The Origin Recognition Complex (ORC) is a critical component of replication initiation. We have previously reported generation of an Orc2 hypomorphic cell line (Δ−/) that expresses very low levels of Orc2 but is viable. We have shown that Chk2 is phosphorylated, suggesting that DNA damage checkpoint pathways are activated. p53 was inactivated during the derivation of the Orc2 hypomorphic cell lines, accounting for their survival despite active Chk2. These cells also show a defect in the G1 to S-phase transition. Cdk2 kinase activation in G1 is decreased due to decreased Cyclin E levels, preventing progression into S-phase. Molecular combing of bromodeoxyuridine-labeled DNA revealed that once the Orc2 hypomorphic cell lines, accounting for their survival despite active Chk2.

The ORC itself is of great interest, as it defines the region in the genome where replication will begin. This function is vital, as evenly spaced initiations are important to complete replication of the entire genome as quickly as possible. In fact, ORC was initially identified in budding yeast based on its ability to interact with the yeast origin sequence (ARS) (2). ORC is formed of six subunits, Orc1−6, that range in size from 97 to 28 kDa. It has been shown that the ORC subunits interact with each other, albeit with different affinities (14, 15). These studies show that Orc2 and Orc3 seem to form a core subcomplex with which other ORC members interact.

To study ORC in human cells, we have previously generated an Orc2 hypomorph cell line (containing one hypomorph Orc2 allele and one null Orc2 allele) was found to be viable, presumably because of compensatory changes in other genes, and was able to proliferate for many generations. The low levels of Orc2 did, however, prevent replication of an exogenous plasmid containing a single Epstein-Barr virus origin. We believe that this difference in replication ability stems from a threshold effect; the levels of Orc2 were not high enough to support chromosome replication, but not episome replication.

To study ORC in human cells, we have previously generated an Orc2 hypomorph cell line, in which Orc2 is only being expressed from one allele at 10% of wild type levels (22). This Orc2 Δ−/− cell line (containing one hypomorph Orc2 allele and one null Orc2 allele) was found to be viable, presumably because of compensatory changes in other genes, and was able to proliferate for many generations. The low levels of Orc2 did, however, prevent replication of an exogenous plasmid containing a single Epstein-Barr virus origin. We believe that this difference in replication ability stems from a threshold effect; the levels of Orc2 were high enough to support chromosome replication, but not episome replication.

In this study, we have examined the effects of low Orc2 on the replication ability of the cellular chromosomes, as well as the cell as a whole.

**Materials and Methods**

**Cell Culture**—HCT116, HCT116 p53−/−, and Δ−/− (HCT116 Orc2 Δ−/−) cells were maintained in McCoy’s 5A medium (Cellgro), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Standard tissue culture growth conditions and methods were used.
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Western, Northern, and RT-PCR—Western blots were performed as described. In this case, cells were lysed in 50 mM Tris, pH 7.4, 0.2% Nonidet P-40, 150 or 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 1/100 Protease Inhibitor Mixture (Sigma). Orc3, -4, and -6, Cdk1, and RPA34 and RPA70 antibodies have been previously described (23–26). Rabbit antibodies against Orc5 were raised using His6-tagged recombinant Orc5 (75–686). p27 (C-19), p21 (C-19), and MCM7 antibodies were purchased from Santa Cruz Biotechnology, and Orc2 antibody was purchased from BD Biosciences. p53 antibody (D0–1) was purchased from Santa Cruz Biotechnology, and Orc2 antibody was purchased from Cell Signaling (recognizes pTyr15 on Cdc2 and Cdk2.) Chk2 was purchased from BD Biosciences. p53 antibody (D0–1) was purchased from Santa Cruz Biotechnology, and Orc2 antibody was purchased from Cell Signaling. pTyr15 Cdk2 was detected by immunoprecipitation against Cdk2, followed by Western using pTyr15 Cdc2 from Cell Signaling (recognizes pTyr15 on Cdc2 and Cdk2.) Chk2 was purchased from Sigma, and E2F1 was purchased from Upstate. Total cellular RNA was prepared using the RNAeasy Midi or Mini kit (Qiagen) or TRIzol extraction.

