Simian Virus 40 Large T Antigen Disrupts Genome Integrity and Activates a DNA Damage Response via Bub1 Binding[▽]

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Simian virus 40 (SV40) large T antigen (LT) is a multifunctional protein that is important for viral replication and oncogenic transformation. Previously, infection of monkey or human cells with SV40 was shown to lead to the induction of DNA damage response signaling, which is required for efficient viral replication. However, it was not clear if LT is sufficient to induce the damage response and, if so, what the genetic requirements and functional consequences might be. Here, we show that the expression of LT alone, without a replication origin, can induce key DNA damage response markers including the accumulation of γ-H2AX and 53BP1 in nuclear foci. Other DNA damage-signaling components downstream of ATM/ATR kinases were induced, including chk1 and chk2. LT also bound the Claspin mediator protein, which normally facilitates the ATR activation of chk1 and monitors cellular replication origins. Stimulation of the damage response by LT depends mainly on binding to Bub1 rather than to the retinoblastoma protein. LT has long been known to stabilize p53 despite functionally inactivating it. We show that the activation of a DNA damage response by LT via Bub1 appears to play a major role in p53 stabilization by promoting the phosphorylation of p53 at Ser15. Accompanying the DNA damage response, LT induces tetraploidy, which is also dependent on Bub1 binding. Taken together, our data suggest that LT, via Bub1 binding, breaches genome integrity mechanisms, leading to DNA damage responses, p53 stabilization, and tetraploidy.

Simian virus 40 (SV40) is a small DNA tumor virus, belonging to the polyomavirus family, that induces a productive infection in its natural host, the rhesus macaque, but yields oncogenic transformation in nonpermissive hosts such as rodent cells. The highly multifunctional large T antigen (LT) is the key early protein essential for both driving viral replication as well as inducing cellular transformation. Two other early proteins, small t antigen and 17k T antigen (17k) may perform auxiliary functions during the viral life cycle (39, 69). LT has served as a powerful model system for understanding fundamental cellular processes such as nuclear translocation, transcriptional regulation, eukaryotic DNA replication, immortalization, and malignant transformation (reviewed in references 26 and 37).

LT overrides cellular control mechanisms and reprograms the host cell to create a permissive environment for viral replication. The deregulation of cellular proliferation is dependent on LT's interaction with specific host proteins, among which the tumor suppressors p53 and the retinoblastoma protein (pRB) are the best characterized (reviewed in reference 37). Transformation in vitro and tumor induction in vivo frequently depend on LT binding and functionally inactivating these key tumor suppressors (37). Additional cellular targets such as p300/CBP (23), CUL7 (2), Nbs1 (67), as well as Hsc70 (10, 56), bound through the N-terminal DnaJ domain of LT, contribute to oncogenic transformation and/or viral replication, but their mechanisms of action are less well characterized. Recently, we have identified the Bub1 mitotic spindle checkpoint kinase as being an additional target bound by LT (16). Mutational analysis indicates that this interaction also contributes to oncogenic transformation, but not immortalization, elicited by LT. A key LT mutant defective for Bub1 binding is dl89-97 (16). Our unpublished data, together with previously reported evidence that it dephosphorylates p130 like wild-type (wt) LT (60), argue that the DnaJ domain remains intact in this mutant.

Bub1 is involved in safeguarding the genome, primarily by enforcing the spindle checkpoint that delays anaphase progression when kinetochores lack bipolar attachment to microtubule spindles (38, 40). Interestingly, Bub1 is mutated sporadically in human cancers, and it was previously suggested that the mutation of Bub1 may be a driving force in tumorigenesis via the generation of chromosomal instability and aneuploidy (9). A total loss of Bub1, or the spindle checkpoint, is apparently lethal due to catastrophic mitosis (31, 44), but a weakened checkpoint may contribute to tumorigenesis while cooperating with other genetic changes (33). Notably, LT is known to induce genomic instability manifested in both structural and numerical chromosome changes (11, 27, 48, 49, 57, 66). Frequently, LT-expressing cells are tetraploid or aneuploid and

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harbor structural aberrations in one or more chromosomes. Limited evidence, such as an LT transgenic study and observations with certain temperature-sensitive mutants of LT, suggests that the destabilization of the host genome may contribute to transformation (24, 47, 53).

