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The Nuclear Actin-related Protein of *Saccharomyces cerevisiae*, Arp4, Directly Interacts with the Histone Acetyltransferase Esa1p

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Ten actin-related proteins are known in *Saccharomyces cerevisiae*, classified into Arps1–10 according to their relatedness to actin. Arp4, a nuclear protein, essential for viability of *S. cerevisiae*, is a component of at least three chromatin-modifying complexes, one of which is the histone acetyltransferase (HAT) complex NuA4. Since recent data point to a role for Arp4 in the recruitment to specific sites of interaction, we tested if Arp4 directly interacts with the HAT Esa1p that is the catalytic subunit of NuA4. We observed that Arp4 directly binds to Esa1p, whereas Act1p, which is also a component of the NuA4 complex, does not interact with Esa1p. The interaction of Arp4 and Esa1p was not abolished by a deletion of one or both of the specific insertions present in the *ARP4* gene. We propose that the interaction of Arp4 with Esa1p is crucial for proper functioning and targeting of the NuA4 complex.

Key words: actin, Arp4, Esa1, histone acetylation, NuA4.

Abbreviations: INO80 (Inositol requiring), a protein complex that exhibits chromatin-remodelling activity and 3′ to 5′ DNA helicase activity; NuA4, nuclear acetyltransferase complex with predominant specificity to acetylate histone H4; SWR1 (sick with rat8 ts), a protein complex that is involved in chromatin remodelling.

Arp4 (originally termed Act3p) is a member of the family of actin-related proteins (Arps). Classes of Arps are numbered according to their similarity to conventional actin, with class 1 being most similar to muscle actin (*1*). Actin and Arps most likely possess a common tertiary structure centred around a highly conserved ATP/ADP-binding pocket (*2*, *3*). On the other hand, the different subfamilies exhibit divergent surface features caused by insertions and point mutations (*4*), suggesting the existence of specific functions for each family member. The ten Arps (Arp1–10) of *Saccharomyces cerevisiae* are located in the cytoplasm (Arp1–3), the nucleus (Arp4–9), or both (Arp10) (*1*, 5, 6).

Gel filtration chromatography experiments revealed that nuclear Arps are essential components of several chromatin-modifying and chromatin-remodelling complexes (*7–10*). However, information about their functions in these complexes is still limited. Arps may be involved in both, the assembly and function of chromatin-modifying complexes.

Interestingly, all nuclear complexes that contain any Arp always contain either at least one additional Arp or actin itself. Arp4 is a subunit of at least three chromatin-modifying complexes: INO80, together with actin, Arp5 and Arp8 (*10*, *11*), SWR1, together with actin and Arp6 (*12*) and NuA4, together with actin (*9*). The histone acetyltransferase (HAT) complex NuA4 consists of at least 12 subunits, virtually all of which are essential for life (*9*, *13–15*). Its catalytic component, Esa1p, is essential for cell cycle progression, gene-specific regulation and has been implicated in DNA repair (*14*, *16*). Arp4 is proposed to play a role in the integrity of the NuA4 complex (*9*), and was recently found to interact with phosphorylated histone H2A (*17*), recruiting the complex to sites of double-strand breaks (DSBs), where acetylation of histone H4 is important for DSB repair. Additionally, genetic studies indicated that Arp4 is involved in transcriptional regulation (*18*, *19*).

It is generally assumed that the 3D configuration of Arp4 resembles more or less the structure of actin, similarly consisting of four domains. The two Arp4 specific insertions are supposed to form additional loops at the surface of the domain according to domain 4 of actin (*2*).

Here we report the ability of native and recombinant Arp4 to directly bind to Esa1 protein *in vitro* and in a two-hybrid assay. We also show that deletion of the Arp4-specific insertions I and II is not lethal, but impairs the resistance to elevated temperature, hydroxyurea (HU) and methyl methanesulphonate (MMS). Sensitivity to the alkylating agent MMS is clearly increased if both, *ESA1* and *ARP4*, are mutated.