Pulse-Chase—Logarithmic cells were starved of methionine for 1 h in Dulbecco’s modified Eagle’s medium, supplemented with dialyzed fetal bovine serum. Cells were then labeled with 300 μCi of [35S]methionine for 4 h. Cells were washed to remove unincorporated methionine and were chased with McCoy’s 5A medium supplemented with fetal bovine serum for the indicated lengths of time. Cells were lysed in the same buffer as described above, but with 300 mM NaCl. Orc3 was immunoprecipitated with the above antibody and separated by SDS-PAGE. The gel was then dried and exposed to a phosphorimaging plate for visualization and analysis.

FACS Analysis—Cells were prepared as previously described (27). The analysis was carried out on a BD Biosciences FACs Calibur using CellQuest and FloJo software.

Chromatin Immunoprecipitation—In vivo cross-linking was performed as described in Ref. 28 with some modifications. In brief, 80% confluent HeLa, HCT116, or HCT116 Δ− cells were grown as described above and then treated with formaldehyde (1%). Cross-linked cell nuclei were sonicated 10 times for 30 s each time, and the chromatin size was monitored by electrophoresis (29). This treatment generated fragments of ~20 kb. To further reduce the chromatin size to smaller fragments of 1.5–3.5 kb, DNA was digested with SpnI, HindIII, PstI, and EcoRI restriction endonucleases in NEB2 buffer (100 units of each; New England Biolabs, Beverly, MA) at 37 °C for 6 h. Sheared chromatin-lysed extracts were incubated with 50 μl of protein G-agarose (Roche Applied Science) to reduce background caused by nonspecific adsorption of irrelevant cellular proteins/DNA to proteins. These cleared chromatin lysates were incubated for 4 °C for 6 h on a rocker platform with either 50 μl of preimmune rabbit serum (Santa Cruz Biotechnology) or 5 μg of anti-Or52, anti-Or53, anti-Or54, or anti-Or6 antibodies. Protein G-agarose (50 μl) was added, and the incubation was continued for 12 h. The precipitates were successively washed twice for 5 min with 1 ml of each buffer, lysis buffer, WB1 (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate), WB2 (as WB1 with no NaCl), and 1 ml of TE (20 mM Tris–HCl, pH 8.0, 1 mM EDTA). The precipitates were finally resuspended in 200 μl of extraction buffer (1% SDS/TE). The samples were incubated at 65 °C overnight to reverse the protein/DNA cross-links, followed by a 2-h incubation at 37 °C with 100 μg of proteinase K (Roche Applied Science). Finally, the samples were processed for DNA purification by passing them through QIAquick PCR purification columns (Qiagen, Valencia, CA). PCR reactions were carried out in 20 μl with 1/200th of the immunoprecipitated material with the use of LightCycler capillaries (Roche Applied Science) and the LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science). The real-time PCR quantification was performed as described in Ref. 30, using primer sets LB2 and LB2 C1.

Cell Cycle Analysis—Cells were arrested in 40 ng/ml of nocodazole for 16 h. To release the cells from mitosis, they were washed three times in sterile phosphate-buffered saline and replated in fresh warmed medium. After the desired incubation time, cells were labeled with 10 μM BrdUrd for 1 h and then prepared for FACS or Western blot analysis as above. Alternately, after the desired incubation time, cells were treated with 1.25 μCi of [3H]thymidine for 1 h. Cells were then washed and incubated with cold stop solution (10% trichloroacetic acid, 200 mM sodium pyrophosphate). Cells were washed with 95% ethanol and solubilized (1% SDS, 10 mM NaOH.) The resulting solution was spotted on Whatman paper, dried, and counted on a Beckman LS 6000 scintillation counter. To examine cells in S-phase, cells were treated with 2 mM thymidine for 12 h, released into untreated medium for 12 h, and further treated with 1 μg/ml of aphidicolin for 12 h.

Chromatin Fractionation—Chromatin fractions were isolated as described previously (31). Briefly, cells (2 × 109) were lysed in 100 μl of CSK buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) containing 0.5% Triton X-100, 1 mM ATP, 1 mM Na3VO4. Lysates were incubated on ice for 20 min and then centrifuged at 1500 rpm for 5 min at 4 °C. Supernatant (S1) was removed, and pellets were washed with 1 ml of lysis buffer and centrifuged again. Pellets were incubated in 100 μl of lysis buffer containing 1 mM CaCl2 and 120 units of micrococcal nuclease ( Worthington) for 10 min at 37 °C and centrifuged. Supernatant (S2, chromatin-bound fraction) was removed, and pellets were washed with 1 ml of lysis buffer and centrifuged again. Pellets were boiled in 100 μl of 1 × sample buffer (P2).

