The ORC1 Cycle in Human Cells

I. CELL CYCLE-REGULATED OSCILLATION OF HUMAN ORC1*

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Components of ORC (the origin recognition complex) are highly conserved among eukaryotes and are thought to play an essential role in the initiation of DNA replication. The level of the largest subunit of human ORC (ORC1) during the cell cycle was studied in several human cell lines with a specific antibody. In all cell lines, ORC1 levels oscillate: ORC1 starts to accumulate in mid-G1 phase, reaches a peak at the G1/S boundary, and decreases to a basal level in S phase. In contrast, the levels of other ORC subunits (ORCs 2–5) remain constant throughout the cell cycle. The oscillation of ORC1, or the ORC1 cycle, also occurs in cells expressing ORC1 ectopically from a constitutive promoter. Furthermore, the 26 S proteasome inhibitor MG132 blocks the decrease in ORC1, suggesting that the ORC1 cycle is mainly due to 26 S proteasome-dependent degradation. Arrest of the cell cycle in early S phase by hydroxyurea, aphidicolin, or thymidine treatment is associated with a decrease in ORC1, indicating that ORC1 proteolysis starts in early S phase and is independent of S phase progression. These observations indicate that the ORC1 cycle in human cells is highly linked with cell cycle progression, allowing the initiation of replication to be coordinated with the cell cycle and preventing origins from refiring.

*This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The replication of chromosomal DNA in eukaryotes is limited to once per cell division cycle. This control appears to be achieved mainly by the regulation of replication origins so that they fire only once per cell cycle. The origin recognition complex (ORC), identified in budding yeast as a protein complex that binds origins, consists of six gene products (1–5). In addition to ORC, several factors highly conserved among eukaryotes are involved in initiation (6–8). The sequential assembly of these factors on origin-ORC complexes precedes initiation, as has been shown in yeast and *Xenopus* systems. For example, mini-chromosome maintenance (MCM) proteins are loaded onto origins in the presence of ORC and CDC6, which establishes the pre-replicative complex (pre-RC) necessary for subsequent protein assembly (9–13). After origin firing, the pre-RC changes to a post-replicative form by the dissociation of MCM from the complex (12, 14, 15). These associations provide an important mechanism that 1) ensures that replication origins fire at precise times and 2) prevents re-initiation.

Budding yeast ORC is a static complex that is maintained at a constant level and remains bound to origins throughout the cell cycle (4, 5). Thus, MCM loading in yeast is mainly regulated by other factors such as cell cycle-regulated Cdc6 or CDK kinase activities (7, 8, 16). It is also known that the phosphorylation status of ORC subunits correlates with the timing of pre-RC formation, suggesting a role for ORC phosphorylation in MCM loading (16). Similar phosphorylation of ORC subunits was found in a *Xenopus* egg extract system, suggesting a conserved mechanism for the regulation of ORC functions (17, 18). Recent studies have elucidated possible mechanisms for the regulation of ORC activity in mammals. In human and hamster cells, ORC1, the largest subunit, is ubiquitinated in S phase (19–21). In human cells, ORC1 is poly-ubiquitinated by the SCF<sub>skp2</sub> ubiquitin ligase complex and degraded through the 26 S proteasome pathway (19). Indeed, the cellular content of ORC1 is greatly reduced in S phase (19, 22). In hamster cells, ORC1 is only mono-ubiquitinated and may be regulated by a mechanism other than degradation (21). Indeed, the cellular ORC1 level remains constant in hamster cells (21, 23). These data suggest that the activity of mammalian ORC is regulated by ubiquitination of ORC1, but ubiquitinated ORC1 has different fates in different species or cell lines.