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MATERIALS AND METHODS

Plasmids and Strains

Bacteria

For bacterial expression, the full-length yeast *ARP4* coding sequence was amplified by polymerase chain reaction (PCR) from plasmid pIUACT3 (*3*). Using primers arp4fwdNdeI and arp4revXhoI, an N-terminal *Nde*I restriction site was created, integrating the start-ATG of the gene, and the stop codon was replaced by an *Xho*I restriction site (arp4fwdNdeI: GGCGGTGAAAAGTATAC ATATGTCCAATGCTGCTTTG, arp4revXhoI: CGTTTT CTACTCGAGTCTAAACCTATCGTTAAGCAATC). After *Nde*I/*Xho*I digestion, the PCR product was subcloned into the bacterial expression vector pET23a (Novagen). To delete the insertions I and II, mainly distinguishing Arp4 from actin (*2*), we performed additional PCRs, replacing insertion I (C_{598}-T_{669}, P_{200}-Y_{223}), and insertion II (A_{886}-T_{1137}, K_{296}-N_{379}), with GCC GCG (coding for two alanines), and GCA GCC GCG GCT GCT (coding for five alanines) respectively. For bacterial expression, PCR products were subcloned into pET23a, yielding plasmids pET23arp4ΔI, pET23arp4ΔII and pET23arp4ΔI/II. *ESA1*, *esa1L254P* and *esa1-414* were amplified from plasmids pLP949 and pLP952, respectively (*14*), and subcloned (including the stop codon) into pET28b (Novagen) for expression in *E. coli*.

Yeast

The genotypes of all strains used in this study are listed in Table 1. For transformation into yeast, done by the lithium acetate procedure (*20*), all *arp4* alleles were subcloned into the CEN-vector YCplac33 (*21*). Chromosomal mutations of *ESA1* were inserted by recombination using the 'two-step gene replacement' method following a standard protocol (*22*). Chromosomal integration of the *esa1L254P* and *esa1-414* containing plasmids pLP949 and pLP952, respectively (*14*), into the *ESA1* locus of the haploid strain K700, was achieved by prior digestion with *Bst*EII. Haploid strains containing double mutations were derived from spores of diploids resulting from mating single-mutant strains.

All plasmids and alleles were verified by sequencing (VBC Genomics, Vienna).

Two-hybrid Analysis—The BacterioMatch® Two-Hybrid System Vector Kit (Stratagene) was used to examine the interaction of Arp4 with Esa1p and Act1p. The coding sequences of *ARP4*, *ESA1* and *ACT1* were amplified by PCR to add appropriate flanking restriction sites and cloned into the target vector pTRG (*ARP4* with *Not*I/*Xho*I), and the bait vector pBT (*ESA1* with *Not*I/*Bgl*II; *ACT1* with *Not*I/*Xho*I), respectively. The obtained plasmids Arp4pTRG, Esa1pBT and Act1pBT were sequenced (VBC Genomics, Vienna) to verify the integrity of the coding sequences and the correctness of the construction. The plasmids were then used for interaction studies according to the manufacturer's manual. In brief, reporter strain competent cells were co-transformed with bait and target plasmids and cultured on selective LB-TCK (12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, 50 μg/ml kanamycin) and LB-CTCK (LB-TCK plus 250 μg/ml carbenicillin) agar plates. The strain itself is resistant to kanamycin. Resistance to tetracycline and chloramphenicol is diagnostic for the uptake of the plasmids pTRG and pBT, respectively. Colonies on LB-CTCK agar should only appear as a consequence of the interaction of bait and target protein. All controls were included according to the protocol.

Expression of Recombinant Proteins—Recombinant proteins, corresponding to yeast Arp4 and Esa1p were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene). His-tagged versions of recombinant

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

Strain	Relevant genotype	Source
K699[a]	*MAT**a***	K. Nasmyth
K700[a]	*MATα*	K. Nasmyth
AK300[a]	*MAT**a***/α	A. Karwan (*24*)
AK314[a]	*MAT**a***/α *ARP4*/*Δarp4::LEU2*	A. Karwan (*3*)
AK329[a]	*MATα; Δarp4::LEU2* (Ycplac33ARP4)	A. Karwan (*3*)
LK120[a]	*MATα; Δarp4::LEU2* (Ycplac33arp4△I)	This study
LK122[a]	*MATα; Δarp4::LEU2* (Ycplac33arp4△II)	This study
LK124[a]	*MATα; Δarp4::LEU2* (Ycplac33arp4△I/△II)	This study
LK200[a]	*MATα; esa1L254P*	This study
LK210[a]	*MATα; esa1-414*	This study
LK218[a]	*MATα; esa1L254P* (YCplac33ARP4)	This study
LK220[a]	*MATα; esa1L254P* (YCplac33arp4△I)	This study
LK222[a]	*MATα; esa1L254P* (YCplac33arp4△II)	This study
LK224[a]	*MATα; esa1L254P* (YCplac33arp4△I/△II)	This study
LK228[a]	*MATα; esa1-414* (YCplac33ARP4)	This study
LK230[a]	*MATα; esa1-414* (YCplac33arp4△I)	This study
LK232[a]	*MATα; esa1-414* (YCplac33arp4△II)	This study
LK234[a]	*MATα; esa1-414* (YCplac33arp4△I/△II)	This study
AK603[b]	*MATα; pep4-3*, *ura3-Δ1*, *leu2-Δ1*, *trp1* (pFL1/6-1)	This study