DNA Combing and Detection by Fluorescent Antibodies—Cells were synchronized at very early S-phase by treatment with 2 mM thymidine for 12 h, release into untreated medium for 12 h, and further treatment with 1 μg/ml of aphidicolin for 12 h. Cells were released into medium containing 50 μM BrdUrd for the indicated time points. Cells were then washed in medium containing 100 μM thymidine and incubated in 10 μM thymidine until 8 h post release. Cells were then harvested with trypsin/EDTA and suspended in 1% agarose. Agarose blocks were incubated with ESPK (0.5 mM EDTA, 1% sarkosyl, 2 mg/ml proteinase K (Fisher)) for 24 h twice. Blocks were then washed with TE/phenylmethylsulfonyl fluoride and stored in 0.5 mM EDTA.

DNA from whole cells was extracted and combed on silanised coverslips as described (32). Combed DNA was dehydrated in a series of ethanol (70, 90, and 100%), denatured with 1N NaOH for 30 min, again dehydrated, and blocked in a blocking solution (1 × phosphate-buffered saline, 0.1% Tween, 1% bovine serum albumin) for 1 h. BrdUrd was detected with an anti-BrdUrd antibody (Abcys), followed by an anti-rat antibody conjugated with AlexaFluor 488 and an anti-goat AlexaFluor 488 antibody (33). Total DNA was visualized afterward by an anti-guanosine antibody (Argene), followed by an anti-mouse AlexaFluor 594 (Molecular Probes). Antibody incubations were in general for 30 min and were separated by three-four washes with 1 × phosphate-buffered saline, 0.1% Tween. Coverslips were mounted in Vectashield® solution. To analyze data, images of the combed DNA molecules were acquired by a Leitz DC300F camera associated with the LeicaFW4000 software and measured by ImageGauge 4.2 software. Fields of view were chosen at random in the AlexaFluor 594 channel and then photographed under the AlexaFluor 594 and AlexaFluor 488 filters. The replication extent of each sample was defined as the sum of all eye lengths divided by the total length of the molecules. Fork density is the total
ORC and Pre-RC Loading Is Decreased in Orc2 Hypomorph Cells—In yeast, Orc2 is required for DNA replication initiation (35). To investigate ORC loading at a specific origin, chromatin immunoprecipitation was performed using several ORC subunits. The associated chromatin was assayed by quantitative PCR using primers in the Lamin B2 region, a previously reported human origin (36). Orc2, 3, 4, and 6 associated with the Lamin B2 origin in HeLa cells, but not a nearby control region, whereas normal rabbit serum did not associate with either region (Fig. 2A).

The experiment was then performed on HCT116 +/+ and Δ− cells, which revealed a decrease of ORC loading on Lamin B2 in Δ− cells (Fig. 2B). The amount of cross-linked DNA molecules pulled down in HCT116 cells was 3- to 7.7-fold higher than in the Δ− cells. This indicates that origin association of the entire ORC (including Orc4 and Orc6, whose levels are not changed) is dependent on the presence of Orc2. This extends our earlier observation that Orc2 loading decreases in a chromatin fractionation experiment (22). Interestingly, origin specificity (the ratio of ORC signal at Lamin B2 versus the control region) was retained in Δ− cells despite the low levels of ORC binding.

ORC association with origins recruits Cdt1 and Cdc6, which in turn recruit the MCM2–7 complex to complete a functional pre-RC (5, 6). To investigate pre-RC loading, we fractionated cells and examined MCM7 levels in the chromatin fractions. Chromatin-bound MCM7 was drastically decreased in the Δ− cells (Fig. 2C). As chromatin-loaded MCM2–7 is required not only to recruit additional factors for replication but also to unwind the DNA for fork firing, the decrease in pre-RC loading suggests that replication may be affected in the Δ− cells, as chromatin-loaded MCM2–7 is required not only to recruit additional factors for replication but also to unwind the DNA for fork firing.

