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The CDK regulators Cdh1 and Sic1 promote efficient usage of DNA replication origins to prevent chromosomal instability at a chromosome arm

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ABSTRACT

Robustness and completion of DNA replication rely on redundant DNA replication origins. Reduced efficiency of origin licensing is proposed to contribute to chromosome instability in CDK-deregulated cell cycles, a frequent alteration in oncogenesis. However, the mechanism by which this instability occurs is largely unknown. Current models suggest that limited origin numbers would reduce fork density favouring chromosome rearrangements, but experimental support in CDK-deregulated cells is lacking. We have investigated the pattern of origin firing efficiency in budding yeast cells lacking the CDK regulators Cdh1 and Sic1. We show that each regulator is required for efficient origin activity, and that both cooperate non-redundantly. Notably, origins are differentially sensitive to CDK deregulation. Origin sensitivity is independent on normal origin efficiency, firing timing or chromosomal location. Interestingly, at a chromosome arm, there is a shortage of origin firing involving active and dormant origins, and the extent of shortage correlates with the severity of CDK deregulation and chromosome instability. We therefore propose that CDK deregulation in G1 phase compromises origin redundancy by decreasing the number of active and dormant origins, leading to origin shortage and increased chromosome instability.

INTRODUCTION

DNA replication and chromosome segregation occur once per cell cycle and are mutually exclusive in eukaryotic cell cycles to prevent genome variations in progeny. Maintaining intact genomes also requires complete DNA duplication, contributed by redundancy amongst the many origins of DNA replication on each chromosome which vary in their efficiency and timing of firing during S phase (1–3). Origins are activated in two steps (for review see (4)). Firstly, in G1 phase during the origin-licensing step, the Orc1-6, Cdc6 and Cdt1 proteins are recruited to origins and load a double hexameric ring of the Mcm2-7 proteins around dsDNA, to form pre-replicative complexes (pre-RC). The Mcm2-7 proteins form the catalytic core of the essential DNA helicase at replication forks, but are inactive when loaded at origins during G1 phase. Secondly, in S phase the GINS (Go, Ichi, Ni, San; Sld5, Psf1, Psf2, Psf3) complex and the Cdc45 protein are recruited to Mcm2-7 to form the active CMG (Cdc45-Mcm2-7-GINS) helicase (5,6) allowing further recruitment of polymerases and other factors. This leads to the formation of a replication bubble with two polar replisomes that progress in opposite directions, until they either reach telomeres at chromosome ends or else meet a converging fork to terminate replication. Some of the licensed origins remain dormant and are either passively replicated by progressing forks from neighbouring origins, or else provide a backup in case of inactivity of flanking origins or failure or stalling of neighbouring forks (7,8). This flexible pattern of redundant origin usage favours replication completion by the end of S phase.

During the initiation of DNA replication, cyclin-dependent kinase (CDK)/cyclin complexes regulate both steps of origin activation. For instance in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), CDK activity inhibits origin licensing which is thus restricted to G1 phase when CDKs are low. Subsequently, CDK activation is necessary for origin firing during S phase. This double control allows time to license sufficient origins in G1 phase while avoiding origin re-licensing and re-replication (9–11). The low CDK activity in G1 phase is achieved by many levels of control. In addition to repressed cyclin expression, B-type cyclins are ubiquitylated and degraded by the Anaphase Promoting Complex/Cyclosome (APC/C)-Cdh1 E3 ligase

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(12–16), and any remaining CDK/cyclin complexes are bound by CDK inhibitors (CKIs), such as the CIP/KIP families that include in mammals p21Cip1/Waf1 (17,18), and p27Kip1 (19). The budding yeast Sic1 protein performs a similar role as a CKI (20,21). Proliferative stimuli through G1-CDKs commit cells to terminate the licensing period and enter the cell cycle by APC/C-Cdh1 inhibition and CKI degradation (for review see (9,22)). Hence, the activation of a normal set of replication origins relies on the scheduled regulation of CDK both in G1 phase and also during the transition to S phase.

Deregulation of CDK is frequent in cancer cells (23) and leads to unscheduled CDK activity even in G1 phase. This can drive cells into a premature G1 exit and an aberrant replication leading to replicative stress and DNA damage, and the activation of DNA damage repair (DDR) pathways as barriers to genome instability (24–26). Continued aberrancies in repeated cell cycles would impose a selective pressure in favour of DDR defective clones that are unable to deal properly with damage on DNA, leading to genome instability (27,28).

Although it seems clear that CDK deregulation leads to DNA replication abnormalities that drive the acquisition of genome instability, the molecular nature of such abnormalities and how can they mechanistically cause genome instability is largely unknown. Diminished origin activity is likely to be important, since mutation of budding yeast pre-RC components, or reduced origin density, cause chromosomal instability (29–31). Similarly, decreased levels of Mcm2-7 components in metazoans promote minimal licensing and limit dormant origin numbers, increase DNA damage, impair chromosome stability especially in conditions of replicative stress and increase the occurrence of cancer in mammals (32,33). Moreover, in budding yeast and mammalian cells, CDK deregulation in G1 phase reduces origin activity and firing efficiencies, correlating with increased chromosomal rearrangements (34–38). Insertion of multiple origins reduces the local rates of gross chromosomal rearrangements (GCR) in *S. cerevisiae* cells that overexpress the G1-cyclin Cln2, presumably by increasing the efficiency of origin firing, and fork density (35). However, important questions remain unanswered regarding the links between CDK deregulation, origin licensing and genome instability. Firstly, the licensing and firing activity of few individual origins have been studied, and only in yeast (34), so that the general applicability of the model remains unclear. Secondly, it remains unaddressed if reduced origin activity spontaneously concentrates at unstable chromosome regions in CDK-deregulated cells, and if the extent of origin inefficiency reflects instability. Thirdly, reduction of origin firing efficiency is not uniform amongst the studied origins, posing the question of which origins are most sensitive to CDK deregulation (34). Indeed, not all chromosome regions are equally prone to genome instability, and oncogenic cell cycles show increased instability at fragile sites in the form of loss of heterozigosity (24–26). Moreover, common fragile sites display origin paucity in correlation with increased fragility (39,40). Finally, origin-licensing inefficiency may not be limited to active origins, and the behaviour of dormant origins upon CDK deregulation remains to be explored.

Here, we model CDK deregulation in G1 phase and analyse origin activity in budding yeast cells. Whereas previous studies focused on cells lacking the CDK inhibitor Sic1, we have also analysed cells lacking the APC/C activator Cdh1. We report the firing efficiency of a variety of origins upon CDK deregulation to address if particular subtypes are more sensitive than others to CDK deregulation. We also study if reduced origin usage colocalises with increased GCRs at a chromosome region. We conclude that the CDK regulators Cdh1 and Sic1 cooperate in promoting origin redundancy to prevent a regional shortage of active origins and maintain chromosome stability.