To elucidate the mechanisms that regulate ORC activity in mammals, and especially in humans, we determined the levels of ORC subunits in several human cell lines throughout the cell cycle. From systematic studies in which we synchronized these cells by a variety of methods, we conclude that ORC1 exhibits cell cycle-dependent oscillation, a phenomenon we have termed the ORC1 cycle. The ORC1 cycle ensures the dynamic assembly of ORC and MCM on chromatin in G<sub>1</sub> phase, which may correspond to the formation of the pre-RC in human cells, as demonstrated in an accompanying paper (24).
EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). To impose cell cycle arrest by the double-thymidine block method, HeLa S3 cells were incubated in the presence of 2.5 mM thymidine for two periods of 24 h with an intervening incubation of 12 h in the absence of thymidine. For M phase arrest, cells were subjected to the double-thymidine block procedure and then cultured in the presence of 150 μg/ml TN16 (WAKO, Japan) for 12 h. Synchronous growth following arrest was achieved by two washes with PBS and subsequent culturing without reagents. Telomerase-immortalized human retina pigment epithelial cells (TERT-RPE1 cells, Invitrogen) were synchronized by serum starvation. TERT-RE1 cells (50% confluent) were incubated with Dulbecco's modified Eagle's medium without FCS for 84–96 h and then released from starvation by the addition of 10% FCS. Hydroxyurea (HU) was included at 2.5 mM. Synchronized growth was monitored by flow cytometry (BD Biosciences) of propidium iodide-stained cells (25), by immunoblot analysis of cyclin E and cyclin A levels, and by 5-bromo-2'-deoxy-uridine (BrdUrd) incorporation. Incorporated BrdUrd was detected using the 5-Bromo-2'-deoxyuridine Labeling and Detection Kit I (Roche Applied Science, Germany). Briefly, cells were labeled with BrdUrd for 10 min and detected using a monoclonal antibody against BrdUrd and a fluorescein-conjugated secondary antibody, as specified by the manufacturer. Cells were also stained with 4,6-diamidino-2-phenylindole (DAPI) as described previously (25). The fluorescent signals were visualized with a Leica DMRLB2 and captured with Photometrics PXL.

Expression of ORC1-FLAG in Human 293 Cells—The ORC1 cDNA with a C-terminal fusion to the 3xFLAG sequence (5'-GACTACAGACCCAGACCCAGACCCAGACCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAAG-3') and an N-terminal fusion to an artificial Kozak sequence (5'-GGCCGCCGCCGCC-3') was inserted downstream of the CMV promoter of pcDNA3.1zeo (Invitrogen). The resulting plasmid (pcDNA-ORC1-FLAG) was transfected into 293 cells with FuGENE 6 (Roche Applied Science, Germany), and clones expressing ORC1-FLAG were obtained by selection on zeocin. A clone (1C2) expressing a level of ORC1-FLAG equivalent to the endogenous level of ORC1 was chosen for the experiments shown in Fig. 4C.

Preparation of Cell Extracts—For whole cell extracts, cells plated on dishes were washed three times with PBS, suspended in modified CSK buffer (0.1% TX-100mCSK: 100 mM NaCl, 10 mM Pipes (pH 7.0), 300 mM sucrose, 0.1% Triton X-100 (Triton), 1 mM MgCl₂, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 4% SDS, 0.1% bromphenol blue, and 20% glycerol). Trichloroacetic acid fixation was done as described previously (25). Proteins from HeLa S3 cells were fractionated with Triton as previously described (25).

Antibodies—Antibodies against human ORC1 (#209) and human ORC5 (#241) were generated from New Zealand White rabbits immunized with recombinant ORC1 protein fragments (amino acid residues 1–698) tagged with His₅ (5'-CACCACACCCACCCACCCACCC-3') or full-length ORC5 tagged with glutathione S-transferase at their respective N termini. The peptides were expressed and purified from Escherichia coli by standard methods. To obtain nonspecific antibodies, the crude antisera were affinity-purified with antigens. Anti-human ORC2 and MCM7 monoclonal antibodies, 3B7 and 4B4, were purchased from MBL (Japan), and anti-human ORC3 and ORC4 antisera were kindly provided by Dr. A. Dutta (26, 27). Rabbit anti-murine MCM3 (mMCM3) antiserum, which reacts with human MCM3, has been described previously (26, 27). Anti-FLAG monoclonal antibody (M2) was purchased from Sigma. Immunoblotting with these antibodies was done as described previously (25).

