SUPPLEMENTARY INFORMATION

Supplementary materials and methods

Yeast strains. All *Saccharomyces cerevisiae* strains were congenic with W303. The following ARS sequences were used for genomic translocation: *ARS605* (Chr6, nucleotides 135948-136185); *ARS607* (Chr6, nucleotides 199341-199527); *ARS609* (Chr6, nucleotides 256270-256459); *ARS409* (Chr4, nucleotides 212367-212672); *ARS305* (Chr3, nucleotides 39449-39718); *ARS501* (Chr5, nucleotides 549537-549805); *ARS737* (Chr7, nucleotides 888340-888605); *ARS607Δ1* (Chr6, nucleotides 199341-199506) and *ARS607Δ2* (Chr6, nucleotides 199341-199485). In addition, two T to C point mutations were made in one of the Fkh1/2 binding sites in *ARS607Δ1* sequence with positions Chr6:199490 and 199495 resulting in *ARS607Δ1-3'mut* sequence. The other Fkh1/2 binding site was mutated in *ARS607Δ1-5'mut* sequence, where Chr6:199416 G nucleotide was replaced with T. In *ARS607Δ1-5'mut-3'mut* sequence both Fkh1/2 sites were mutated. In *ARS607B3toFkh* mutant the B3 box (Chr6, nucleotides 199494-199502) was replaced with an alternate Fkh binding site sequence ATAAACAAA. Similar Fkh1/2 binding site mutations were also introduced to *ARS305* and *ARS737* sequences. In *ARS305-5'mut* sequence one Fkh1/2 binding site was mutated by replacing G (Chr3, nucleotide 39566) and T (Chr3, nucleotide 39568) nucleotides with C-s. Both Fkh1/2 binding sites were mutated in *ARS737-5'mut-3'mut* sequence where G to C (Chr7, nucleotide 888409), T to C (Chr7, nucleotide 888411), T to G (Chr7, nucleotide 888488) and A to G (Chr7, nucleotide 888490) substitutions were made. In addition two strains were made where two Fkh1/2 binding sites were introduced to *ARS609* sequnce. In *ARS609-5'Fkh-3'Fkh* strain AATTTAG (Chr6, nucleotides 256356-256362) sequence was mutated to TGTTTAT and also TATACTA (Chr6, nucleotides 256435-256441) sequence was mutated to GTAAATA, that introduced two Fkh1/2 binding sites to the *ARS609-5'Fkh-3'Fkh* sequence. To create *ARS609-5'Fkh-3'B3* sequence, first TCTTTAT (Chr6, nucleotides 256400-256406) sequence was replaced with TGTTTAC and second, CAGCGTAAGGTAAATATTATGG sequence from *ARS607* was inserted into *ARS609* after A in position Chr6, nucleotide 256459. These manipulations introduced two Fkh1/2 binding sites to *ARS609-5'Fkh-3'B3* sequence.

All origins were inserted into genomic loci by two step gene replacement protocol. First, *URA3* gene was inserted into desired locus, then it was replaced with ARS sequence by homologous recombination and counter-selection on 5-FOA plates. All ARS sequences were inserted into *VPS13* locus (Chr12:60425-60426) in different strains. Several other loci were chosen primarily for insertion of *ARS607*: *DBP11* (Chr10:265455-265456); *CLD1* (Chr7:715223-715605); *HXK1* (Chr6:256227-256481). Mutated variants of *ARS607, ARS305, ARS737* and *ARS609* were studied in either *VPS13* or

HXK1 loci. In *CLD1* and *HXK1* loci native origins *ARS728* and *ARS609* were replaced, respectively. For detection of Cdc45 and Pol2 proteins, triple 1E2 epitope tag (Icosagen) was inserted into C-terminus of *CDC45* and *POL2* genes, respectively. Fkh1 and Orc2 proteins were tagged with C-terminal triple 5E11 epitope tag (Icosagen). For efficient α-factor arrest, the *BAR1* gene was also deleted in all strains. Genotypes of the strains used in this study are summarized in Table S1.