MATERIALS AND METHODS

Yeast strains and growth conditions

The yeast strains in this study are based on W303-1a or S288C backgrounds (Supplementary Table S1), and derive from the parental YAC36 and RDKY3615, respectively. Cells were routinely grown at 25ºC in YP media supplemented with 40 mg/L adenine and 2% glucose (YPAD). *GAL1,10p-SIC1* cells were always grown in 1% raffinose, 0.3% galactose YP media supplemented with 40 mg/L adenine (YPARG), and before analysis were transferred to YPAD for 4 h to repress Sic1 expression (the minimal time required by an asynchronous cell culture to stabilise the cell cycle). Cell cycle arrests were performed in nocodazole (15 μg/mL for 210 min) or alpha-factor (40 ng/mL for 3 h in *bar1Δ* strains). Samples for flow cytometry were fixed and processed as described previously (41), analysed using a FACSCalibur flow cytometer (BD Biosciencies), and plotted using the Cell Quest software (BD Biosciencies). The *GAL1,10p-SIC1* strain was constructed by replacing 19 bp immediately before the *SIC1* ATG with a *TRP1-GAL1,10p* cassette, so the only *SIC1* copy in the cell is under the control of the *GAL1,10* promoter. *HphNT* was inserted in the parental strains at coordinates 71495–71533 of chromosome VI (according to the *Saccharomyces* Genome Database, SGD, http://www.yeastgenome.org) to construct YAC177 and YAC188; the remaining strains derive from these two and are isogenic for this insertion except YAC571. To construct the *SIC1p-SIC1* strains, the *SIC1* gene together with both flanking intergenic regions (SGD, coordinates 286446–287933, chromosome XII) was amplified by PCR, cloned into the pGEM-T Easy system I vector (Promega) and fully sequenced. The SacII-SalI fragment containing *SIC1p-SIC1* was subcloned into pRS305 (42) to generate the plasmid pAC738 that linearised with NarI was targeted to *LEU2*, and single integrants were selected by Southern analysis. The *ars507Δ::ARS305* strains were constructed by first deleting the 234 bp of *ARS507* (SGD, coordinates 59283–59516, chromosome V) with the *URA3* cassette from pRS306 (42), what completely abolishes origin firing activity (data not shown), followed by a replacement of *URA3* with 234 bp of *ARS305* (43) (SGD, coordinates 39382–39615, chromosome III).

Two-dimensional DNA gel electrophoresis

Samples of chromosomal DNA for 2D gels were prepared as previously described (41) and from 5 to 15

μg of digested DNA was employed per gel. DNA was digested with the following enzymes to generate the indicated origin-containing fragments: MluI-ClaI (4.84 kb *ARS504.2*, 6.33 kb ClaI-ClaI *proARS506*), ClaI-EcoRI (5.49 kb *ARS607*, 3.85 kb *ARS522-501*, 5.36 kb *ARS302-ARS303-ARS320*, 5.93 kb *ARS503-ARS504*, 1.81 kb EcoRI-ClaI *ARS608*, 4.23 kb ClaI-ClaI *ARS306*), NcoI (5,12 kb *ARS305*, 4.67 kb *ARS416-1*, 2.61 kb *ARS603.5*) and ClaI-SacI-KpnI (2.64 kb ClaI-KpnI *ARS432*, 5.82 kb ClaI-KpnI *ARS1014*, 2.47 kb SacI-KpnI *ARS507*, 2.38 kb KpnI-ClaI *ARS508*, 2.47 kb SacI-KpnI *ars507Δ::ARS305*). The specific probe for each origin corresponds to the following coordinates of the indicated chromosomes (according to the SGD): *ARS306* (73001–73958, chromosome III), *ARS305* (39073–40557, chromosome III), *ARS607* (198958–199845, chromosome VI), *ARS432* (1158713–1159194, chromosome IV), *ARS1014* (417974–418715, chromosome X), *ARS416-1* (462772–463329, chromosome IV), *ARS522-501* (548689–549857, chromosome V), *ARS603.5* (118090–118962, chromosome VI), *ARS302-ARS303-ARS320* (14040–14991, chromosome III), *ARS608* (215755–216554, chromosome VI), *ARS503-ARS504* (8919–9432, chromosome V), *ARS504.2* (10744–11272, chromosome V), *proARS506* (18123–18663, chromosome V), *ARS507* (59965–60438, chromosome V) and *ARS508* (92964–93372, chromosome V). After hybridisation, detection was performed exposing against BAS-MS imaging plates (Fujifilm), and read with a Personal Molecular Imager FX (Bio-Rad). Quantitative determination of origin efficiency was determined as previously described (44,45), by measuring the signal of the whole bubble-arc and the Y-arc for each blot by free-hand contouring of specific signals above the background, employing the Quantity One software (Bio-Rad). Origin efficiency is the frequency of active initiation (bubble arc/(bubble arc + Y arc)). We discarded from the measurement the region of large Ys where passive Ys comigrate with active Ys resulting from bubble resolution, so is a region of mixed active and passive signals. The distinct quality of blots may influence the accuracy of quantification, but our results were largely reproducible in independent repeats and in distinct backgrounds.

Western blot analysis

Yeast proteins were extracted by the TCA method (46) from 2.5×10^8 cells. Sic1 was detected with the rabbit polyclonal anti-Sic1 FL-284 antibody (Santa Cruz Biotechnology Inc.), and Pgk1 with the 22C5D8 mouse monoclonal antibody (Invitrogen). Secondary antibodies employed were the HRP donkey anti-rabbit, and sheep anti-mouse (GE Healthcare), respectively.

GCR assay

The estimation of GCR rates was performed by fluctuation analysis as described (35,47). At least five independent colonies were inoculated from YPAD (control and Δ*cdh1* cells) or YPARG (*GAL1,10p-SIC1*) plates into liquid YPAD or YPARG, as indicated in each experiment, at a density of 2.5×10^5 cells/mL and grown at 25°C until stationary phase (usually 32 to 40 h). Cells were collected, counted in a Neubauer counting chamber (Brand), and 5×10^8 cells were spread per 150 mm Petri dish of synthetic minimal FOA-Can media (1.1 g/L of 5-FOA, and 60 mg/L of sulphate salt L-canavanine) supplemented with glucose except for *GAL1,10p-SIC1* strains where raffinose-galactose was employed. The number of colonies was counted and the median employed to estimate the mutation (GCR) rates, as described previously (35,48).

Pulse field gel electrophoresis

Cells were collected by centrifugation in log phase and prepared employing the CHEF Yeast Genomic DNA Plug Kit (Bio-Rad), following manufacture's instructions. Plugs were loaded into a 1% Megabase (Bio-Rad) agarose gel in 0.5x TBE and run at 14°C in a Gene Navigator System (GE Healthcare Life Sciences) at 180 V during 24 h with pulses of 90, 105 and 120 s for 9:36, 6 and 8:20 h, respectively. DNA was transferred to Hybond-XL membranes (GE Healthcare Life Sciences) and Southern analysis, exposure, and image processing was performed as for 2D gels.