Two-step Immunostaining with Anti-ORC1 and Anti-BrdUrd Antibodies—For the initial staining with the #209 antibody, cells on coverslips were washed with PBS three times, fixed with 1% formaldehyde in PBS for 10 min, and washed three times with PBS. The coverslips were incubated with Tris-buffered saline (TBS) containing 0.1% Triton for 10 min, washed three times with TBS containing 0.1% Tween 20 (TBS-T), incubated with #209 in TBS-T containing 10% FCS overnight at 4 °C, washed three times with TBS-T and incubated with a Cy3-conjugated secondary antibody (Zymed Laboratories Inc., reactive with rabbit IgG) at room temperature for 2 h. After three washes with TBS-T followed by one PBS wash, cells were treated with 1% formaldehyde in PBS for 10 min and washed three times with PBS. Samples were stained with the anti-BrdUrd antibody in the same way. Fluorescent images were collected using a Leica DMRLB2 microscope coupled to a Photometrics PXL capturing system (Fig. 2), or by using an Olympus confocal microscope (Fluoview FV500; Fig. 3). To count BrdUrd- and ORC1-positive cells (Fig. 2), threshold levels were set just above the cytoplasmic background (Iplab Spectrum P 3.1.2c, Scanalytics Inc.), and cells showing nuclear signals were scored as positive.

RESULTS

Detection of Human ORC1 from Human Cell Lysates with Anti-ORC1 Antibodies—We previously characterized ORC1 in human cells using the monoclonal antibody 3A2A, which was raised against a human ORC1 peptide (25). In this study, we prepared a new polyclonal anti-ORC1 antibody, #209, which detected a 100-kDa polypeptide band, as shown by immunoblot analysis (Fig. 1), and precipitated proteins of the same size from human cell extracts (24). We have confirmed that the 100-kDa polypeptide that reacts with #209 is ORC1 by two lines of evidence. First, the amino acid sequences of 16 tryptic peptides produced from the immunoprecipitated band matched those of human ORC1, as shown by LC/MS/MS analysis (24). Second, the 100-kDa band shown in Fig. 1 disappeared after cells were subjected to RNA interference with human ORC1-specific small interference RNAs (24). Therefore, the new antibody is highly specific to human ORC1. On the other hand, although our previous antibody, 3A2A, also reacted specifically with 100-kDa polypeptide(s) in human cells, including ORC1, LC/MS/MS analysis showed that it also cross-reacted with the 100-kDa polypyrimidine tract binding protein-associated splicing factor (data not shown). The broader recognition specificity of 3A2A may affect our previous conclusions concerning human ORC1 (25). Thus, we re-examined human ORC1 with the new antibody and confirmed that most of our original results are valid, except that we now estimate the ORC1 content of HeLa S3 cells to be 1.5 × 10⁴ molecules/cell (data not shown), and the ORC1 level oscillates in a cell cycle-dependent manner as described below. Hereafter, we used the #209 antibody to characterize ORC1 in human cells.

Cell Cycle-dependent Oscillation of ORC1 in HeLa S3 Cells Synchronized by M Phase Arrest—As described under “Experimental Procedures” and above for the #209 antibody, we obtained antibodies specific for ORCs 1–5 that could be used to detect their respective targets in HeLa S3 cell lysates. We analyzed synchronously growing HeLa S3 cells released from a metaphase arrest imposed by treatment with TN16. Cell cycle progression was monitored by counting BrdUrd-positive cells and by analyzing the single-parameter FACS patterns. These profiles indicated that S phase started between 6 and 9 h after release from arrest (Fig. 2). Whole-cell extracts were prepared from cells withdrawn at indicated time points and subjected to immunoblot analysis with antibodies against ORC subunits, cyclins A and E. The latter two proteins were used as repre-
sentative markers for cell cycle transitions (29). It is notable that the cyclin E level correlated with the frequency of BrdUrd-positive cells and that the cyclin A level increased in S phase. ORC1 was at a basal level from metaphase to early G1 (0–3 h), started to increase between 3 and 6 h, peaked at 9 h (G1/S) at a level 10-fold higher than the basal level, and then decreased from S to G2 phase (Fig. 2). In contrast, the levels of the ORC2–5 subunits remained constant over time. The oscillation of ORC1 observed by immunoblotting was confirmed by counting #209-positive cells. The ORC1-positive proportion of cells increased during G1 up to the G1/S boundary (3–9 h) and decreased during S phase (9–15 h). These two results clearly indicate that the increase in ORC1 abundance occurred slightly earlier than the increases in the cyclin E level and in the frequency of BrdUrd-positive cells, which both peaked at 15 h (Fig. 2).