Although LT clearly binds and inactivates p53 by blocking its site-specific DNA binding activity, it also potently stabilizes the wt p53 protein (19, 20, 43). This has been an enigma in the field for over 20 years, because the rationale and exact underlying mechanisms remain unclear. Stabilization of p53 follows genotoxic damage and is some times triggered by its N-terminal phosphorylation (5, 55). Interestingly, LT was recently shown, independent of pRB binding, to induce p53 phosphorylation at Ser15, Ser20, and Ser37, sites that are phosphorylated in response to DNA damage (7).

These observations may be explained by the recent reports that SV40 infection of permissive cells induces a DNA damage response (DDR) via the ATM (54, 70) or the ATR (42) pathway. The ATM and ATR kinases are master signal transducers in response to DNA damage (1). Following DNA damage, ATM phosphorylates p53 directly on Ser15, whereas ATR can phosphorylate both Ser15 and Ser37 (1). The activation of the DDR is normally coupled to cell cycle arrest at G_1, S, or G_2 checkpoints. For that, ATR and ATM in turn activate the downstream effector kinases chk1 and chk2, respectively. An emerging theme is that viruses, in unique ways, have adopted strategies to inactivate or exploit cellular DDRs (34). In the case of SV40 and mouse polyomavirus, the induction of a DDR is beneficial for the viral life cycle, since ATM inhibition blocks viral replication (18, 54, 70).

While previous studies of this topic have used the whole virus, we have analyzed the consequences of stably expressing LT alone in normal human fibroblasts. Here, we show for the first time that LT in the absence of a viral replication origin is sufficient to induce DDR signaling via ATM/ATR kinases. Interestingly, this stimulation of the DDR is dependent mainly on Bub1 binding rather than binding to pRB. Likely as a consequence of the induced DDR, p53 is phosphorylated and stabilized by LT. The stabilization of p53 requires its phosphorylation on Ser15. Concomitant with DDR activation, we also find that LT induces significant tetraploidy via Bub1 binding. We hypothesize that tetraploidy and DDR induction could contribute significantly to both long-term increases in the oncogenic phenotype from genetic alterations and stimulation of viral replication.

MATERIALS AND METHODS

Cell culture. U2OS cells, stable derivatives expressing LT or dl89-97, and Phoenix amphotropic cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) from Cellgro and 1% penicillin-streptomycin. ATM-deficient GM05823 human fibroblasts (AT5BI; Coriell Institute) and ATR-hypomorph Seckel syndrome F02-98 cells (kindly provided by P. Andreassen) were cultured in the same medium but using HyClone FCS. BJ/tert cells and derivatives expressing LT or mutants thereof were grown in 80% Dulbecco's modified Eagle's medium and 20% medium 199 (Invitrogen) supplemented with 10% HyClone FCS and 1% penicillin-streptomycin. Cell lines inducibly expressing wt p53 or the Ser15Ala, Ser37Ala, and Ser15Ala/Ser37Ala mutants in the background of the spontaneously immortalized p53-deficient MDAH-041 human fibroblasts were a gift from G. Stark (5). These cell lines were grown as outlined above but with the addition of 1 μg/ml doxycycline to repress p53 expression until the start of the experiment. All cells were maintained at 37°C with 5% CO_2.

Viral infections. Retrovirus was packaged by transfection of 12 μg DNA into Phoenix amphotropic cells using the 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES) calcium phosphate protocol as previously described (28). Virus was harvested 48 h posttransfection, passed through a 0.45-μm filter, and stored at −80°C in aliquots until usage. Cells were infected overnight with 1 ml of retroviral supernatant, 3 ml of complete medium, and 8 μg/ml of polybrene. Selection was initiated at 48 h after infection by the addition of 5 μg/ml of blasticidin (Invitrogen) or 3 μg/ml of puromycin (Calbiochem). Pools of retrovirally infected cells were established after selection for 5 to 7 days. These pools were used between 1 and 2 weeks after the original infection to limit secondary effects arising from extended culturing. Lentiviral packaging and infection were performed as previously described (28).