Chk2 Is Phosphorylated in Δ− Cells—The low levels of pre-RC are expected to lead to replication stress. This could cause fork stalling (and checkpoint activation) as forks extend from more sparsely firing origins. To test for checkpoint activation, we looked at phosphorylation of Chk1 at serine 317, a target of the DNA damage modulator ATR (37). We also looked at phosphorylation of Chk2 at threonine 68, a target of the other major modulator, ATM (38). Chk1 phosphorylation, although induced by hydroxyurea, is not increased in Δ− cells (data not shown). However, we found that Chk2 phosphorylation at Thr-68 is increased in the Δ− cells (Fig. 3A). It therefore appears that a DNA damage checkpoint is activated in these cells.

To determine the downstream effects of Chk2 phosphorylation, we examined several known targets. Cdc25C is phosphorylated by Chk2 at serine 216 (39, 40), which serves to inhibit its normal role in activating cell cycle progression.
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Cyclin B/Cdc2 (41), thus preventing mitotic entry. Phosphorylation of Cdc25C was slightly decreased in Δ−/− cells, indicating it is not a target of the active Chk2 (supplemental Fig. S2a). Cdc25A is also phosphorylated by Chk2, which, in turn, would lead to increased Tyr-15 phosphorylation on Cdk2 (43, 44). We did not, however, observe any decrease in the amount of Cdc25A in the Δ−/− cells (it was actually increased) (supplemental Fig. S2a). Likewise, the total amount of Tyr-15 phosphorylation on Cdk2 was less in Δ−/− cells, the opposite of what is expected if Cdc25A were inactive (supplemental Fig. S2b). Therefore, Cdc25A and the inhibitory phosphorylation of Cdk2 do not seem to be affected by activated Chk2 in the Δ−/− cells.

Perhaps the most well studied target of Chk2 is p53. Chk2 stabilizes p53 by phosphorylating it at serine 20 (45, 46). We were surprised to see not seem to be affected by activated Chk2 in the Δ−/− cells. Surprisingly, p53 was lost in the Δ−/− cells, indicating this DNA damage response pathway is completely disrupted in these cells at a step after Chk2 activation. To determine at which point p53 loss occurred compared with Orc2 loss, we looked at p53 levels in each of the Δ−/− precursor cell lines. Surprisingly, p53 was lost in the Δ−/+ cells created after Cre-mediated recombination (Fig. 3, B and E). Induction with γ irradiation failed to induce p53 or p21 in Δ−/− cells, indicating this DNA damage response pathway is completely disrupted in these cells at a step after Chk2 activation.

To distinguish these possibilities, we analyzed the percentage of cells entering S-phase by BrdUrd labeling at different time points following release from nocodazole arrest. These cells were fixed, labeled with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody, stained with propidium iodide, and analyzed using two-color FACS (Fig. 4B). HCT116 and p53−/− cells incorporate BrdUrd similarly at each time point, indicating there is no S-phase entry defect in the p53−/− cells. In contrast, a much smaller percentage of Δ−/− cells incorporate BrdUrd at every time point. Cells enter S-phase, as shown by an increase in this percentage as time passes, but they do so with a significant delay compared with controls.

Recent work has described a possible role for mammalian Orc2 in mitosis (20). The failure of S-phase entry we observed may stem from a
mitotic deficiency due to low levels of Orc2. However, we did not observe any defect in mitotic exit as determined by chromatid condensation. Nocodazole-arrested HCT116 and Δ−/− cells showed equal DNA condensation in nocodazole and equal levels of decondensation 3 h after release (Fig. 4C). This indicates that there is no delay in exit from mitosis and that the S-phase entry delay is caused by a G1−S transition defect.