The ORC1 Level Decreases before S Phase in Individual Cells—More precise observations of cells stained with the #209 antibody by confocal microscopy showed that ORC1 appeared as punctate signals in HeLa S3 nuclei, suggesting that it associates with nuclear structures (Fig. 3). The number of dots in a cell was correlated with its position in the cell cycle by simultaneously staining for BrdUrd. As indicated in Fig. 3, more than 500 ORC1-specific dots were dispersed throughout the nucleus in G1 phase cells, but this number decreased in parallel with cell cycle progression (Fig. 3, early S and late S). These data strongly suggest that the decrease in ORC1 abundance that precedes S phase is a result of changes in protein levels in individual cells. Interestingly, ORC1 signals did not overlap with BrdUrd signals, suggesting that ORC1 is not present at sites of DNA synthesis.

Cell Cycle-dependent Oscillation of ORC1 Observed under Various Conditions—Next, we asked whether the oscillation of ORC1 in HeLa S3 cells described above is cell type-specific. We tested two human cell lines, 293 (an embryonic kidney cell line transformed with adenovirus type 5, Fig. 4A) and HEp-2 (a larynx carcinoma cell line, Fig. 4B). Synchronization experiments demonstrated that HEp-2 cells enter S phase with a schedule similar to that of HeLa S3 cells, but 293 cells enter S phase slightly earlier, as indicated by FACS analysis and the frequency of BrdUrd-positive cells. For both cell lines, ORC1-specific oscillations were observed, and the ORC1 level peaked earlier than the increase in the frequency of BrdUrd positive cells, although ORCs 2–5 remained at constant levels throughout the cell cycle, as seen for HeLa S3 cells (Fig. 4, A and B). Next we tested whether oscillation could be observed for FLAG-

**FIG. 2.** Quantitative analysis of ORC subunits in synchronously growing HeLa S3 released from M phase arrest. HeLa S3 cells were released from M phase arrest by TN16 treatment and harvested at the indicated time points. FACS profiles (upper left panels, first row) and staining patterns with 4,6-diamidino-2-phenylindole (DAPI, second row) or antibodies to ORC1 (#209, third row) and BrdUrd (fourth row) are shown. The ratio of ORC1 (closed circles) and BrdUrd (open circles)-positive cells to total DAPI-stained cells is shown graphically, upper right. Quantitative immunoblot analysis of ORC subunits, cyclin A and cyclin E (left panels, fifth to eleventh rows) was performed on whole cell lysates prepared from 1 × 105 cells at each indicated time point. Relative band intensities of ORC1 (closed circles), cyclin A (open squares), and cyclin E (open triangles) with the highest values taken as 1 are shown graphically at lower right.

**Oscillation of the ORC1 Protein in Human Cells**

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**Fig. 3.** Confocal microscopic observations of ORC1 and BrdUrd signals in HeLa S3 cells. Confocal microscopic images of HeLa S3 cells visualized by phase contrast (Phase) or by staining with the #209 (ORC1) and anti-BrdUrd (BrdUrd) antibodies and their merged images (Merge; ORC1 is red and BrdUrd is green) are shown. Cell cycle phases were determined by patterns of BrdUrd staining. Typical results for G1, early S, and late S phases are shown.
tagged ORC1 ectopically expressed from a CMV-promoter in 293 cells. For a cell line expressing FLAG-tagged ORC1 at a level similar to that of endogenous ORC1 (data not shown), we observed oscillation in the levels of the FLAG-tagged protein as that of endogenous ORC1 (Fig. 4C).

We further tested a different synchronization method to confirm ORC1 oscillation. HeLa S3, 293, and HEp-2 cells were arrested in early S phase for 24 h by the double-thymidine block method, and cellular protein contents were studied as described above after release from arrest (time 0) (Fig. 5, A–C). In all cases, ORC1 was at a basal level at time 0 and remained there until the beginning of BrdUrd incorporation. As was seen in M phase synchronization experiments, the ORC1 peak appeared earlier than the increase in the frequency of BrdUrd-positive cells. Again, the levels of ORC2–5 were constant.