Plasmids. The retroviral expression vector pLB(N)CX, a modification of pLNCX (Clontech), was previously described (7). A derivative expressing wt LT was kindly provided by J. DeCaprio. The dl89-97 mutant cDNA was excised from another vector, and ends were blunted and then cloned into the HpaI site of pLB(N)CX. The 17k K1 and 17k K1/dl89-97 constructs in pBabe-puro were previously described (28). The lentiviral short hairpin RNA (shRNA) constructs targeting ATM/ATR were obtained from the RNAi Consortium (Cambridge, MA) via W. Hahn.

Immunofluorescence. BJ/tert stable lines were seeded and allowed to attach on polylysine-coated glass coverslips for 48 h. Cells were briefly washed with phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. After two washes with PBS, cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS and subsequently blocked for nonspecific binding for 45 min in 10% goat serum (Invitrogen) with 0.5% bovine serum albumin. Afterwards, cells were incubated in dilutions of primary antibody for 1 h, followed by three washes with PBS for 5 min each. Secondary antibody, applied for 30 min, was either Cy3 linked (Jackson Immunoresearch), Alexa 488 conjugated (Invitrogen), or both for dual staining. After three washes with PBS, the cells were stained briefly with DAPI (4′,6′-diamidino-2-phenylindole) and mounted onto glass slides using mounting medium (BMM-01; Fisher).

Cells were visualized using an Olympus microscope with either a 40× or 100× objective. Images were acquired using Spot Advanced software and processed with Adobe Photoshop 7.0 software.

Antibodies, immunoprecipitations, and Western blotting. The LT monoclonal antibodies PAb419 and PAb423 were previously described (16). The following other antibodies were used: p53 DO-1 or PAb1801 (Santa-Cruz Biotechnology), phospho-p53 (phospho-p53 antibody sampler kit; Cell Signaling), phospho-Rad17 Ser645 (Cell Signaling), phospho-chk2 Thr68 (R&D Systems), γ-H2AX (JBW301 monoclonal; Millipore), phospho-ATM Ser1981 monoclonal (a gift from C. Bakkenist) (64), 53BP1 (Ab-1; Calbiochem), Claspin/phospho-Claspin Thr916 (kindly provided by J. Chen) (12, 36), ATRIP (R. Freire, unpublished data), TopBP1 (Bethyl Laboratories), ATM (2C1; GeneTex), Nbs1 (Cell Signaling), phospho-chk1 Ser317 (R&D Systems), chk1 (G-4; Santa-Cruz Biotechnology), chk2 (clone 7; Millipore), hemagglutinin (HA.11; Covance), actin, tubulin, and vinculin (Sigma).

Lysates were prepared as follows. Briefly, cells were washed in cold PBS and extracted in TEB LT extraction buffer (20 mM Tris [pH 7.5], 137 mM NaCl, 10% glycerol, 1% Nonidet P-40) supplemented with protease (leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride) and phosphatase inhibitors (NaF, Na_3VO_4, and microcystin LR [Calbiochem]). After centrifugation to clear cell debris, lysates were immunoprecipitated by the addition of antibody and mixing for 2 h at 4°C, followed by the capture of immune complexes by the addition of protein A-Sepharose (GE Healthcare) and incubation for another 45 min while mixing at 4°C. Beads were washed three times with TEB lysis buffer and eluted with Laemmli buffer, and subsequently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was performed using 4 to 12% NuPAGE gels (Invitrogen). When whole-cell lysates were analyzed, the samples were normalized for total protein (Bio-Rad assay). For analysis of phospho-p53 by Western analysis subsequent to the immunoprecipitation of total p53, antibody-conjugated beads were used (DO-1-agarose beads from Calbiochem or PAb1801 from Santa Cruz) to eliminate immunoglobulin G heavy-chain interference.

