mutational defect after HU treatment than the predicted from the individual *arp4* mutant.

The potential of HU treated cells to recover after replicative stress was further assayed by FACS analyses. Aliquots from the four studied strains were compared by FACS prior and immediately after the stress. Additionally HU treated cells were washed in YPD and were left to recover by cultivation at optimal conditions for 18 h. At certain time points (1 h 30 min, 3 h, 16 h and 18 h) cells were FACS analysed. Results are shown on (Suppl. Fig. 1A). As could be seen from the histograms most HU treated cells were blocked in G0/G1 after the stress with this effect being more pronounced for *hho1delta* and *arp4 hho1delta* mutants. Both mutants had approximately 90% of its cells blocked in G0/G1 while *arp4* and the wild type had approx. 60%. The recovery after stress surprisingly showed that *hho1delta* and *arp4 hho1delta* mutant cells much faster exit from G0/G1 block. The transition of these cells from G0/G1 into G2/M was observed on the 3rd hour while for WT and *arp4* cells this happened much later - on the 16th hour. These observations reveal the complex HU cell survival phenotype occurs because of the strong positive epistatic Hho1p - Arp4p interaction. On one hand, the results show an enhanced sensitivity of the mutants lacking the gene for the linker histone to HU and on the other, a faster recovery after the stress. Taken together these results confirm that the interaction

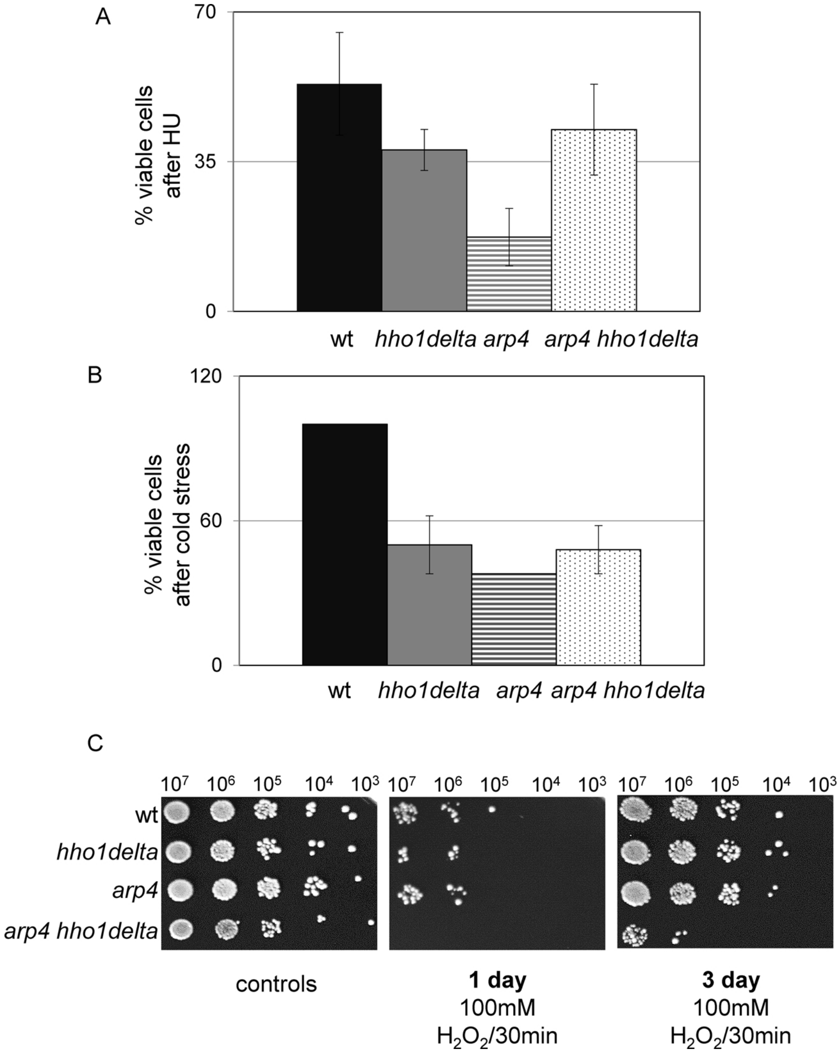

Fig. 4. Potential of cells to survive different stress conditions.

Cells from control cultures and cells exposed to replicative, cold temperature and oxidative stress were plated on solid rich media and were cultured at 30 °C for three days. CFUs (colony forming units) or spot assays were used to quantify the survived cells. Three repetitions of the experiment have been done and values for cellular survival show MEAN ± STDV.