[a]These strains have the W303-1A genetic background, *ade2-1*, *trp1-1*, *can1-100*, *leu2-3*, *112*, *his3-11*, *15*, *ura3*, *ssd1*. [b]AK603 is derived from the protease deficient strain 20B12.

proteins were purified by affinity chromatography on Ni^{2+} columns, following the protocol of the manufacturer (Novagen, pET system manual). Proteins were solubilized, purified and stored in buffers containing 6 M urea. The molecular masses of the purified recombinant proteins were analysed by SDS–PAGE.

Purification of Arp4 from S. cerevisiae—Native protein was purified from the protease deficient strain AK603 [(*23*); see Table 1], overexpressing Arp4. Yeast extracts were prepared as described by Weber *et al*. (*24*). The protein was bound to an anti-Arp4 column, the antibodies coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia). Fractions were eluted by pH shift (50 mM glycine–HCl, pH 3), neutralized immediately (2 M Tris) and analysed by SDS–PAGE.

Overlay Assays—Esa1 proteins (purified protein or bacterial extracts) and actin were run on SDS–PA gels (10%) and blotted to transfer membranes (Immobilon-P, Millipore). Unspecific binding was blocked by incubation with 5% dry milk powder in PBST (0.1% Tween 20) for 2 h at room temperature. Arp4 proteins were overlayed in PBST containing 2% dry milk powder overnight at 4°C, followed by immunodetection according to the western blot procedure.

Western Blot Analysis—Western blot analysis was performed with a polyclonal rabbit antiserum against Arp4 (*24*), diluted 1 : 40,000 and with a polyclonal rabbit anti-actin antibody (Sigma A 2066), diluted 1 : 500. Immunoreactivity was detected by horseradish peroxidase (HRP) reaction, using HRP-conjugated secondary antibodies (anti-rabbit IgG, Promega), diluted 1 : 40,000 and a chemiluminescence-developing reagent (ECL system, Amersham Biosciences).

Spot Assays—To determine hypersensitivity to drugs and non-permissive temperature, yeast strains were grown in liquid yeast peptone dextrose medium (YPD), diluted and plated at tenfold dilution steps onto solid YPD, containing additional agents: formamide (FA), HU and MMS. Each strain was pipetted in four dilutions, the spots containing 20,000, 2000, 200 and 20 cells, respectively. All plates were incubated for 3–5 days at temperatures as specified.

RESULTS

Arp4 Directly Interacts with Esa1p In Vitro—Arp4 and actin have previously been shown to be stable subunits of the chromatin-modifying complex NuA4 (*9*). The HAT Esa1p is the catalytic subunit of this complex (*13*).

We were interested if there is a direct interaction of Arp4 with Esa1p. To assess the binding ability, we initially performed a two-hybrid assay. Using the BacterioMatch® Two-Hybrid System Vector Kit (Stratagene), *ARP4* was cloned into the target vector pTRG whereas *ESA1* and *ACT1* were cloned into the bait vector pBT. After transformation into the reporter strain, positive protein–protein interactions were reported by the expression of an ampicillin-resistance cassette, thus enabling cells to grow on carbenicillin plates. Rates were calculated from the number of colonies on plates with carbenicillin (LB-CTCK agar) in relation to the number of colonies appearing on the control plates (LB-TCK agar). For the combination of Arp4 and Esa1p, we observed a significant interaction rate of about 20% carbenicillin-resistant colonies, suggesting positive interaction between these two proteins (Fig. 1). Coexpression of Arp4 and Act1p only yielded about 8% (Fig. 1). This is not significant since it is within the range of the background, which was determined for all three proteins by expression in the presence of the empty second plasmid. These control experiments yielded about 4.5% for Arp4pTRG and 7 and 9% for Esa1pBT and Act1pBT, respectively (Fig. 1).