Fork direction analysis

DNA samples were prepared as for 2D gels and digested with the following enzymes to generate the fragments: NheI-NheI (5.01 kb, chr. III, coordinates 68091–73104 '*upstream ARS306*'), BamHI-HindIII (3.63 kb, chr. VI, coordinates 195438–199068 '*upstream ARS607*') and HindIII-HindIII (3.80 kb, chr. IV, coordinates 1161197–1165004 '*downstream ARS432*'). Electrophoresis, DNA transfer, hybridisation and signal detection were identical to 2D gels except for the in-gel digestion between both dimensions that was performed as indicated (49) using PstI (chr. 3, 71542), XbaI (chr. 6, 198128) and KpnI (chr. 4, 1164170), respectively. The specific probes correspond to the following coordinates and chromosomes (SGD): *upstream ARS306* (68661–69685, chr. III), *upstream ARS607* (196158–196887, chr. VI) and *downstream ARS432* (1161848–1162567, chr. IV). Signal quantification was performed as described (57) employing the same software as for 2D gels.

RESULTS

Cdh1 is important for efficient initiation of DNA replication

Inefficient origin activity has been suggested to contribute to genome instability in cells lacking Cdh1 (50–52), and *Cdh1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) show less efficient DNA replication and reduced levels of Mcm4 and Mcm5 at chromatin, as well as increased chromosome aberrations (38). However, no direct analysis of origin activity has been reported previously for cells lacking Cdh1, and plasmid loss in Δ*cdh1* yeast cells is not reverted with extra origins (50) as is often the case in mutants with defective origin activation.

Therefore, we made a direct comparison of origin firing and genome instability in yeast cells lacking either Cdh1 or Sic1. We deleted the *CDH1* gene in budding yeast, and Δ*cdh1* cells displayed the known cell cycle distribution and synthetic interaction with cells lacking Sic1 (Supplementary Figure S1). Given the reported elevated GCR rates of Δ*sic1*

cells (34,53), we instead employed cells conditionally expressing Sic1 under the control of the *GAL1,10* promoter to minimise the undesired selection of genomically modified clones. Repression of the promoter mimics the deletion of *SIC1* in cell viability (20,34), although with milder phenotypes (Supplementary Figure S1). *GAL1,10p* repression largely removes Sic1 (Supplementary Figure S2), but not completely (residual amounts are detectable in Western blots after very sensitive detection (data not shown) consistent with its milder phenotypes) so hereinafter we refer to *GAL1,10p-SIC1* repression as Sic1-depletion or *sic1* cells. Accordingly, in contrast to the lethality of Δ*cdh1* Δ*sic1* cells (15,16), *sic1* Δ*cdh1* cells were viable although very sick (Supplementary Figure S1). However, the Sic1-depletion was sufficient to render Δ*47cdc6* Δ*cdh1 sic1* cells inviable (54,55) (data not shown).

We measured the firing efficiency (frequency of firing in the population of cells) of individual origins in their natural loci by 2D 'neutral–neutral' agarose gel electrophoresis of replication intermediates (56) in isogenic control, Δ*cdh1*, *sic1* and double mutant *sic1* Δ*cdh1* cells (Figure 1). The relative intensities of the external active bubble-arc and the internal passive fork-arc determine the firing efficiency of the origin (Figure 1A). Control and Δ*cdh1* cells were collected after asynchronous growth to log phase in YPAD, and compared with asynchronous *sic1* cells after repressing Sic1 expression for 4 h in YPAD (the minimum time cells require to display and maintain the cell cycle distribution of cells lacking Sic1; Supplementary Figure S1). We firstly analysed three well-known early efficient origins, ARS306, ARS305 and ARS607, and confirmed in control cells the reported high efficiency (45,57,58) (Figure 1B, upper panels). Sic1-depleted cells showed differential loss of firing efficiency amongst origins regarding control cells, with ARS306 losing around more than 15% of efficiency while ARS305 was not noticeably affected (Figure 1B, second row). This non-uniform origin sensitivity was previously described for Δ*sic1* cells for ARS305 and ARS306, although with higher efficiency losses in the complete absence of Sic1 (34), consistent with the stronger severity of Δ*sic1* cells, and was reproduced by ourselves (Supplementary Figure S3). To generalise this observation we extended this analysis to two more origins, ARS432 and ARS1014, randomly chosen amongst the earliest in their chromosomes and not studied previously by 2D gels (as compiled in the DNA Replication Origin Database, OriDB, (59)). Similar to ARS305, ARS1014 also displayed normal efficiency in *sic1* cells, whereas ARS432 lost around 30% of efficiency (Figure 1B). These results were confirmed in independent repeats (Supplementary Figure S4A) and also in other strain backgrounds (Supplementary Figure S4B and see below).

Importantly, Δ*cdh1* cells also showed loss of firing efficiency of ARS306, ARS607 and ARS432 compared to control cells, whereas ARS305 and ARS1014 were unaffected (Figure 1B, third row). Origin inefficiencies in Δ*cdh1* cells were more modest than in *sic1* cells. To confirm further the observed inefficiencies in origin activity in Δ*cdh1* cells, we analysed the direction of replication fork movement at origin-adjacent regions by using a modification of the 2D gel electrophoresis that includes an in-gel digestion of replication intermediates between both dimensions (49). This approach differentiates the amount of forks progressing in both directions (departing from or arriving to the origin) therefore reflecting origin activity. Hence, reductions in origin activity can be evaluated by the increase of forks moving toward the origin (60). We analysed the direction of fork movement at fragments immediately upstream ARS306 or ARS607, and downstream of ARS432 in wt and Δ*cdh1* cells, and the results are shown in Figure 1(C). In control cells only forks departing from the origin are detected close to ARS306 and ARS607, as previously reported and consistent with their elevated firing efficiency (49,58). ARS432 has more moderate origin activity, and the amount of replication forks adjacent to this origin was similar in both directions in control cells. In contrast, in Δ*cdh1* cells an arc of forks moving toward ARS306 was now visible (Figure 1C(i), right panel) consistent with the reduced ARS306 activity seen in our 2D gels. Similarly at the site adjacent to ARS432, we observed a decreased intensity of the arc of forks departing from the origin (Figure 1C(iii), right panel). Consistent with our earlier finding that ARS607 is less affected than the above two origins in Δ*cdh1* cells (Figure 1B), we did not observe variations in fork direction between wt and Δ*cdh1* cells for this origin (Figure 1C(ii)). Hence, the direction of fork progression at regions adjacent to origins in Δ*cdh1* cells is altered only when the origin efficiency is altered compared to control cells, and accordingly no variation is observed at a region adjacent to ARS305 (Supplementary Figure S5). This is consistent with previous reports of trinucleotide repeat instability in association with decreased origin usage and changes in fork direction (61).