Table S1. Strains used in the study

Strain	Genotype
AKY541	*W303 MAT A GAL-VPS13-ARS605::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY542	*W303 MAT A GAL-VPS13-ARS609::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY543	*W303 MAT A GAL-VPS13-ARS607::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY545	*W303 MAT A GAL-VPS13-ARS409::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY691	*W303 MAT A CLD1::URA3 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY693	*W303 MAT A HXK1::URA3 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY697	*W303 MAT A DPB11::ARS607 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY698	*W303 MAT A HXK1(ARS609Δ)::ARS607 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY703	*W303 MAT A CLD1(ARS728Δ)::ARS607 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY704	*W303 MAT A GAL-VPS13-ARS305::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY705	*W303 MAT A GAL-VPS13-ARS501::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY706	*W303 MAT A GAL-VPS13-ARS737::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY710	*W303 MAT A GAL-VPS13-ARS607::TRP1 POL2-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY716	*W303 MAT A GAL-VPS13-ARS607Δ1::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY717	*W303 MAT A GAL-VPS13-ARS607Δ2::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY727	*W303 MAT A FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY729	*W303 MAT A GAL-VPS13-ARS607Δ1::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY732	*W303 MAT A GAL-VPS13-ARS607Δ1-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY735	*W303 MAT A GAL-VPS13-ARS609-5'Fkh-3'B3::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY736	*W303 MAT A GAL-VPS13-ARS607Δ2::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY738	*W303 MAT A GAL-VPS13-ARS607Δ1-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY746	*W303 MAT A GAL-VPS13-ARS607::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY750	*W303 MAT A GAL-VPS13-ARS607Δ1-5'mut::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY751	*W303 MAT A GAL-VPS13-ARS607Δ1-5'mut-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY761	*W303 MAT A GAL-VPS13-ARS607::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY762	*W303 MAT A GAL-VPS13-ARS607Δ1::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY763	*W303 MAT A GAL-VPS13-ARS607Δ2::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY764	*W303 MAT A GAL-VPS13-ARS607Δ1-5'mut::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY765	*W303 MAT A GAL-VPS13-ARS607Δ1-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY766	*W303 MAT A GAL-VPS13-ARS607Δ1-5'mut-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY767	*W303 MAT A GAL-VPS13::TRP1 CDC45-3x1E2tag::spHIS5 ORC2-3x3F12tag::kanMX bar1Δ::hphMX6*
AKY768	*W303 MAT A GAL-VPS13-ARS737-5'mut-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY769	*W303 MAT A GAL-VPS13-ARS607-B3toFkh::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY770	*W303 MAT A GAL-VPS13-ARS305-5'mut::TRP1 CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY771	*W303 MAT A HXK1(ARS609Δ)::ARS607Δ1-5'mut-3'mut CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY772	*W303 MAT A HXK1:: ARS609-5'Fkh-3'Fkh CDC45-3x1E2tag::spHIS5 bar1Δ::hphMX6*
AKY778	*W303 MAT A HXK1:: ARS609-5'Fkh-3'Fkh CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY784	*W303 MAT A GAL-VPS13-ARS737-5'mut-3'mut::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*
AKY785	*W303 MAT A GAL-VPS13-ARS305-5'mut::TRP1 CDC45-3x1E2tag::spHIS5 FKH1-3x5E11tag::natMX6 bar1Δ::hphMX6*

Table S2. Primers used in the study

Primer name	Sequence
VPS13_F	TGATTCTATAAAGCTGGCAACGT
VPS13_R	CTAAATACCGAATCCCTGGAAAA
ARS_305_Nat_F	TTAAAGTAACTTACACGGGGGCT
ARS_305_Nat_R	TGGTAGCACTTTGATGAGGTCTCTA
ARS_605_Nat_F	GCCCATTGGAATCACTTTTCA
ARS_605_Nat_R	TCAATAATGACGCAATTATGGAAA
ARS_607_Nat_F	GAGACTTACACATTATTCGGCACA
ARS_607_Nat_R	TTCGGTACGACACAAAAACAACT
ARS_609_Nat_F	CGGGGTTGTTTAACAATAAACGT
ARS_609_Nat_R	ACAATCACTCATGTGCATTGCA
ARS_409_Nat_F	CAAAGTAAGTCAAACCCAATTCACA
ARS_409_Nat_R	GGCGCTAGTATCACAATTGCTACT
ARS_737_Nat_F	TGCTTATTAAGGGTCTAGGACATTT
ARS_737_Nat_R	ACTTTTGCTTAAGCGGCAGAAT
ARS_501_Nat_F	TGAAGATGACATTGCTCCTTTATTA
ARS_501_Nat_R	GCTCAATTTTATATACGCATATGCA
CLD1_F	TTCGCCGCTATTTTTCTTCTTA
CLD1_R	CACAGAAATGAATTGTTGAAATCGT
HXK1_F	CCTGAAACAACTGGCATGGAA
HXK1_R	ATCACTCATGTGCATTGCACCT
DBP11_F	CCTGGCGACAATAGGAACCA
DBP11_R	ACTTCAATGTTTTCTGCCTGCA
PAU1_F	TGGCCCAATACTACATGTTCCA
PAU1_R	GGGACACCGGTGATCATTCT