Subsequently, we made an overlay assay to verify the direct interaction of Arp4 with Esa1p. We overlayed recombinant forms of Arp4, purified to near homogeneity by affinity chromatography, onto extracts of bacteria overexpressing Esa1 proteins, purified Esa1 proteins and actin, immobilized on membranes. As a control, we also applied the native protein, purified from *S. cerevisiae* to the same assay. Antibody specificity was verified afore, checking all purified proteins used for cross reactivity. Figure 2 shows a SDS–PAGE of the set of purified proteins, as well as the immunostain with anti-Arp4 serum. The observed bands of Arp4ΔI, Arp4ΔII and Arp4ΔI/II correlate with the expected sizes of 53.1, 46.8 and 44 kD, respectively. No cross reaction with Esa1 proteins or actin was detected. The serum specifically recognized the Arp4 proteins. The main overlay experiments yielded the result that Arp4 significantly bound to Esa1WT, Esa1L254P and Esa1-414, overexpressed in bacterial extracts (Fig. 3) as well as to the purified Esa1 proteins (Fig. 4). Esa1L254P carries a single leucine to proline substitution at position 254 (within an α-helix). In Esa1-414 a deletion of a single nucleotide at position 1887 leads to a frameshift mutation in codon 414, altering 10 amino acids before terminating the open reading frame 22 amino acids prematurely (*14*).

Whereas the recombinant version of Arp4 bound similarly to all three Esa1 proteins (Fig. 3), native Arp4 preferentially bound to Esa1WT (Fig. 4). Binding of native Arp4 to Esa1L254P and Esa1-414 was weaker, but still significant.

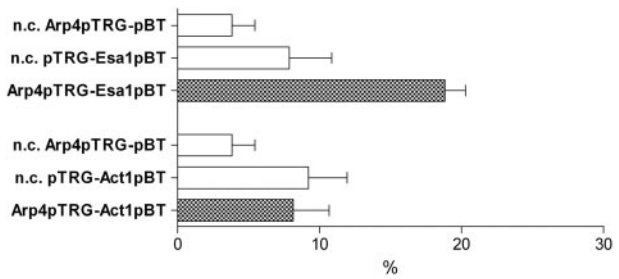

Fig. 1. **Two-hybrid analysis of the interaction of Arp4 with Esa1p and Act1p.** Bars represent percentages of transformants appearing on LB-CTCK agar plates, proportionately to the number of transformants on control plates (LB-TCK agar). Note that the rate is significantly indicating interaction of Arp4 with Esa1p (Arp4pTRG–Esa1pBT), whereas it is only similar to the background for Arp4 with Act1p (Arp4pTRG–Act1pBT). Negative controls (n.c., empty bars) represent transformations with only one expressing plasmid (target or bait) together with the empty second vector (bait or target) as indicated.

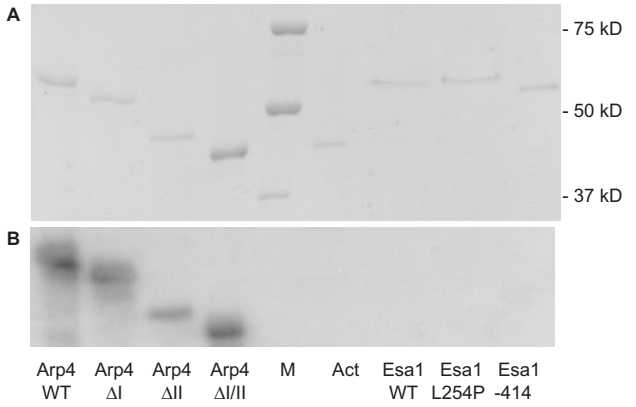

Fig. 2. **Arp4 antiserum specifically recognizes Arp4 proteins, but does not cross-react with actin (Act) or Esa1 proteins.** Purified Arps, actin and Esa1 proteins (400 ng per lane) were run on SDS–PAGE and coomassie stained (A), or blotted and immunostained (B), as described in 'MATERIALS AND METHODS'. Protein-standard (M) sizes are indicated.

Neither native Arp4, nor the recombinant forms of the protein ever exhibited any binding to actin (Figs. 3 and 4).

Arp4 Insertions I and II are not Necessary for Binding to Esa1p—Arp4 possesses two specific insertions of 24 and 84 amino acids, respectively (*2*). These insertions are predicted to be located at the outside of the molecule and potentially responsible for specific interactions. To examine possible binding capacities of these insertions, Arp4 mutant proteins, lacking one of the two insertions, or both, were overlayed onto Esa1 proteins and actin (Fig. 3). None of the two insertions was necessary for the interaction of Arp4 with Esa1 proteins. Binding data obtained with Arp4ΔI, Arp4ΔII and even the double-deletion mutant (Arp4ΔI/ΔII), were similar to those of Arp4. As a control we performed overlay assays using a peptide comprising amino acids S_{269} to D_{399} (insertion II plus adjacent amino acids) and could verify that it does not bind to Esa1 proteins (data not shown).