Altogether these results indicate that differential sensitivity of origins is a common response of cells lacking CDK regulators. We conclude that cells require Cdh1 to promote efficient firing of a subset of origins during the initiation of DNA replication.

The differential origin sensitivity to CDK deregulation is independent of normal origin efficiency, firing timing and chromosomal location

Origins differ in their time of firing during S phase, ranging from very early to mid or late, and in their efficiency from very efficient to dormant (45,57,58,62). In control cells, the five early origins analysed in this study range in efficiency (Figure 1B; 20–40% higher in ARS305, ARS306 and ARS607 regarding ARS432 and ARS1014) and firing timing (Supplementary Table S2), with some being sensitive to CDK deregulation and others resistant (Figure 1B). We also analysed a variety of other origins with varying efficiency that fire in mid-S or late-S phase, or that are dormant (Supplementary Table S2). ARS1 (ARS416), ARS501 (ARS522) and ARS603.5 fire with diverse efficiencies in mid-S phase in control cells. As for the early origins studied above, they showed differential sensitivity in Δ*cdh1* and *sic1* cells, with ARS522 being resistant to CDK deregulation, and ARS603.5 and ARS416 being sensitive (Figure 1D). Similarly, sensitivity in mid-S origins is highly variable from 14 to 35% in *sic1* cells (ARS607 and ARS603.5), and from 10 to 21% (ARS416 and ARS432) in Δ*cdh1* cells (Figure 1D). The tested dormant origins remained inactive in both mutants (Figure 1E). Despite similar origin-sensitivity

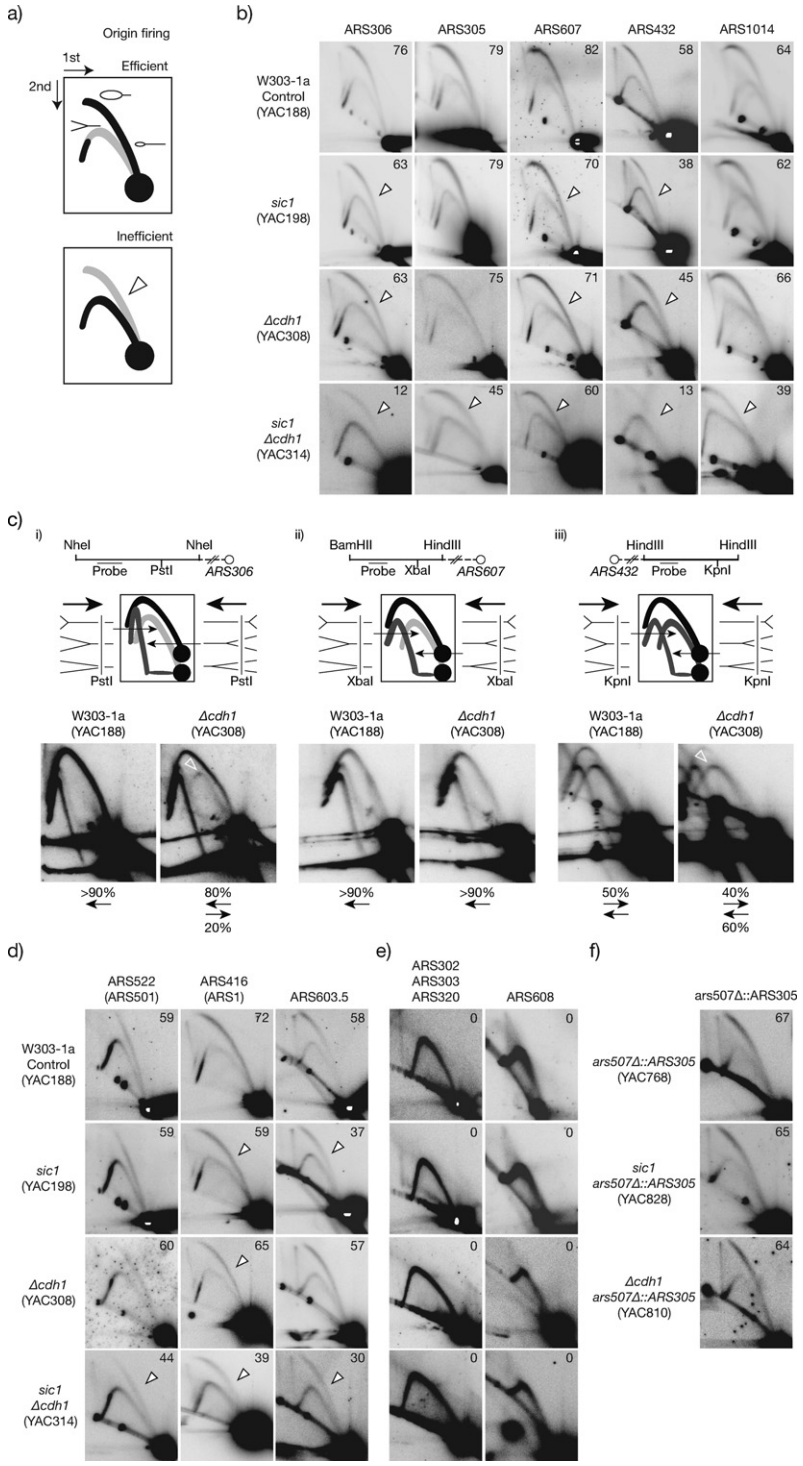

Figure 1. Differential origin firing sensitivity in cells lacking the CDK regulators Sic1 and Cdh1. (**a**) Scheme of efficient and inefficient origin firing observed by 2D gels. The external arc is formed by fragments containing a bubble and reveals origin firing activity, and is termed active- or bubble-arc. The internal arc is formed by forks (Ys) that passively replicate the fragment and indicates passive replication, so is referred as passive-, or Y-arc. The relative intensity of both arcs (open arrowhead) denote origin efficiency. (**b**) 2D blots of early firing origins in control, *sic1*, Δ*cdh1* and double mutant *sic1* Δ*cdh1* cells. Origin efficiencies are shown in the right-upper corners. (**c**) Analysis of fork direction at origin-adjacent regions upstream to ARS306 (i) and ARS607 (ii), and downstream to ARS432 (iii). Upper panels: maps of the analysed restriction fragments containing the enzymes employed before the 1st dimension (above) and for in-gel digestion (below the chromosome lane), the region recognised by the hybridisation probe and the relative position of ARSs. Middle panels: diagrams of rightward- and leftward-moving forks, the expected products after the in-gel digestion, and the observed migration patterns. Lower panels: hybridisation blots of control and Δ*cdh1* cells, including the percentage of signal of forks moving in each direction (when detected). (**d**) Origin firing activity of mid-S phase, and (**e**) dormant origins. (**f**) Efficiency of ARS305 when replacing ARS507 at chromosome V. Open arrowheads show loss of efficiency relative to control cells. See Material and Methods for details of chromosome coordinates, restriction enzymes and probes employed for each origin. Diverse spots appear below the internal arc by unspecific probe hybridisation, or are remaining signals from preceding hybridisations.

patterns in both mutants, origin efficiency was reduced to a greater degree in *sic1* compared to Δ*cdh1* cells. In addition, ARS603.5 was sensitive to CDK deregulation in *sic1* cells but normal in Δ*cdh1* cells. Overall, these data indicate that Cdh1 contributes less than Sic1 to the regulation of origin activity.