A: Replicative stress induced by hydroxyurea (HU).

B: Cold temperature stress induced by incubating of cells at 10 °C for one night.

C: Oxidative stress induced by treatment of cells in 100 mM H_2O_2.

between Hho1p and Arp4p is multifaceted and complicated. Namely, the knock-out of the *HHO1* gene in *arp4* mutant cells partly suppresses *arp4* mutation and allows cells to recover faster after HU stress which is confirmed by the results from viability experiments after this stress.

3.3.2. *Cold temperature tolerance is reduced in the three studied chromatin mutants*

Other type of stress used in our experiments was cold temperature stress. We wanted to check the cold temperature tolerance of the cells by culturing them at 10 °C for 14 h. Subsequently, equal numbers of cells (10^2 cells) were plated on complete media and were left to grow at 30 °C for three days. Cellular viability was assessed by counting the number of viable cells per each strain calculating it as a percentage of the non-treated viable cells. As can be seen on Fig. 4B the three mutants recovered slowly and quite ineffectively. Only half of *hho1delta* cells were viable, 38% of *arp4* and 48% of the double mutant cells. Apparently, the lack of proper chromatin organization in the three mutant strains affected the ability of these cells to withstand cold stress conditions. In order to follow the way cells adapt to this type of stress we took aliquots from cold temperature stressed cells and by cultivating them in fresh SDC media we allowed them to recover for a period of 5 h. Cells were FACS analysed immediately after the stress and at three time points during recovery after the stress has been induced. The time points were 1 h and 30 min, 3 h and 4 h and 30 min and the results are displayed on Suppl. Fig. 1B. All four strains exhibited a delay in the transition of cells through the cell cycle. The cells of WT and *arp4* mutant showed insignificant recovery after the stress (see Fig. 4B and Suppl. Fig. 1B *arp4*). Both *hho1delta* cells and the double mutants though manifested higher sensitivity to cold stress in comparison to the wild type recovered more successfully which was another proof of the already observed positive epistasis between the two mutations in regard to stress tolerance and recovery after it.

3.3.3. *Oxidative stress affects rigorously the double mutant arp4 hho1delta cells*

The studied yeast strains were exposed to oxidative stress during the time course of their chronological lifespan. Logarithmically growing cells (day 1st) and cells which have already entered the stationary phase (3rd day) from the four strains have been subjected to oxidative stress. Cells were treated with 100 mM H_2O_2 for 30 min and were allowed to recover on YPD agar plates for three days (Fig. 4C). We have selected these time points following other authors' and our own previous results which demonstrate that day 1st is the most indicative for exponentially growing young yeast cells while day 3rd under our conditions marks the first days of the chronological ageing of yeast cells (Longo and Fabrizio, 2012; Uzunova et al., 2013). As can be seen from Fig. 4C all studied yeast strains designated as young in our experiment (day 1st) exhibited high sensitivity to oxidative damage. The most pronounced sensitivity to H_2O_2 and to some extent a well pronounced synergistic phenotype demonstrated the young cells of the double mutant which was not able to recover at all. Old cells (day 3rd) appeared to tolerate more oxidative stress. All four strains demonstrated ability to recover faster after oxidative damage when compared to the cells in day 1st. However, after H_2O_2 was used as a stressor during the time course of their chronological lifespan the double mutant old cells again showed higher sensitivity in comparison to the other mutant cells and the wild type itself.

3.4. *Chromatin mutants express CDC28 and RAD9 differently after induction of replicative and cold temperature stress*

To examine some of the molecular mechanisms underlying the observed faster recovery after replicative stress of the *arp4 hho1delta* double mutant as well as the higher cold temperature sensitivity of the three mutants we have studied the expression levels of two genes, *CDC28* and *RAD9*. Results are shown on Fig. 5. There was no detectable change in the expression levels of *CDC28* mRNA in the wild type cells regardless HU treatment. All control (untreated) probes of the three chromatin mutants have demonstrated an interesting trend of *CDC28* expression profile having lower concentrations of *CDC28 mRNA*. When these mutants were treated with HU a slight increase in *CDC28* expression rate was observed in *hho1delta* and *arp4* mutants, respectively 2 and 1.3 times higher than in the wt control. However, when comparing treated with untreated single mutants, a significant rise in *CDC28* level was detected in the former. The double mutant did not change the expression rates of *CDC28* gene under these particular conditions (Fig. 5A). The expression of *RAD9* (Fig. 5B) in the control cells prior and after HU stress remained particularly unchanged. Again the expression rate of the gene of interest in all untreated chromatin mutants was slightly lower than in the wild type cells. When chromatin mutants were treated with HU we observed up to 3 times higher expression rate of *RAD9* gene in *arp4* mutant than in the WT (Fig. 5B, *arp4,* patterned box). While in *hho1delta* and the double mutant it was levelled down around 2 and 1,2 times, respectively (Fig. 5B, see *arp4,* patterned box). The supposed change in the expression levels in the mutants of the same two stress-responsive genes was also examined after cold temperature stress. The *CDC28* mRNA levels after cultivation of cells for 14 h at 10 °C showed decreased expression rates in all of the strains in comparison to the WT control (Fig. 5C). *RAD9* mRNA levels increased only in the double mutant *arp4 hho1delta* cells, with 2.4 times than in the cold stressed wild type cells (Fig. 5D).