Likewise, actin did not bind to Esa1 proteins (data not shown). This is especially remarkable, since we observed that the two Arp4-specific insertions were not involved in the interaction of Arp4 to Esa1 proteins. Apart from these insertions, Arp4 shares a number of crucial amino acids with actin and the 3D structure of the molecule is supposed to be very similar to that of actin, reported by Kabsch *et al*. (*25*).

Phenotypic Analysis of S. Cerevisiae arp4 and esa1 Mutant Strains—ARP4 and *ESA1*, both are essential for viability of *S. cerevisiae* (*2*, *26*). To determine distinct phenotypes caused by mutations in the two genes, we tested mutant strains for hypersensitivity to several conditions, including the DNA-damaging agent MMS, HU, FA, ultraviolet radiation (UV) and elevated temperature (37°C). For construction of yeast strains depending on mutated *ARP4*, we used AK314, a heterozygous diploid strain, carrying one wild type and one knockout allele, disrupted with *LEU2*. AK314 was transformed with centromeric plasmids (YCplac33)

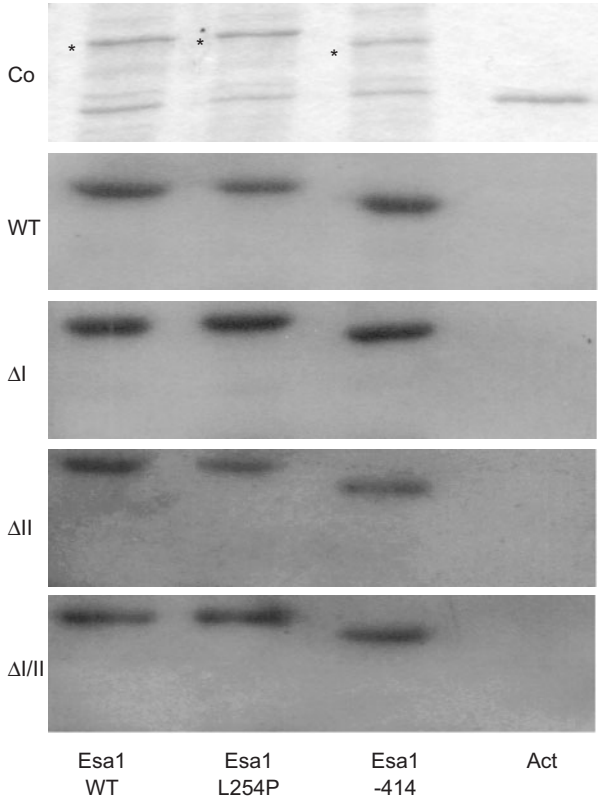

Fig. 3. **Purified recombinant Arp4 protein (wild type, WT) and the deletion variants missing insertions I or/and II bind to Esa1 proteins, but not to actin.** Bacterial extracts containing approximately 1 μg of overexpressed Esa1 protein (Esa1WT, Esa1L254P, Esa1-414), and 1 μg of actin (Act) were run on SDS-PAGE and blotted. After blocking, the blots were each overlayed with 3 ml (5 μg/ml) of Arp4WT (WT), Arp4ΔI (ΔI), Arp4ΔII (ΔII) and Arp4ΔI/II (ΔI/II), respectively, and immunostained. (Co), coomassie staining of a gel, Esa1 proteins marked by asterisks.

containing wild type *ARP4*, *arp4ΔI*, *arp4ΔII* or *arp4ΔI/ΔII*. Surprisingly, subsequent sporulation and tetrad dissection established that all haploid progeny could survive. Therefore, the Arp4 variants lacking one or both insertion(s) are sufficient to provide viability upon standard growth conditions. By the choice of appropriate spore clones we obtained the haploid strains AK329, LK120, LK122 and LK124, respectively (Table 1). These strains were then used for spot assays.

Whereas lack of one of the two insertions had nearly no visible effect, deletion of both insertions caused severe thermosensitivity (Fig. 5A). Furthermore, the *arp4ΔI/ΔII*-cells were markedly hypersensitive to MMS and HU, emphasizing the integral role of Arp4 in DNA repair mechanisms. No hypersensitivity to FA (Fig. 5A) or UV (data not shown) was observed.