Chromosomal location can affect origin efficiency and also the timing of firing (reviewed in (63)). Two consecutive origins separated about 35 kb, ARS305 and ARS306, behave differently (resistant and sensitive, respectively) in Δ*cdh1* or *sic1* cells, despite normally having similar efficiency and timing (Supplementary Table S2). However, their chromosomal position and particular *cis*-acting elements are unique, so we investigated whether sensitivity was influenced by these particular origin characteristics. The known ARS305 *cis* elements are contained in a sequence of 234 bp (43,64). We tested whether moving this region to a sensitive locus would change the resistance of the origin to CDK deregulation, by making an exact replacement of the 234 bp ARS507 origin sequence on chromosome V (Supplementary Table S2 and Figure 2 show is sensitive to CDK deregulation) with ARS305 (see Material and Methods). In this way we found that ars507Δ::ARS305 maintains similar efficiency as the endogenous ARS305 on chromosome III (Figure 1F; compare with the ARS305 panel in B) and notably, efficiency of ars507Δ::ARS305 was not altered in Δ*cdh1* or *sic1* cells. These data indicate that ARS305 resistance to CDK deregulation is independent of its location, but rather is inherent to its *cis*-acting elements present in the 234 bp region. From these results overall we conclude that origin sensitivity to CDK deregulation is independent of normal origin efficiency, firing timing, or chromosomal location, suggesting that is influenced by the specific origin configuration of *cis* elements.

Origin firing shortage and GCR rates coincide at the left arm of chromosome V in Δ*cdh1* and *sic1* cells

In human cells, fragile site instability is associated with a paucity of initiation events (39). Previous work with budding yeast showed that elevated Cln2, lack of Sic1, or lack of Cdh1 increased the rate of GCRs at the left arm of chromosome V (34,35,47,50,53). We observed similar GCR rates, indicating once again that Cdh1 is important but less critical than Sic1 to prevent chromosomal rearrangements (Table 1). Moreover, Sic1-depleted cells displayed reduced GCR rates than Δ*sic1* cells (12 and 30 times the control, respectively). We reasoned that if reduced origin activity in CDK-deregulated cells favours chromosome rearrangements, then both should coincide spatially. Hence, we analysed the pattern of origin usage on the first 100 kb of the left arm of chromosome V (Figure 2A). The efficiency of these origins has not been previously analysed by 2D gels (see OriDB), but two early and active origins were identified by genome-wide studies in this chromosome arm, ARS507 and ARS508 (62,65). We firstly analysed their efficiency in the same cells employed to estimate the rate of GCRs. In control cells, both origins were active and similarly highly efficient, however, while ARS508 remained unaffected in Δ*cdh1* and *sic1* cells, ARS507 had reduced efficiency (Figure 2B). Notably, ARS507 was less affected in Δ*cdh1* cells, correlating with the observed GCR rates. We extended the analysis to W303–1a cells with similar results (Figure 2C), confirming that differential origin tolerance to CDK deregulation is origin specific, independently on the strain background.

The remaining origins in the arm are located near the telomere (Figure 2A). Telomere-adjacent regions in budding yeast chromosomes generally have origins that fire late with low efficiency, or that are dormant origins that likely function as a backup if replication of adjacent origins is delayed (7). We did not detect origin activity by 2D gels for the telomere proximal origins ARS503, ARS504 and proARS506 in any of the strains, consistent with them being dormant (Figure 2C; lack of specific probes prevented analysis of ARS502). In contrast, we detected a weak origin firing at ARS504.2 in control cells indicating that it is an active origin, but the activity was lost in *sic1* cells, showing that ARS504.2 is sensitive to CDK deregulation in these cells (Figure 2C). Significantly, firing at ARS504.2 actually increased in Δ*cdh1* cells (Figure 2C). This is probably due to the milder CDK deregulation reducing efficiency of ARS507 but not blocking licensing of ARS504.2, which thus is able to fire in at least some cells due to delayed arrival of forks from internal origins. Altogether, these results show qualitative and quantitative differences in the pattern of origin usage that would be predicted to affect the stability of this chromosome arm. The pattern of origin usage in the chromosome arm correlates well with the rate of GCR in the region, with the greatest GCR defect in *sic1* cells that have the lowest origin firing in the chromosome arm, and a much milder effect in Δ*cdh1* cells that retain more origin activity overall. We conclude that shortage of active origins coincides spatially with increased chromosome rearrangements in CDK-deregulated cells.

Cdh1 and Sic1 cooperate in promoting efficient origin firing at all origins

As CDK overexpression or premature CDK activation in G1 phase blocks origin licensing and the initiation of DNA replication (66,67), the sensitivity of only a subset of origins in cells lacking Sic1 or Cdh1 might reflect the fact that cells only experience moderate CDK upregulation. Worsening CDK deregulation should thus reduce origin firing efficiency further, and accordingly increase chromosomal instability. In contrast to Δ*sic1* Δ*cdh1* cells that are lethal (15,16), we analysed double mutant Sic1-depleted Δ*cdh1* cells that are alive although very sick due to strong CDK deregulation, with a greatly decreased length of G1 (54) (Supplementary Figure S1A). When assessing the pattern of origin usage on the left arm of chromosome V we observed that ARS508 was less efficient than in control or single mutant cells, ARS507 inefficiency increased compared to the single mutants and, similar to *sic1* cells, there was no detectable firing of the telomeric proximal origins (Figure 2C). This meant that the density of origin firing in the left arm of chromosome V was severely reduced. The efficiency of all other origins examined, including those resistant to CDK deregulation in single mutants, was now reduced (Figure 1B, D and E, lower panels), a result reproduced also in the S288C strain background (Figure 2B and Supplementary

Table 1. The lack of Sic1 and Cdh1 increase GCR rate

Relevant genotype (Strain)	Cells analysed	GCR rate	(*)	(**)
Control (YAC177)	8.21×10^{10}	2.70×10^{-9}	(1.0)	
Control (YAC177)	4.16×10^{10}	5.84×10^{-9}		(1.0)
sic1 (YAC217)	5.03×10^{9}	3.44×10^{-8}	(12.7)	
GAL1-SIC1 (YAC217)	1.28×10^{10}	1.27×10^{-8}		(2.2)
Δ*sic1* (YAC571)	2.35×10^{9}	8.31×10^{-8}	(30.8)	
Δ*cdh1* (YAC300)	1.28×10^{10}	5.64×10^{-9}	(2.1)	

The rates shown are the probability of mutation per cell per division.
*Fold increase versus control cells in glucose media.
**Fold increase versus control cells in raffgal media.
All strains are derivative of RDKY3615 (47).
See Supplementary Table S1 for detailed genotypes.