This and the above-described results confirmed the initial hypothesis that both Hho1p and Arp4p interact not only physically but also genetically and that their intrinsic interplay is important for building and maintaining chromatin structure and consequently for the overall cellular morphology as well as stress response and adaptation. Such a synergistic interplay between the combination of the two mutations when we check tolerance of the cells to oxidative stress together with the strong positive epistatic phenotype when replicative and cold temperature stress survival is studied is not surprising as misregulation of chromatin remodelling is connected with major differences in gene expression which inevitably compromise most cellular functions (Magnani et al., 2012). This is highly important as such alterations often lead to abnormalities which could result even in cancer development (Cho et al., 2004).

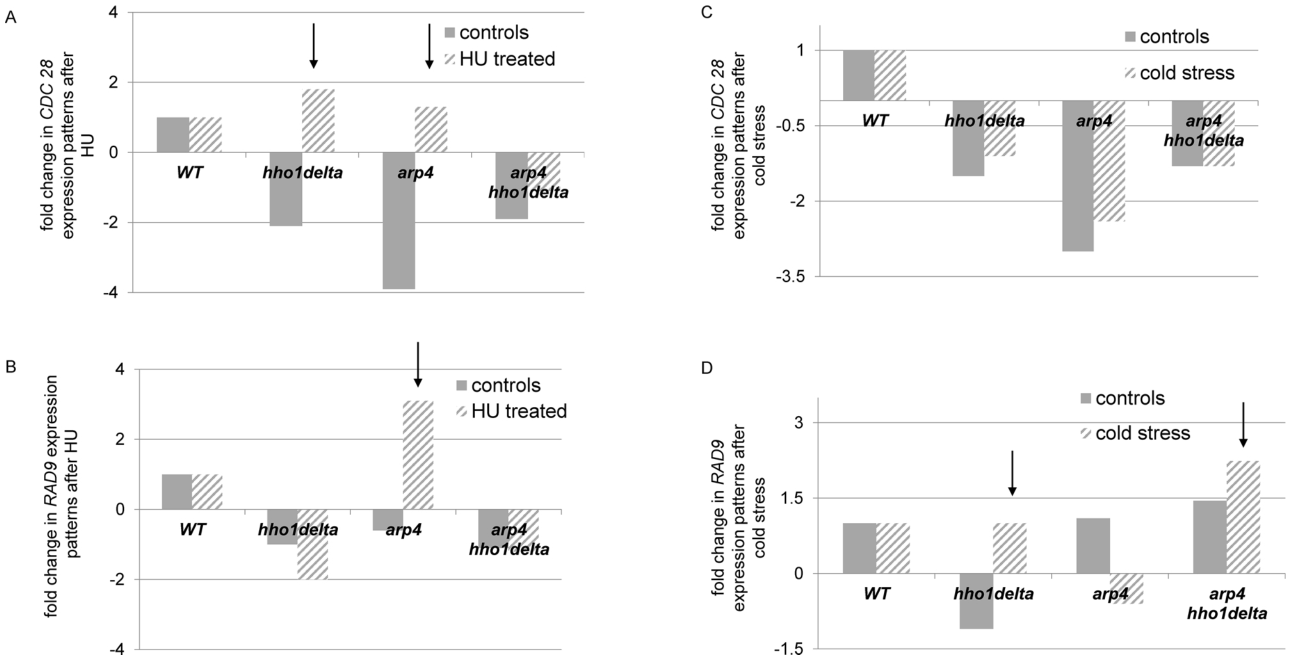

Fig. 5. Reverse transcription qPCR (RT qPCR) analysis of *CDC28* and *RAD9* transcript levels after induction of replicative or cold temperature stress. Immediately after the induced stress the expression levels of *CDC28* and *RAD9* have been studied by RT-qPCR. cDNA in each sample was normalized to the reference gene *ScACT1*. Relative quantification expression levels were calculated by comparative Ct method *via* Rotor-Gene Q Series Software 1.7 and results are presented as graphs in which every bar stands for the gene of interest transcript levels of two technical replicates.