Chromosomally Esa1-mutated strains were obtained by gene replacement of the wild type *ESA1* gene with either the *esa1L254P* or the *esa1-414* allele, yielding strains LK200 and LK210, respectively (Table 1). Similar to the *arp4* double mutation, *esa1L254P* and *esa1-414* caused thermosensitivity, and did not provoke a phenotype after treatment with FA (Fig. 5B) or UV

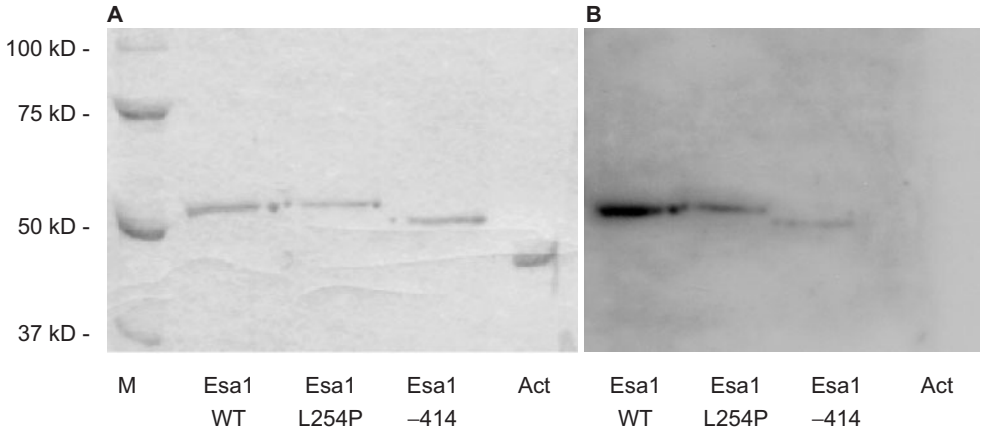

Fig. 4. **Binding of native Arp4 to wild-type (WT) Esa1p is more intense than to Esa1L254P and Esa1-414.** No interaction with actin (Act) could be observed. Equal amounts (approximately 500 ng per lane) of Esa1 proteins and actin were run on SDS–PAGE and coomassie stained (A), or blotted, blocked, overlayed with native Arp4 (3 ml, 5 μg/ml), and immunostained (B). Protein-standard (M) sizes are indicated.

(data not shown). In contrast to the *arp4ΔI/ΔII* double-mutant, both *esa1* mutants were not hypersensitive to HU (Fig. 5B). Only one mutant, *esa1-414*, was hypersensitive to MMS (Fig. 5B).

To be able to examine the consequences of double deficiencies for Arp4 and Esa1p together, we constructed a set of strains based on two strains that contained one of the chromosomal *esa1* mutations together with the *LEU2*-disrupted *arp4* knockout allele. These strains were transformed with CEN-plasmids carrying wild type *ARP4* or one of the *arp4*-mutations *ΔI*, *ΔII*, or *ΔI/ΔII* (strains LK218, 220, 222, 224, 228, 230, 232, 234; Table 1). Strains expressing both proteins—Arp4 and Esa1p—in mutated variants, exhibited thermosensitivity, similarly to *arp4ΔI/ΔII*, *esa1L254P* and *esa1-414*. A slightly increased sensitivity to the ribonucleotide reductase-inhibitor HU was observed for strains expressing either Esa1L254P or Esa1-414, respectively, together with Arp4ΔI/ΔII (Fig. 5C and D). A clear additive relationship of *esa1* mutant alleles concerning MMS hypersensitivity was obtained with the Arp4 insertion I deletion but not with the insertion II deletion (Fig. 5C and D).

DISCUSSION

Arp4 is a subunit of the HAT complex NuA4. At least one of its functions is assigned to the recruitment of this complex to phosphorylated histone H2A at sites of DNA double-strand breaks (*17*). We were interested if Arp4 directly binds to the catalytic subunit of the NuA4 complex, Esa1p, and in the potential role of the Arp4-specific insertions I and II. Furthermore, we investigated the consequences of simultaneous mutations in both proteins, Arp4 and Esa1p.

Considering the finding of Downs *et al*. (*17*), that Esa1p itself is not binding to phosphorylated H2A, an association with Arp4 could potentially ensure the availability of the acetyltransferase at the right place. Here, we investigated if Arp4 interacts with Esa1p. Our data, obtained from a two-hybrid assay and an overlay assay, revealed a direct interaction of Arp4 and Esa1p. Expression in bacteria suggested interaction of Arp4 with Esa1p, but not with Act1p. As a second piece of evidence, purified recombinant and native Arp4 were able to bind to Esa1p *in vitro*, what further confirms this interaction. Whereas purified recombinant Arp4 equally binds well to all Esa1 proteins, native Arp4 preferentially binds to Esa1WT, compared to Esa1L254P and Esa1-414. This is most likely an effect of the missing posttranslational modification in bacteria. Actin, another component of the NuA4 complex, does not bind to Esa1p, or Arp4, respectively.