Figure S4B). However, differential origin inefficiency was maintained, supporting the conclusion that origins are differentially tolerant to inhibitory CDK. The decreased firing density at the left arm of chromosome V should increase the rate of GCRs accordingly, but the compromised viability of the double mutants (Supplementary Figure S1) precluded an accurate estimation of GCR rates (data not shown). Instead, we measured the incidence of chromosome breakage by pulse field gel electrophoresis (PFGE) and Southern analysis to detect chromosome fragments (Figure 3A). We collected control, single mutant and double mutant cells grown to log phase in glucose-based media to repress *SIC1* in *GAL1,10p-SIC1* cells, and observed that single mutants showed increased breakage at chromosomes III and V relative to control cells, consistent with their higher instability. Moreover, breakage was further increased in the double mutant (Figure 3B and C). We conclude that the level of CDK deregulation in G1 phase directly influences the extent of reduced origin efficiency and chromosomal instability. Moreover, Cdh1 and Sic1 cooperate non-redundantly for efficient firing of origins and the prevention of chromosome breakage.

Origin inefficiency and gross chromosomal instability in Δ*cdh1* cells is suppressed by increased *SIC1* gene dosage

Defects in origin activity and increased GCR rates in Δ*cdh1* cells are likely to result from elevated levels of mitotic cyclins and M-Cdk1 activity (15,16), but the accumulation of the many other APC/C-Cdh1 substrates raised the question of the relative contribution of CDK deregulation to those defects. To determine if origin inefficiency in Δ*cdh1* cells is dependent on increased B-cyclin associated CDK activity in G1 phase, we duplicated the *SIC1* dosage under the control of its promoter (*SIC1p-SIC1*). The duplicated *SIC1* expression will preserve in every cell cycle the normal regulation of the Sic1 protein, which accumulates in G1 phase and is degraded outside of G1 phase, thus allowing the analysis of GCR rates. In control cells, the *SIC1* duplication does not significantly modify the GCR rate (Table 2), cell viability, nor the cell cycle profile that display a normal length of G1 phase (Figure 4A and B). In addition, the *SIC1p-SIC1* integration suppressed the GCRs and other defects associated with the depletion of Sic1 (Figure 4; Table 2). Significantly, Δ*cdh1 SIC1p-SIC1* cells also reverted the rate of GCRs (Table 2), cell viability, length of G1 phase and origin firing efficiency (Figure 4), consistent with previous findings (54,68). These results strongly suggest that increased Cdk1/Clb activity causes most of the origin firing inefficiency and GCR rates in Δ*cdh1* cells. We cannot, however, exclude that increased Sic1 levels are compensating the stability of certain Cdh1 substrate(s) indirectly through CDK regulation.

DISCUSSION

Our approach of studying in parallel the firing efficiency of a variety of origins in budding yeast cells lacking the CDK regulators Cdh1 and Sic1 allowed us to analyse the patterns of origin usage in CDK-deregulated cells and, at the same time, to directly address the relationships between the overall origin firing and the rates of chromosomal rearrangements in a chromosome arm.

We show that Cdh1 is required for optimal origin firing efficiency during initiation of DNA replication in budding yeast. The APC/C-Cdh1-dependent degradation of licensing-inhibitory factors including cyclin A, cyclin B and geminin in metazoans and M-cyclins in yeasts (9,10,69,70) supports the relevance of Cdh1 for origin licensing and efficient origin use in S phase. Previous studies suggested that loss of Cdh1 compromises licensing leading to genome instability (38,50,52,54,71,72), but no direct analysis of origin activity had been reported in cells lacking Cdh1. Here, we show that origin firing efficiency is reduced in Δ*cdh1* cells and, importantly, that the degree of origin sensitivity to the lack of Cdh1 is not uniform amongst origins. Despite the many APC/C-Cdh1 substrates, we show that duplicating the *SIC1* dosage in Δ*cdh1* cells suppresses firing inefficiency and GCR rates, strongly supporting the idea that Cdk1/Clb inactivation is the major Cdh1 target for chromosome rearrangements and origin activity in budding yeast. This is consistent with CDK inactivation being the only essential function of APC/C for origin resetting (68). Cdh1 can also be relevant in metazoans for normal origin activity given the conserved APC/C and Cdh1 roles in targeting the origin-licensing inhibitors cyclin A and B and geminin. However, we imagine that this scenario will not be as simple in metazoans where Cdh1 also targets the licensing proteins Cdc6, Orc1 and Cdt1 in G1 phase for proteolysis (73–75).

Importantly, differential origin sensitivity is not a particularity of the lack of Cdh1. Previous studies showed that Δ*sic1* cells display differential origin sensitivity (34), and we show that the pattern of origin sensitivity is mostly the