A) RT qPCR of *CDC28* transcript levels after replicative stress.

B) RT qPCR of *RAD9* transcript levels after replicative stress.

C) RT qPCR of *CDC28* transcript levels after cold temperature stress.

D) RT qPCR of *RAD9* transcript levels after cold temperature stress.

4. Discussion

4.1. The cells without the linker histone and bearing a mutation in ARP4 die young and possess premature ageing phenotypes

Cellular ageing is generally defined as the progressive decline in the resistance to stress and other cellular damages, causing a gradual loss of cellular functions and resulting eventually in cell death. During cellular lifespan cells are persistently exposed to different environmental and endogenous stimuli causing DNA damage. Fortunately, cells possess conserved and effective DNA repair pathways by which they can maintain genomic integrity (Attikum and Gasser, 2005; Liu et al., 2012). In the processes of DNA repair chromatin remodelling is crucial, as the organization of eukaryotic DNA into chromatin presents a natural barrier to all events happening on DNA. Studies on normal ageing and human premature ageing syndromes have suggested that accumulated damages might lead to accelerated ageing (Burtner and Kennedy, 2010; Liu et al., 2005). Many hypotheses have been proposed to understand the underlying mechanisms of the ageing process, and these include the disease theory, free radical theory and DNA damage accumulation theory. However, the existence of these theories does not unify all known mechanisms that underlie ageing and yet does not explain the role of the higher-order chromatin structure organization in this process.

In order to study the role of higher-order chromatin organization in maintaining genome stability during ageing we have investigated the way three chromatin mutants age in regard to their compromised chromatin organization. The used model organism is the yeast *S. cerevisiae* and the studied chromatin mutants are *hho1delta*, *arp4* and *arp4 hho1delta,* all well-known with their impaired chromatin structure organization at its upper levels (Georgieva et al., 2008, 2015b; Uzunova et al., 2013). Results have shown that the disturbed interaction between the linker histone and Arp4p (a constituent of several chromatin remodelling complexes) led cells to premature ageing phenotypes and early death in the timeline of their chronological ageing. The double mutant cells were severely affected by the lack of the linker histone and the mutation in Arp4 which proved our hypothesis that chromatin remodelling indeed governs and controls chromatin dynamics in regard to the process of ageing and that this is executed by healthy interactions with the linker histone. Double mutant cells not only die young but also exhibit abnormal growth accompanied by premature ageing phenotypes like impaired proliferation capacity and altered vacuolar morphology. These results are logic in the light of our previous and other authors' studies which do indeed propose that ageing involves global remodelling of chromatin (Adams, 2007; Cavalli and Misteli, 2013) and that the proper chromatin organization is important for cellular ageing (Uzunova et al., 2013). Recent studies on human models of ageing further support our findings. They have demonstrated that human ageing is associated with remodelling of genomic repetitive elements, including attrition of telomeric and NOR-rDNA repeats, as well as reorganization of centromeric DNA elements (Ren et al., 2017). Having in mind that the linker histone is predominantly associated with the ribosomal DNA locus in yeast cells (Levy et al., 2008) it is very logic to assume that this chromatin protein is crucial for the process of ageing. This role is apparently highly supported by the cooperative work between H1 and chromatin remodelling as in the case of Arp4p which is a major constituent of three chromatin modifying complexes in yeast (Harata et al., 1999, 2000).