Orc2-deficient Cells Have Cell Cycle Progression Defects—Δ−/− cells released from a nocodazole arrest appear to have difficulties transitioning from G1 to S-phase. We therefore examined several cell cycle factors important for G1 progression. Rb protein is a known regulator of the transition from G1 to S-phase. Its hyperphosphorylation before S-phase by cyclin-dependent kinases (CDKs) causes its dissociation from the transcription factor E2F, which allows transcriptional activation of many genes required for cell cycle progression (for review, see Ref. 48). After release from nocodazole, Δ−/− cells have more hypophosphorylated Rb compared with wild type cells. (Fig. 5A) We also observed less phosphorylation of two specific sites on Rb, serine 780 and serine 807/808, in the Δ−/− cells. The persistence of the inhibitory form of Rb indicates that cell cycle progression through G1 is delayed in the Δ−/− cells. We therefore examined several cell cycle factors important for G1 progression. Rb protein is a known regulator of the transition from G1 to S-phase. Its hyperphosphorylation before S-phase by cyclin-dependent kinases (CDKs) causes its dissociation from the transcription factor E2F, which allows transcriptional activation of many genes required for cell cycle progression (for review, see Ref. 48). After release from nocodazole, Δ−/− cells have more hypophosphorylated Rb compared with wild type cells. (Fig. 5A) We also observed less phosphorylation of two specific sites on Rb, serine 780 and serine 807/808, in the Δ−/− cells. The persistence of the inhibitory form of Rb indicates that cell cycle progression through G1 is delayed in the Δ−/− cells. We therefore examined several cell cycle factors important for G1 progression. Rb protein is a known regulator of the transition from G1 to S-phase. Its hyperphosphorylation before S-phase by cyclin-dependent kinases (CDKs) causes its dissociation from the transcription factor E2F, which allows transcriptional activation of many genes required for cell cycle progression (for review, see Ref. 48). After release from nocodazole, Δ−/− cells have more hypophosphorylated Rb compared with wild type cells. (Fig. 5A) We also observed less phosphorylation of two specific sites on Rb, serine 780 and serine 807/808, in the Δ−/− cells. The persistence of the inhibitory form of Rb indicates that cell cycle progression through G1 is delayed in the Δ−/− cells. We therefore examined several cell cycle factors important for G1 progression. Rb protein is a known regulator of the transition from G1 to S-phase. Its hyperphosphorylation before S-phase by cyclin-dependent kinases (CDKs) causes its dissociation from the transcription factor E2F, which allows transcriptional activation of many genes required for cell cycle progression (for review, see Ref. 48). After release from nocodazole, Δ−/− cells have more hypophosphorylated Rb compared with wild type cells. (Fig. 5A) We also observed less phosphorylation of two specific sites on Rb, serine 780 and serine 807/808, in the Δ−/− cells. The persistence of the inhibitory form of Rb indicates that cell cycle progression through G1 is delayed in the Δ−/− cells. We therefore examined several cell cycle factors important for G1 progression. Rb protein is a known regulator of the transition from G1 to S-phase. Its hyperphosphorylation before S-phase by cyclin-dependent kinases (CDKs) causes its dissociation from the transcription factor E2F, which allows transcriptional activation of many genes required for cell cycle progression (for review, see Ref. 48). After release from nocodazole, Δ−/− cells have more hypophosphorylated Rb compared with wild type cells. (Fig. 5A) We also observed less phosphorylation of two specific sites on Rb, serine 780 and serine 807/808, in the Δ−/− cells. The persistence of the inhibitory form of Rb indicates that cell cycle progression through G1 is delayed in the Δ−/− cells.
normal in the δ− cells once they have entered S-phase. Therefore, cells that enter S-phase have enough Cyclin E/Cdk2 kinase activity to phosphorylate Rb and promote the G1→S transition.

Once δ− cells enter S-phase, they seem to be able to proceed normally with replication. We have shown that initiation at β-globin and c-Myc origins was normal in δ−/− cells (22) but could not rule out that these origins were exceptional. To look at origin firing globally, we used DNA combing on cells that have entered S-phase (32). Briefly, cells were synchronized with thymidine/aphidicolin and released in the presence of BrdUrd. Genomic DNA was combed and prepared for immunofluorescent microscopy. DNA lengths labeled with BrdUrd were compared with total lengths to determine the rate at which new forks appeared during the first 160 min of S-phase. Finally, total DNA amounts were normalized by the percentage of cells actually entering S-phase as determined in Fig. 6A. The average fork density (all three time points together) is similar among the three cell lines (Fig. 6C). These results indicate that similar numbers of forks are firing in the cell lines. Furthermore, the replication progression (percentage of DNA replicated at a given time) is also very similar between δ−/− and wild type cells (Fig. 6C). These results suggest that once δ− cells enter S-phase, they are able to fire forks relatively normally despite very low levels of pre-RC loading.