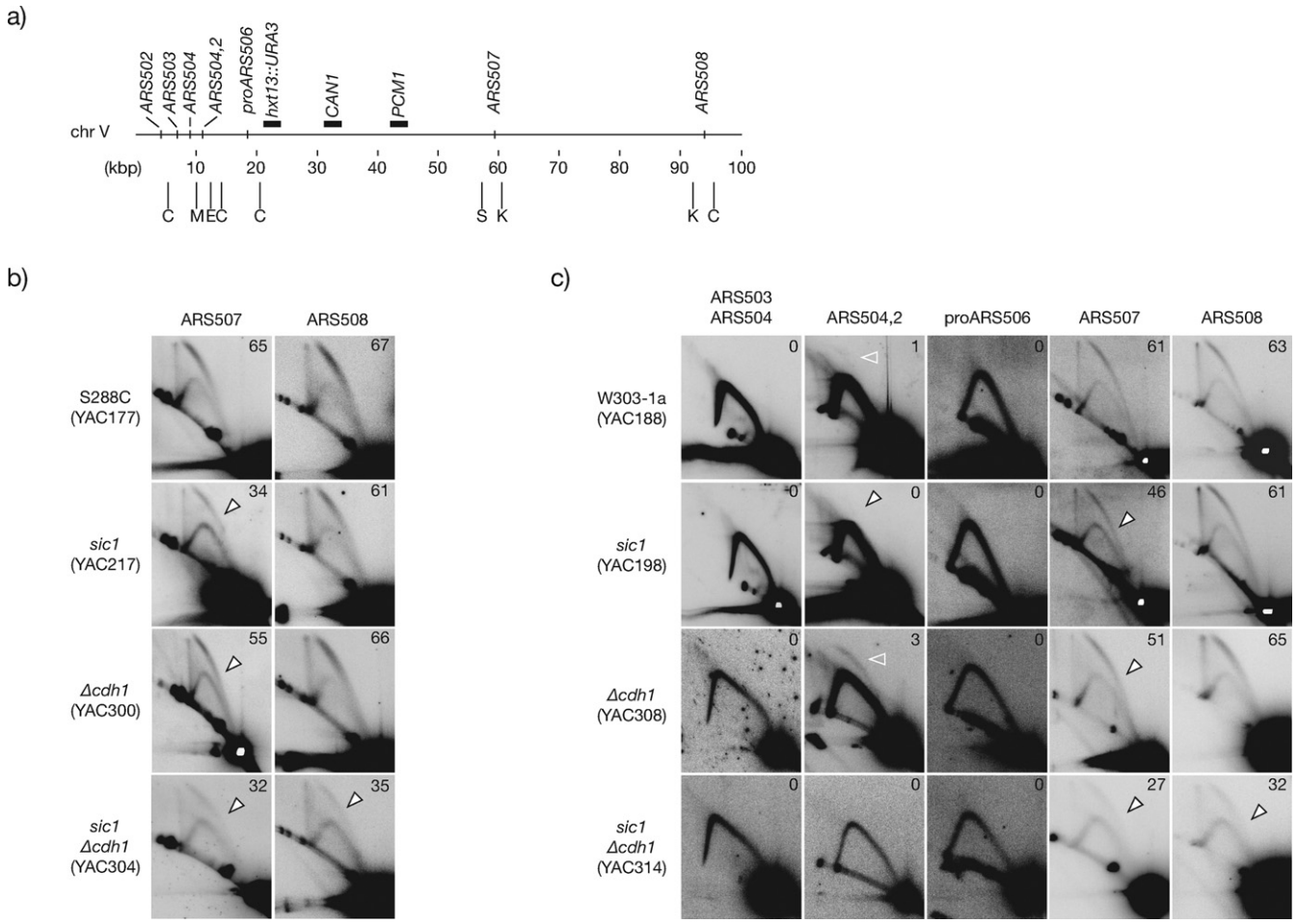

Figure 2. Overall origin firing efficiency at the left arm of chromosome V in CDK-deregulated cells. (**a**) Map of the first 100 kbp of chromosome V including the relative position of all confirmed and likely origins (according to the OriDB and the SGD). Horizontal black bars above the chromosome denote the relative position of markers for the GCR assay (*URA3* and *CAN1*), and the first essential gene from the left telomere (*PCM1*) relevant for the GCR assay. Below the chromosome, vertical bars indicate the position of the restriction enzymes employed to digest DNA for 2D gel analysis: C, ClaI; M, MluI; E, EcoRI; S, SacI; K, KpnI. (**b**) Firing efficiency analysis of ARS507 and ARS508 in S288C control, *sic1*, Δ*cdh1* and s*ic1* Δ*cdh1,* double mutant cells. (**c**) Firing efficiency of the indicated active, dormant and likely origins at the left arm of chromosome V in W303–1a control and CDK-deregulated cells. Open arrowheads indicate loss of efficiency relative to control cells. Open white arrowheads mark the bubble arc at ARS504.2. See Material and Methods for details of chromosome coordinates and probes employed for each origin.

Table 2. The GCR rate of Δ*cdh1* cells is suppressed by one extra copy of *SIC1*

Relevant genotype (Strain)	Cells analysed	GCR rate	(*)
SIC1p-SIC1 (YAC800)	9.25×10^{10}	2.19×10^{-9}	(0.8)
sic1 SIC1p-SIC1 (YAC801)	9.95×10^{10}	5.16×10^{-9}	(1.9)
Δcdh1 SIC1p-SlC1 (YAC803)	1.07×10^{11}	2.26×10^{-9}	(0.8)

The rates shown are the probability of mutation per cell per division.
*Fold increase versus YAC177 in Table 1.
All strains are derivative of RDKY3615 (47).
See Supplementary Table S1 for detailed genotypes.

same in cells lacking either Cdh1 or Sic1, although Sic1 contributes more than Cdh1 for efficient origin activity in budding yeast. Origin usage is a reflect of the extent of CDK deregulation. All origins are sensitive to more severe CDK deregulation in the double mutant *sic1* Δ*cdh1*, consistent with previous studies of strong CDK deregulation (76), although differential sensitivities are maintained amongst origins. Hence, in budding yeast the CDK regulators in G1 phase, Cdh1 and Sic1, cooperate to activate exceeding origin numbers for efficient origin activation. We presume that the differential tolerance of origins to CDK deregulation adds more redundancy and robustness to replication initiation.

Differential origin responses have been previously reported upon mutation of CDK-inhibitory controls in pre-RC components, resulting in re-replication (77–79), but the