Supplementary Figure 1.
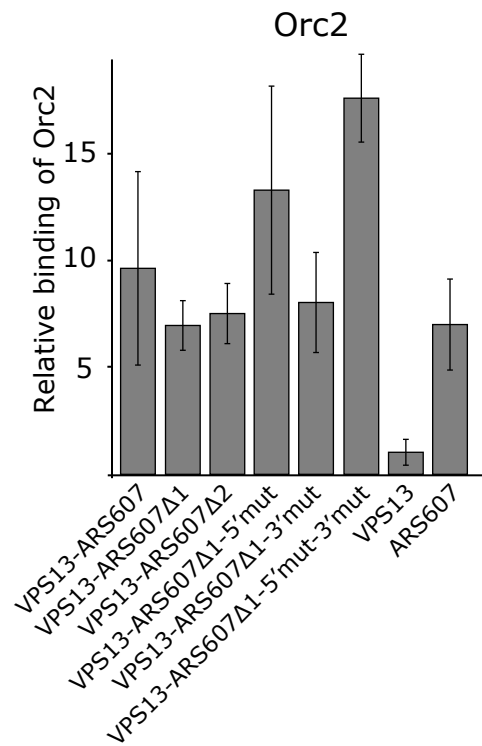
Supplementary Figure 1.
Binding of Orc2 protein to mutated ARS607 sequences in the VPS13 locus was determined by the ChIP assay in G1-arrested cells. Orc2 binding to genuine ARS607 and origin-free VPS13 loci are shown for reference. Error bars indicate standard deviation of 3 experiments.
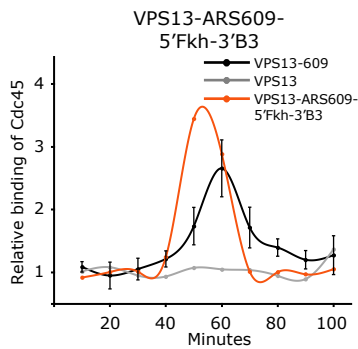
Supplementary Figure 2.

A

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ARS609               CAATTCGCTTTATGCTCTTTTTTTTTTGTGAATTTAGCCAATTCCGGAAAAAACATAAAAA
ARS609-5'-3'Fkh      CAATTCGCTTTATGCTCTTTTTTTTTTGTGTGTTTATCCAATTCCGGAAAAAACATAAAAA
ARS609-5'Fkh-3'B3    CAATTCGCTTTATGCTCTTTTTTTTTTGTGAATTTAGCCAATTCCGGAAAAAACATAAAAA

ARS609               AATGGTTATGCATTCTTTATTTTGAAGAATGAACTAGATATACAGTAATATACTAAATTG
ARS609-5'-3'Fkh      AATGGTTATGCATTCTTTATTTTGAAGAATGAACTAGATATACAGTAAGTAAATAAATTG
ARS609-5'Fkh-3'B3    AATGGTTATGCATTGTTTACTTTGAAGAATGAACTAGATATACAGTAATATACTAAATTG

ARS609               TCAGCTCTCGGCATAATTCAATGGCCGGAAAGTAG
ARS609-5'-3'Fkh      TCAGCTCTCGGCATAATTCAATGGCCGGAAAGTAG
ARS609-5'Fkh-3'B3    TCAGCTCTCGGCACAGCGTAAGGTAAATATTATGG
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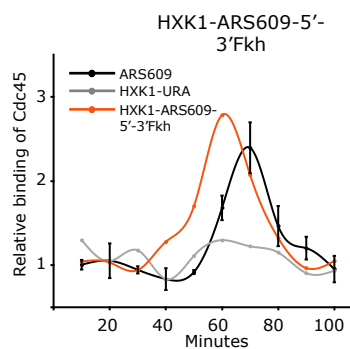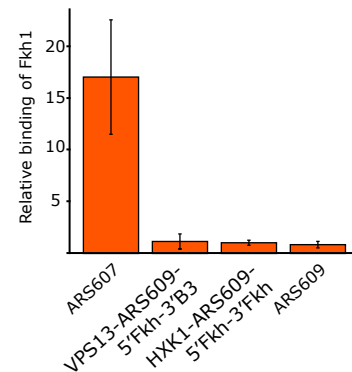

B

VPS13-ARS609-5'Fkh-3'B3

C

HXK1-ARS609-5'-3'Fkh

D

Supplementary Figure 2.
Introduction of Fkh1/2 consensus binding sequences to *ARS609* is not sufficient to change its activation time. **(A)** The sequences of modified *ARS609* loci. Two Fkh1/2 sites were created by site-directed mutagenesis in the *ARS609-5'-3'Fkh* construct. One Fkh1/2 site was created by mutagenesis and another was added as 22 bp sequence from *ARS607* B3 box region in the *ARS609-5'Fhk-3'B3* construct. The Fkh1/2 sites are shown in bold, the sequence originated from *ARS607* is underlined and the ARS consensus sites (ACS) are marked with yellow. **(B)** *ARS609-5'Fhk-3'B3* origin was inserted into *VPS13* locus and the binding of Cdc45 to the origin was determined by the ChIP assay throughout the S phase. The binding of Cdc45 to wt *ARS609* in *VPS13* and to origin-free *VPS13* loci are shown for reference. **(C)** *ARS609* was mutated in its genuine locus (*HXK1*) to create the *ARS609-5'-3'Fkh* origin. The binding of Cdc45 to the origin was determined throughout the S phase. Wt *ARS609* and origin-free *HXK1 (HXK1-URA)* loci are shown for reference. **(D)** Binding of Fkh1 protein to mutated *ARS609* loci was determined by the ChIP assay in G1-arrested cells. Fkh1 binding to genuine *ARS607* and *ARS609* loci are shown for reference. Error bars indicate standard deviation of 3 experiments. *VPS13-ARS609-5'Fkh-3'B3* on (B) and *HXK1-ARS609-5'-3'Fkh* on (C) are averages of two independent experiments.