We used a relatively mild lysis method in the preceding experiments, by first suspending cells in Triton buffer and subsequently mixing them with SDS buffer. Thus, it is formally possible that the decrease in ORC1 might be due simply to degradation during sample preparation, because ORC1 has been reported to be very unstable (21, 23). To address this concern, we tested more direct extraction conditions: 1) cells in culture dishes were directly lysed with SDS buffer, and 2) cells were first fixed with trichloroacetic acid and then lysed with SDS buffer (Fig. 5D). In both cases, the extent of degradation of ORC1 in extracts prepared from thymidine-blocked cells was the same as in extracts prepared by our original method, indicating that the levels of ORC1 observed in our cell lysates reflect the levels of ORC1 in human cells.

Further studies done with TERT-RPE1 cells (telomerase-immortalized human retina pigment epithelial cells) arrested in G0 by serum starvation for 4 days showed that the level of ORC1 was also low in these cells (Fig. 6). When the cells were stimulated with serum, the level of ORC1 started to increase at 12 h, peaked at 18 h, and decreased in the following S phase (18–27 h, Fig. 6). This result indicates that the ORC1 cycle also occurs in synchronized non-cancer cells. Therefore, irrespective of cell types, antibodies for detection and syn-
Cell Cycle-dependent Degradation Controls the ORC1 Cycle

It has been shown that there are E2F sites upstream of the ORC1 gene and that ORC1 expression is cell cycle-regulated (30). This observation suggested that E2F dependence of ORC1 expression may control ORC1 accumulation during G1 phase. However, we demonstrated that FLAG-tagged ORC1 expressed...
from a CMV promoter exhibited cyclical expression, indicating that transcriptional control makes only a minimal contribution to the regulation of ORC1 levels in cancer cells (Fig. 4C). We also observed that the reduced level of ORC1 in S phase-arrested cells was increased by treatment with a proteasome-specific inhibitor, MG132 (Fig. 7). This result indicates that the ORC1 cycle includes the periodic degradation of ORC1 mediated by the ubiquitin-26 S proteasome pathway. Recently, it has been reported that ORC1 is poly-ubiquitinated by Skp2 during S phase and degraded via the 26 S proteasome pathway in human cells (19).

ORC1 Degradation Is Programmed to Occur Prior to or Early in S Phase—As described above, ORC1 is present at a basal level in cells arrested in early S phase by thymidine, although the ORC1 level is high in early S phase cells released from M phase arrest. The S phase arrest-specific degradation of ORC1 was also observed during arrest promoted by hydroxyurea (HU) or aphidicolin treatment (data not shown). These results suggest that the programmed degradation of ORC1 is initiated before the early S phase arrest point, causing the protein to decrease to a basal level in arrested cells. To study the transition in the level of ORC1 in cells approaching the early S phase arrest point, HeLa S3 cells were first arrested in M phase and then released in the presence of HU (Fig. 8). FACS analysis indicates that these cells made the transition from M to G1 between 0 and 6 h, but exhibited a 2N DNA content until at least 21 h, indicating that they were arrested in early S phase by HU treatment. Under these conditions, the ORC1 cycle was apparent, indicating that the program of ORC1 degradation turns on prior to or at early S phase. On the other hand, although an increase in the level of MCM3 in the chromatin fraction paralleled the increase in ORC1, MCM3 bound to chromatin remained at high levels even after ORC1 was degraded (Fig. 8). This result suggests that, although MCM3 is loaded onto chromatin in an ORC1-dependent manner (24), its subsequent fate is independent of ORC1 degradation.

DISCUSSION

It is well known that the eukaryotic replication initiation factor, ORC, remains at a constant level in chromatin throughout the cell cycle of various species (4, 5, 7, 8, 31–33). However, thus far we have shown that, in four different cell lines subjected to three synchronization methods, human ORC1 exhibits a cell cycle-specific oscillation in abundance, as judged by immunoblotting and immunostaining analyses. On the other hand, the other ORC subunits, ORCs 2–5, remain at constant levels throughout the cell cycle. These same observations have been reported for HeLa and Raji cells by others (19). Taking these results into consideration, we conclude that, in human cells, the levels of ORC1 oscillate, a phenomenon we have termed “the ORC1 cycle” (8). Previous studies had reported that the level of ORC1 was constant throughout the human cell cycle (25, 34), an observation that may be explained by the cross-reactivity of the human ORC1 antibody with other protein(s), as we have demonstrated here.