DISCUSSION

One of the most immediate ramifications of low Orc2 levels is the equal decrease of Orc3 protein and, to a lesser extent, Orc5 protein. These decreases are not transcriptional, as Orc3 and Orc5 mRNA levels are unchanged between HCT116 and δ−/− cells. The half-life of Orc3 is significantly reduced in the δ−/− cells, but this instability is not caused by proteasomal degradation. Therefore, Orc3 seems to be more prone to non-proteasomal forms of degradation in the absence of Orc2. Biochemical studies of the human ORC proteins show a tight binding between Orc2 and Orc3 (14, 15); this interaction may serve to stabilize Orc3. When Orc3 cannot bind to Orc2, residues normally buried in the Orc2/3 interface would be exposed. This could result either in degradation by an unknown active mechanism or loss of structural integrity, allowing the protein to be degraded by housekeeping mechanisms. Dhar et al. (14) also reported that Orc5 is able to bind Orc2/3, forming a stable subcomplex. The proteasome-independent decrease in Orc5 levels may also be due to a decrease in stability resulting from the absence of binding partners. The other ORC subunits are unaffected by loss of Orc2/3 (or Orc5), supporting the notion that Orc1, Orc4, and Orc6 form relatively stable interactions with Orc2,3,5.

As several members of the ORC are decreased in δ−/− cells, it is not surprising that the origin loading of these subunits is decreased. However, Orc4 and Orc6 loading is drastically reduced despite unchanged protein levels, which indicates that their chromatin loading is dependent on the presence of the Orc2,3,5 subcomplex. The lack of ORC origin binding also results in decreased MCM2−7 loading on chromatin in human cells, which confirms results seen in other model systems.

The overall decrease in Orc2 seems to cause several unique problems for replication. δ−/− cells released from mitosis begin incorporating labeled nucleotides later than controls, and the total level of labeling is decreased despite equal starting numbers of cells (Fig. 4A). Fewer δ−/− cells enter S-phase, and those cells that do enter seem to do so later than control cells. This difficulty in entering S-phase is not due to any mitotic defect resulting from low ORC but is a defect in the G1→S transition.

Cyclin E protein and message levels are decreased in G1−/− cells, and Cdk2 kinase activation is significantly delayed. This kinase activation is one of the critical events in the G1→S transition. In addition to phosphorylating various factors required for replication fork loading, Cyclin E/Cdk2 also phosphorylates Rb, which allows release of the transcription factor E2F. E2F is then free to activate transcription of a variety of important cell cycle genes, including Cyclin E, and many different replication factors (59). We found that the δ−/− cells have a large population of hypophosphorylated Rb, further preventing entry into S-phase. Additionally, E2F levels are low, suggesting that the G1→S signal amplification between Rb, E2F, and the CDKs is disrupted in the δ−/− cells. Because these proteins form a complex, cross- and autoregulatory amplification signal cascade, it is difficult to identify the initiating event. However, despite previous reports of Cyclin D/Cdk4 involvement in Ser−780 phosphorylation on Rb (an initial event required before further phosphorylation), we find the Cdk4-associated kinase activity is unchanged in the two cell lines. This suggests that although Cyclin D/Cdk4 may be essential for Ser−780 phosphorylation, it is not sufficient. An additional factor seems to be missing, preventing phosphorylation of Ser−780 in cells with low Orc2 levels. The resulting G1 delay explains why fewer δ−/− cells enter S-phase and could also account for the overall slower apparent proliferation rate of these cells. Despite this G1 delay, it appears that once cells exit G1−, they can proceed through S-phase relatively normally. δ−/− cells released from a thymidine/aphidicolin arrest have no cyclin/CDK defect, have equivalent fork firing, and show normal Rb phosphorylation. We imagine that some cells are able to amplify the G1→S signal cascade, eventually increasing Cyclin E protein levels and thus Cyclin E-associated kinase activity. Once the kinase activity reaches a threshold, a given cell is able to enter S-phase.

It is interesting to speculate which ORC-dependent events are important for cell cycle progression through G1. One possibility is that pre-RC loading onto chromatin is monitored so that the cells only proceed into S-phase when a minimum threshold is reached. Allowing the δ−/− cells extra time in early S-phase may give them the opportunity to reach such a threshold, at which point the origins fire and replicate normally. Even if a pre-RC loading threshold exists, the proliferation of the δ−/−
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