Western blotting was conducted using standard protocols for electrophoretic transfer onto nitrocellulose. Conditions for blocking were the use of either 5% nonfat dry milk in TBS-Tween (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 1 h or according to the manufacturer's specific instructions. Immunoblots were probed overnight with primary antibody dilutions. After washes in TBS-Tween and secondary antibody, the signal was visualized by enhanced chemiluminescence (Western Lightning chemiluminescence reagent; Perkin-Elmer Life Sciences) or imaging using a Licor Odyssey system. Some quantitations were performed by densitometry using ImageJ software.

Inhibitors and DNA-damaging agents. Adriamycin (doxorubicin; Sigma) was used at 0.2 µg/ml, hydroxyurea (Calbiochem) was used at 2 mM, wortmannin (Sigma) was used at 10 µM, LY294002 (Cell Signaling Technology) was used at 20 µM, and caffeine (Sigma) was used at 5 mM. γ irradiation was conducted using 10 Gy.

Cycloheximide chase. For determinations of p53 stability, cycloheximide was added to the cells at a 50-µg/ml final concentration, and the cells were harvested either immediately thereafter (time zero) or after 4, 8, or 24 h of cycloheximide treatment. The p53 levels at each time point were monitored by Western analysis with DO-1 antibody and quantitated using a Bio-Rad FluorImager (FluorS MultiImager).

Chromosome spreads. The microtubule inhibitor colcemid was added to cells at a final concentration of 100 ng/ml for 1.5 h. Cells were harvested by trypsinization and low-speed centrifugation followed by the dropwise addition of hypotonic solution (0.8% sodium citrate). After 10 min of incubation at 37°C, the cell pellet was obtained by centrifugation and treated three times consecutively with Carnoys fixative (75% methanol, 25% glacial acetic acid). Finally, the cell suspension was dropped onto glass slides, and metaphase chromosomes were visualized by Giemsa (Sigma) staining.

Fluorescent in situ hybridization (FISH) analysis. Slides with metaphase spreads were first prepared as outlined above. Subsequently, slides were placed in denaturing solution (70% formamide, 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] [pH 7]) at 73°C for 5 min and then dehydrated in 70%, 85%, or 100% ethanol for 2 min each using a Coplin jar. The denatured probe (10-µl volume) was quickly applied, a coverslip was placed on top, and rubber cement was used to seal it up for overnight incubation in a humidified chamber at 42°C. Fluorescently labeled centromeric probes for chromosomes 6 and 17 were obtained from Vysis (catalog numbers 32-132006 and 32-130017), along with the recommended hybridization buffer. Slides were washed in a solution containing 0.4× SSC–0.3% Nonidet P-40 at 73°C for 2 min followed by washing with a solution containing 2×SSC–0.1% Nonidet P-40 for 1 min. Finally, DAPI solution (Vysis) was applied, and microscopy was performed.