One important question concerns the time of appearance of the ORC1 cycle during evolution, because it does not exist in yeast or frogs. We have not been able to study the behavior of ORC1 in other species, because our antibody is highly specific to human ORC1. However, a variant ORC1 cycle has been reported in Chinese hamster cells; in these cells, the level of ORC1 is constant (21, 23), but the protein is released from the chromatin fraction in S phase (21, 33). Therefore, the level of ORC1 associated with chromatin cycles in both human and hamster cells, regardless of its overall cellular level. In connection with these behaviors, the S phase-specific ubiquitination of ORC1 has been reported for both species (19–21). Interestingly, hamster ORC1 is only mono-ubiquitinated, but human ORC1 is poly-ubiquitinated, and these distinctions may have consequences for the different fates of the two ORC1 proteins in S phase.

In serum-starved quiescent cells, ORC1 is at a basal level, as observed for M or early S phase-arrested cells. This basal level may be maintained by degradation, but E2F regulation might also have a role in keeping the level of ORC1 low in quiescent cells. Indeed, ectopic expression of ORC1 in quiescent Drosophila cells alters the program of proliferation during development (35). In addition, transcription of the ORC1 gene is at a basal level in serum-starved cells, and it can be induced by the addition of serum or by overproduction of the E2F transcription factor (30), suggesting the importance of transcriptional regulation in the maintenance of a low level of ORC1 during G0 arrest.

In yeast, ORC binds to replication origins as a complex of six
proteins throughout the cell cycle, and defects in any of these subunits result in a failure in the initiation of replication, suggesting that an intact ORC is necessary (36, 37). Similarly, if intact ORC is required for human DNA replication, the concentration of ORC1 may be a major factor in the regulation of ORC activity, because the levels of ORCs 2–5 are constant. Human ORC6 appears to associate with ORCs 1–5 only weakly, and its functional roles are currently not well understood (38, 39). As we have demonstrated, the level of human ORC1 protein is under strict cell cycle control, which in turn indicates that ORC activity is strictly controlled by cell cycle progression. Indeed, we have demonstrated that the ORC1–5 complex parallels the accumulation of ORC1 in human nuclei, which is further linked with the loading of MCM onto chromatin. These points are discussed in the accompanying paper (24).

Human ORC1 is at a basal level in cells arrested in early S phase by aphidicolin, HU, or thymidine. Furthermore, the arrested cells can start DNA synthesis without accumulating ORC1 after release from this block. Thus, we propose that ORC1 degradation begins prior to S phase. A similar behavior also was observed for Xenopus ORC1 (XlORC1) when Chinese hamster nuclei were introduced into Xenopus egg extracts (33). XlORC1 loaded onto hamster chromatin was released soon after the assembly of pre-RCs on chromatin. It has been also suggested that XlORC is not required for the progression of S phase once pre-RCs have assembled (33, 40). Thus, it is possible that a basal level of ORC1 is sufficient for origin firing. However, we prefer another possibility: that ORC1 that has accumulated in G1 is used to mark origin sites and is not necessary for subsequent actual firing. Immunostaining experiments showed that ORC1 and BrdUrd signals do not overlap. This result strongly supports the second possibility and furthermore inspires the idea that the removal or degradation of ORC1 is necessary to allow the cell cycle to progress from the initiation reaction to the next stage.

It is important to know what triggers the degradation of ORC1 prior to origin firing. Among candidate processes are the assembly of the pre-RC and the increase in S-CDK or CDC7 kinase activity. Further studies of molecular assemblies, including ORC at specific origins in human cells, will be necessary to address this issue.

Acknowledgments—We thank Drs. Hisao Masukata (Osaka University) and Masatoshi Fujita (National Cancer Center Research Institute) for critical reading of the manuscript and valuable comments. We are also grateful to Dr. Anindya Dutta (Harvard Medical School) for providing antibodies.

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