Both, Arp4 and Esa1p, are indispensable for viability of yeast (*2*, *26*), and essential subunits of the NuA4 HAT complex (*9*, *13*). Recent publications revealed the importance of Arp4 for DSB repair (*27*). Interacting with phosphorylated histone H2A, Arp4 mediates accumulation of the NuA4 complex on H2A P-Ser129-containing sites near DSBs *in vivo*, which allows chromatin remodelling (acetylation of H4 via Esa1p), thus facilitating DSB repair (*17*). The role of Arp4 for targeting was also indirectly supported by the detection of a novel, highly active smaller complex named Piccolo NuA4 (picNuA4). This complex comprises the subunits Esa1p, Epl1p and Yng2p, but not Arp4. Boudreault *et al*. (*15*) proposed that picNuA4 represents a non-targeted histone H4/H2A acetyltransferase activity responsible for global acetylation, whereas the NuA4 complex is recruited to specific genomic loci to perturb locally the dynamic acetylation/deacetylation equilibrium.

We observed that a deletion of both Arp4-specific insertions (I and II) did not cause lethality. The insertions are suggested to be at the surface of the molecule (*2*, *28*), therefore being potential regions of interaction, but so far only one publication exists in which direct binding is assigned to one of the two insertions (*28*). The authors describe the interaction of a peptide, comprising insertion II and flanking amino acids, with yeast core histones, using a yeast two-hybrid assay. This result was later specified by Downs and colleagues (*17*), demonstrating the binding of native Arp4 to phosphorylated, but not