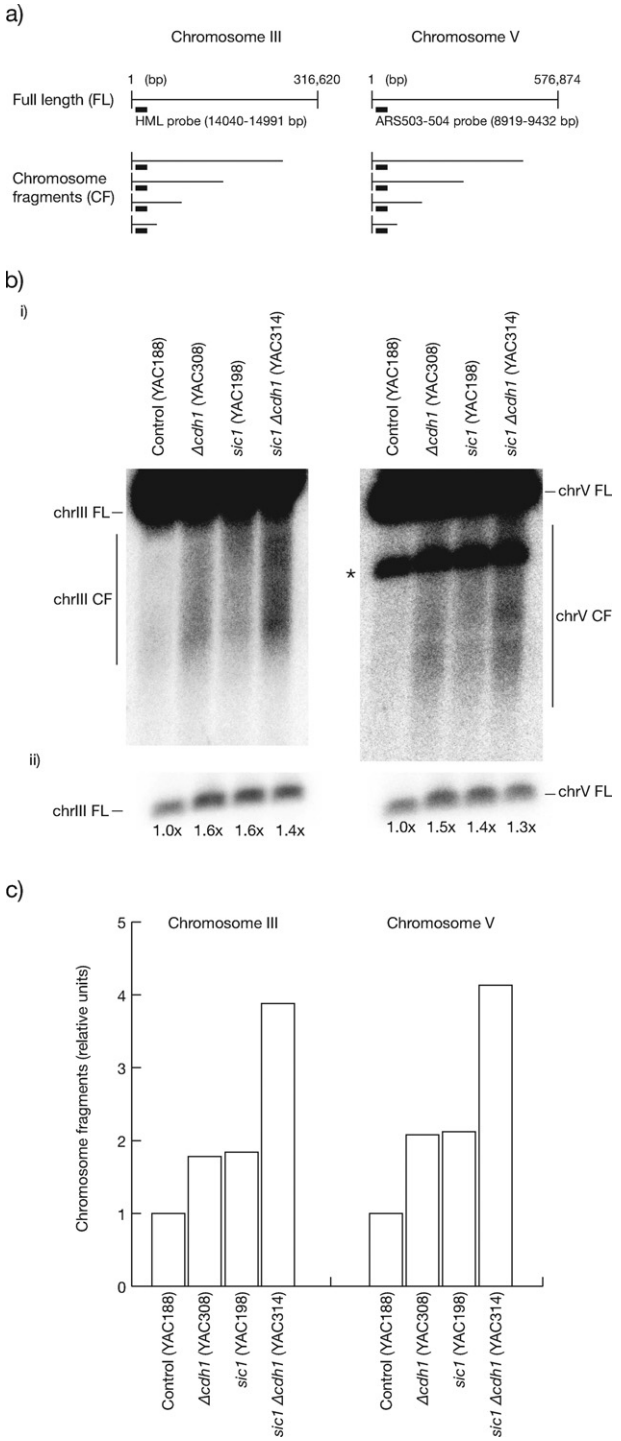

Figure 3. Breakage at chromosomes III and V increase in Δ*cdh1 sic1* cells over single mutants. (**a**) Maps of chromosomes III (left panel) and V (right panel) indicating the expected chromosome fragments recognised by the showed probes. (**b**, panel (i)) PFGE and Southern analysis of chromosomes III and V, including the band corresponding to the full-length (FL) chromosomes, and chromosome fragments (CF), as developed by the specific probes indicated in (a). *, indicates in the PFGE of chromosome V the residual signal of FL chromosome III not fully removed after stripping of the previous hybridisation with the HML probe. (Panel (ii)) Short exposures of the FL bands of blots in (i) showed as loading controls, and quantification of signals relative to control cells. (**c**) Graph representing the quantification of chromosome fragments of PFGEs in (b) (quantified as described in Material and Methods), in units relative to control cells.

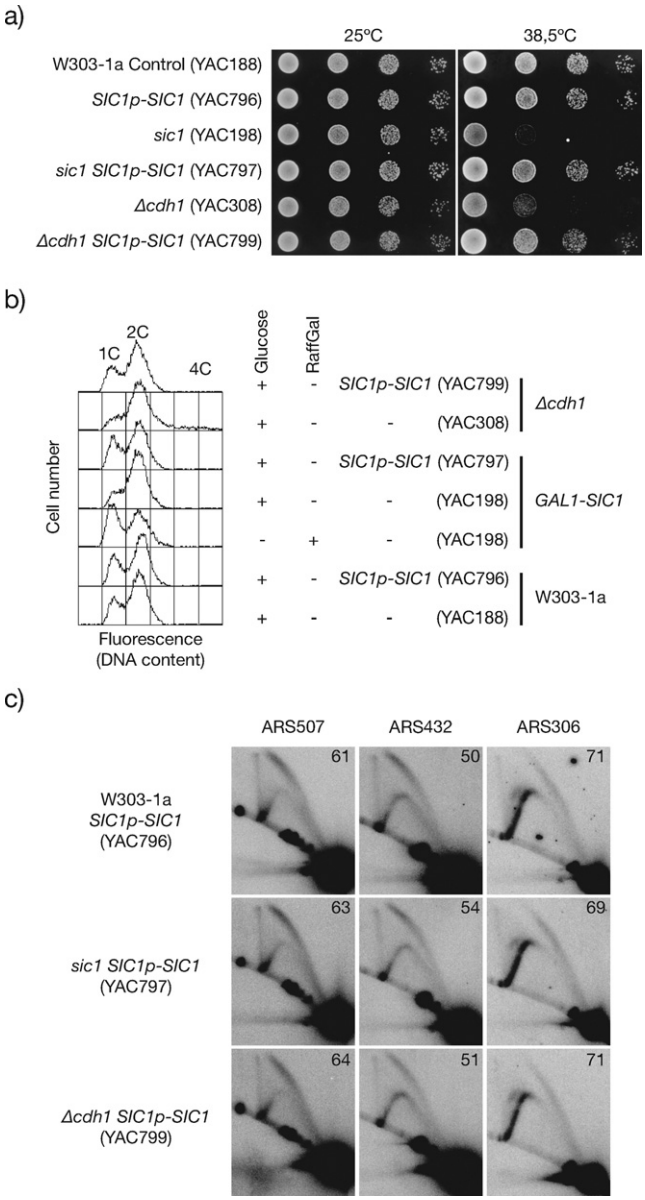

Figure 4. Duplicating the gene dosage of *SIC1* restores origin firing efficiency in Δ*cdh1* cells. (**a**) Dilution spotting of W303–1a control, *sic1* and Δ*cdh1* cells carrying a *SIC1* allele under the control of its own promoter (*SIC1p-SIC1*), at 25°C and 38.5°C, growing in glucose-based rich media plates. (**b**) FACS analysis of asynchronous cells at 25°C in glucose or raffinose-galactose (RaffGal). (**c**) 2D analysis of origin firing efficiency at ARS507, ARS432 and ARS306.

patterns of origin sensitivity are different to those observed in our study. We see that sensitivity is independent on either the timing of origin firing, the origin efficiency, or the chromosomal location. We hypothesise that origin sensitivity to CDK deregulation is instead dependent on unique origin characteristics as the *cis*-acting origin elements that, apart from the ARS consensus site, are highly variable amongst origins and modulate origin activities (80,81). Hence, particular configurations of *cis* elements may result in differential recruitment abilities amongst origins for licensing and/or firing factors in budding yeast, as observed in fis-

sion yeast origins (82), influencing the origin tolerance to CDK deregulations.

Origin redundancy is likely to be a determinant factor for the stability of a chromosome region in CDK-deregulated cells. For chromosome maintenance, normal cells tolerate the loss of a few active origins, but not the additional loss of flanking efficient origins and particularly the loss of dormant origins at chromosome ends (29,60). In CDK-deregulated cells, the coincidence of sensitive origins at a chromosome region can lead to reduce firing to levels compromising chromosome stability. We found, at the left arm of chromosome V, that origin firing shortage increases with the severity of CDK deregulation, in correlation with higher rates of GCRs. Furthermore, the extent of efficiency loss, the number of origins losing activity and the activity of a dormant origin reflect the instability of the chromosome.

Fragile site expression is linked to origin paucity in human cells (39) and reduced Mcm2-7 subunits in primary MEFs decrease the licensing of dormant origins, leading to genome instability and increased cancer incidence (33). Presumably, in CDK-deregulated cells origin firing shortage can compromise the timing and the completion of DNA replication regionally within S phase, agreeing with previous suggestions (24,34–36,83). In mammalian cells, the activity of individual origins upon CDK deregulation remains largely unaddressed. Our preliminary data indicates that origin efficiency is altered in p27Kip1-/- MEFs (J. Sequeira-Mendes, C. Ávila-Zarza, M. Gómez and A. Calzada, unpublished results), but it remains to be established whether this reflects regional changes in origin density or in the timing of firing. The lengthening of S phase in CDK-deregulated cells might also reflect an altered DNA replication dynamics, consistent with increased arrested forks in human cell lines overexpressing cyclin E (84), and with slower replication fork progression and reduced frequency of termination events in Cdh1-depleted cells (85). Addressing the dynamics of DNA replication along the genome in time and space in CDK-deregulated oncogenic cells, and whether it contributes to chromosome instability by untimely termination, are interesting open questions that will require further analysis in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including reference [86].

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