4.2. The lack of proper chromatin structure in the mutant cells make them more prone to different types of stress

The process of ageing is characterized by nine common hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopez-Otin et al., 2013). At a cellular level, these age-dependent declines can arise from the accumulation of DNA damage. Insufficient DNA repair is generally accompanied by oxidative and metabolic stress and mitochondrial dysfunction. In order to trace the role of chromatin structure organization in genome stability during the process of cellular ageing we have used three types of stress on our chromatin mutants. Hydroxyurea, cold temperature and oxidative stress were used in our studies in order to follow the potential of the studied chromatin mutants to survive at different stress conditions. The guiding role of Ino80 in DNA repair and in the potential of cells to overcome stalled replication forks has been proved by certain amount of data (Papamichos-Chronakis and Peterson, 2008). As Arp4p is an essential subunit of this complex responsible for its ATP-dependent remodelling properties, it was of interest to study how cells with mutated Arp4p would behave under replicative stress. The obtained results were quite surprising. The *arp4* mutant cells do exhibit lower survival level after HU stress than the wild type. However, deletion of the linker histone in this mutant enabled the double mutant to recover faster than the wild type after HU treatment. In the case of cold temperature stress double mutant *arp4 hho1delta* presented similar rate of recovery with the single *hho1delta* mutant. Apparently, the lack of the linker histone in *arp4* mutant cells increased the potential of cells to recombine their DNA and thus to exit faster from replication block and other stress conditions. This suggestion is relevant to other authors' data showing that the yeast linker histone suppresses homologous recombination (Downs et al., 2003). The above results were additionally verified by FACS analysis used to study the mutants' recover after replicative stress. Surprisingly, though *hho1delta* and the double mutant *arp4 hho1delta* were most severely affected by HU as almost all of their cells were blocked in G0/G1, they recovered faster after this type of stress. The detected increased expression of *CDC28* in *hho1delta* and *arp4* could explain to some extent these results. *CDC28* is important cyclin-dependent kinase which is associated with normal progression of cells through the phases of cell cycle, with chromosome segregation and with replicative stress (Benton et al., 1993; Lee et al., 2014; Tsuchiya and Fukui, 1986). Apparently, the two mutations in Hho1p and Arp4p make cells more sensitive to stress resulting in change of the expression levels of this kinase which in turn leads to sooner recovery of the double mutant cells from G0/G1 block, especially after HU stress. Recent results point to the fact that elevated expression of *CDC28* could be used as a prognostic factor in nasopharyngeal carcinoma (Lee et al., 2014). Interestingly, our results show too that the revoked interaction between the linker histone and Arp4p sensitizes cells and makes them prone to uncontrolled cellular growth and to major morphological alterations, features characteristic for tumour cells. This in turn strengthens the hypothesis that chromatin structure is a complex epigenetic mechanism, in which many players take part and any misregulation in any one of them or in the interaction among them could potentiate tumour development. This has been proven by the changed expression levels of *RAD9*, which is important for the maintenance of genomic stability and DNA repair. After replicative stress only *arp4* mutants had elevated expression patterns of *RAD9*, thus pointing out to the fact that these cells experienced high genomic instability again stressing on the fact that a broken interaction between Arp4p and Hho1p exposes cells to a risk of genomic instability and inability to deal with different stress conditions. Moreover, the results with the intolerance of the double mutant which lacked the gene for the linker histone and had a point mutation in *arp4* to oxidative stress further prove beyond doubt that when chromatin structure is abrogated as is in *arp4 hho1delta* cells (Georgieva et al., 2015b, 2012b) it affects cellular adaptability to stress *per se*. The two chromatin mutations in the double mutant cells not only lead to altered and highly disordered organization of chromatin at the most intricate levels of its compaction, *i.e.* the chromatin loops and structures above it (Georgieva et al., 2015b; Uzunova et al., 2013), but also lead cells to premature ageing phenotypes.

These results unambiguously prove the cooperative role of linker histones and chromatin remodelling in ageing by their mutual capacity to pertain higher-order chromatin compaction and thus to maintain genome stability and to allow cells to adapt to stress. Undoubtedly, further studies are needed in order to reveal the deep molecular mechanisms through which the cooperation among linker histones and other chromatin proteins influences and directs cells during the timeline of their ageing.

5. Conclusions

- Hho1p interaction with Arp4p is crucial for yeast chronological lifespan.
- Abrogation of the interaction between the yeast linker histone Hho1p and Arp4p leads to premature ageing phenotypes in the mutant cells.
- The cells without the linker histone and bearing a mutation in *ARP4* die young.
- These chromatin mutant cells possess changed vacuolar morphology at early stages of their lifespan.
- The lack of proper chromatin structure in the mutant cells make them more prone to different types of stress.

Contributions

GM, DS and MG designed the experiments. MG, KU, BV conducted the experiments under supervision of GM and MG. MF and PZ participated in results discussions. MG and GM wrote the manuscript.

Conflict of interest statement

None declared.

Acknowledgements

We are indebted to Prof. M. Harata (Tohoku University, Japan) for providing WT (DY2864, ARP4 wild type) and arp4 (DY4285, arp4 mutant) yeast strains used in this study.

This work was supported by the Bulgarian National Research Fund [grant number: DN 11/15] and NATO Science for Peace and Security programme [grant number: NATO SPS MYP G5266].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mad.2018.07.002.

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