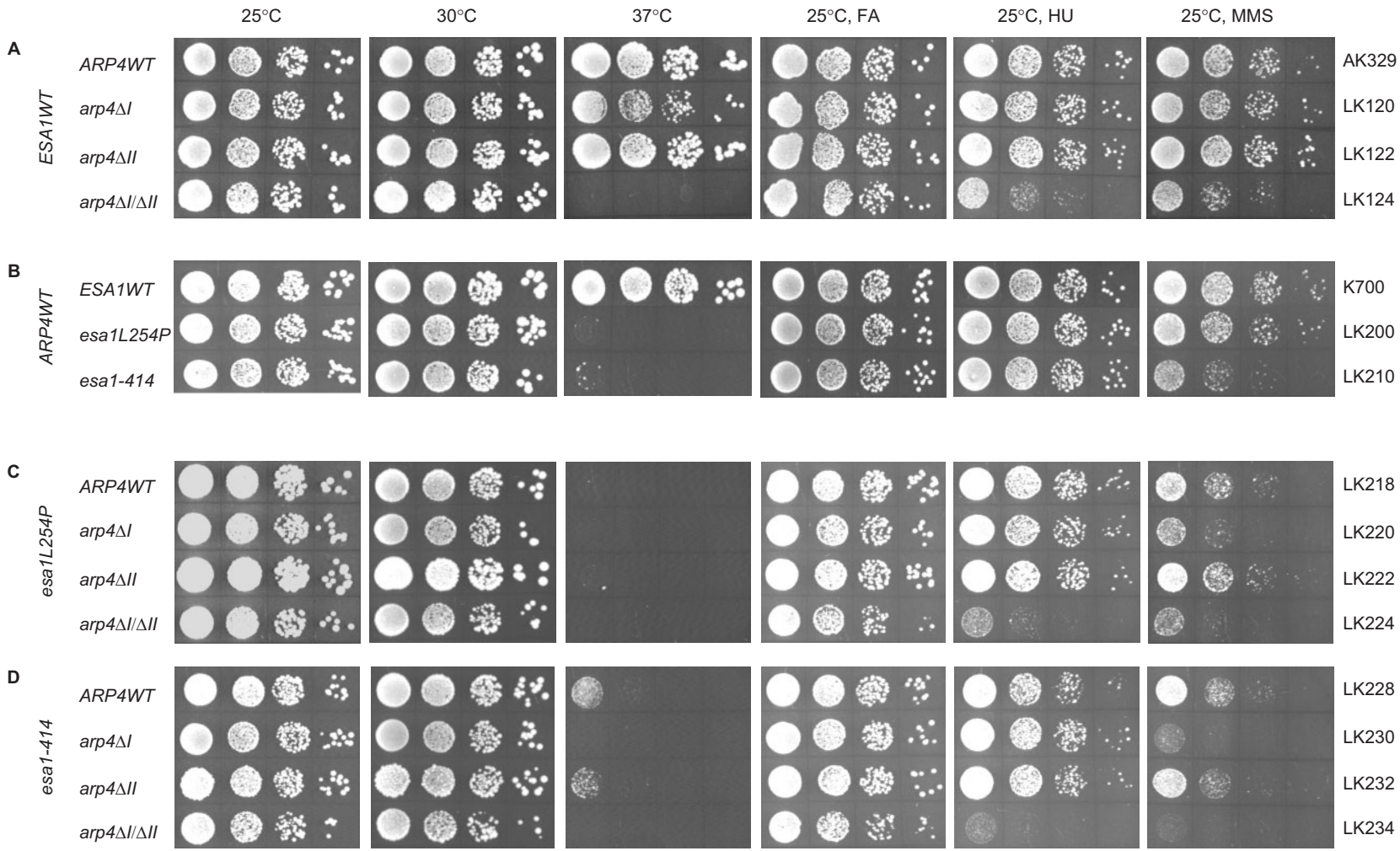

Fig. 5. **Phenotype analysis of *ARP4* and *ESA1* variants.** Strains were grown in synthetic complete medium (SC) at 26°C for two days, 10-fold serially diluted on YPD plates (for incubation at 25, 30 and 37°C), and on FA (1%), HU (85 mM), and MMS (0.02%) plates, respectively, and subsequently incubated at 25°C for 3 days. (A) *ESA1 WT* and *arp4* mutant strains. Deletion of both insertions (I and II) leads to hypersensitivity to MMS, HU and the elevated temperature (37°C). (B) *ARP4WT* and *esa1* mutant strains. Both *esa1* mutations are not able to support growth at 37°C, and *esa1-414* causes hypersensitivity to MMS. (C, D) Effect of *arp4* mutant alleles in an *esa1L254P* (C) or *esa1-414* (D) background. Note the increased MMS sensitivity of the combinations *arp4ΔI/esa1L254P* and *arp4ΔI/esa1-414*, respectively. The respective names of the strains, as listed in Table 1 are denoted on the right side.

to unphosphorylated H2A. Although the insertions are not essential for viability, phenotypic analyses showed that they are nevertheless important for proper functioning of the protein. Deletion of insertion I or II alone did not evoke a phenotypic change. If both insertions together were deleted, the cells were significantly affected. These *arp4ΔI/ΔII* strains were hypersensitive to 37°C, HU and MMS, but insensitive to FA. Cell death at 37°C could be an effect of either impaired function due to improper folding or degradation of the proteins. If the hypersensitivity to the non-permissive temperature would be merely a result of protein degradation, one would expect the strains to be also susceptible to FA, a denaturing solvent that destabilizes non-covalent bindings. Since this was not the case, it is therefore likely, that the impact of the non-permissive temperature was a consequence of functional imbalances following modified protein folding. HU confines the availability of dNTPs for DNA synthesis by inhibiting ribonucleotide reductase (*29*). The hypersensitivity of the double mutation *arp4ΔI/ΔII* to this agent therefore points to a role for Arp4 in DNA replication. MMS induces DNA DSBs (*30*), and an increased sensitivity for it is usually interpreted as a hint for an impaired DSB repair capacity. Taken together, these results strengthen the view of *ARP4* as a pleiotropic gene, being involved in transcription (*18*), DNA replication and repair.

Our finding that only *esa1-414*, but not *esa1L254P*, caused a phenotypic change upon treatment with MMS, highlights the functional importance of the C-terminal region of Esa1p. This is in line with the results of Clarke *et al*. (*14*) who reported that haploid *esa1-414* strains—in a different strain background than we used—were only viable when the mutated gene was plasmid-borne, and therefore presumably present at slightly increased gene dosage. Strains expressing mutated forms of Esa1p in combination with Arp4 lacking insertion I (Arp4ΔI and Arp4ΔI/ΔII) exhibited significantly enhanced hypersensitivity to MMS. This was true for Esa1-414 as well as for Esa1L254P. One possible explanation would be that the Arp4 domain containing insertion I (but not insertion II) is required for a different task during DSB repair than Esa1p as a whole. Such an assumption is in agreement with the above-mentioned finding that Arp4 mediates the recruitment of the NuA4 complex to phosphorylated H2A next to DSBs, enabling the subsequent H4 acetylation via Esa1p.

The potentially extensive important role of Arp4 in DSB repair is supported by the results of Kobor *et al*. (*31*) and Shen *et al*. (*10*). They have shown that two other chromatin-modifying complexes, SWR1 and INO80, respectively, are necessary for survival in the presence of DSB-inducing agents. Both complexes also contain Arp4, which encourages the hypothesis that Arp4 may be the binding component, which coordinates interactions between different chromatin-modifying complexes and chromatin.

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