RESULTS

LT induces key DNA damage response markers via Bub1 binding. Previous reports indicated that whole SV40 virus induces DDR signaling, potentially via both ATM and ATR pathways (42, 54, 70). However, it was not clear if LT by itself is sufficient to induce the response or whether viral replication structures are required. To investigate this possibility, we expressed LT without a viral replication origin in normal human BJ/tert fibroblasts. Specifically, we introduced LT, the Bub1 binding mutant dl89-97, or an empty vector by retroviral transfer followed by blasticidin selection. At 1 week postinfection, the cells were examined by immunofluorescence using an antibody recognizing γ-H2AX, which appears as foci in cells undergoing a DDR (50). Strikingly, the BJ/tert LT cells exhibited distinct and frequently occurring γ-H2AX foci, whereas the vector expressing BJ/tert only infrequently showed foci (Fig. 1A shows representative cells). The BJ/tert dl89-97 cells exhibited γ-H2AX foci at a frequency only slightly higher than that of control cells. Hence, Bub1 binding by LT is linked to increased levels of γ-H2AX focus formation, which is considered to be one of the hallmarks of an activated DDR. However, since γ-H2AX is in some cases linked to DNA repair or cell death rather than damage per se (35), we also examined 53BP1 focus formation, another marker frequently associated with the DDR. As shown in Fig. 1A, LT induced prominent 53BP1 foci, whereas the dl89-97 mutant was largely negative in this assay. The γ-H2AX and 53BP1 foci were colocalized mostly as reported previously in other studies. We also noticed a marked increase in the number of micronuclei when LT was expressed (16.4% of 835 nuclei) (Fig. 1A), whereas dl89-97 exhibited a less pronounced increase (1.9% of 1,000 nuclei) than did control cells (0.4% of 724 nuclei). Although the spe-
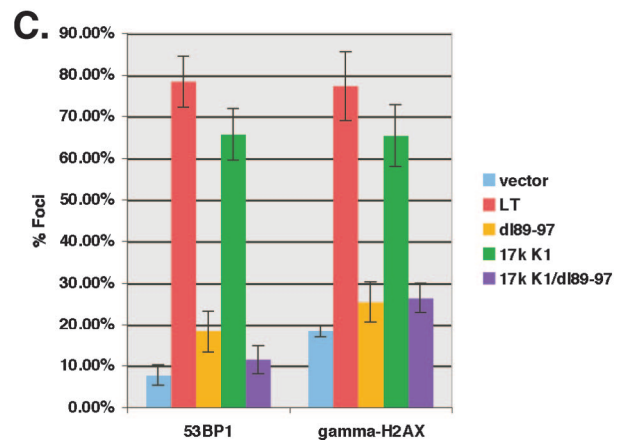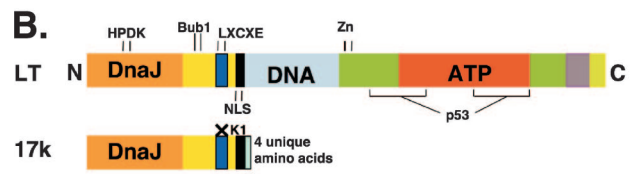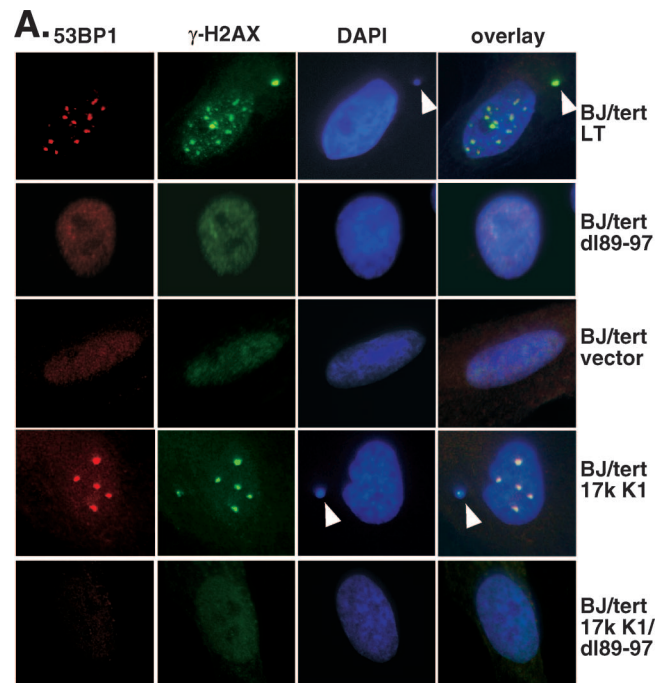

FIG. 1. LT and the 17k K1 mutant induce γ-H2AX/53BP1 focus formation. (A) BJ/tert cells expressing an empty vector, LT, dl89-97, 17k K1, or 17k K1/dl89-97 were stained by immunofluorescence for γ-H2AX and 53BP1. (B) Schematic drawing to compare the salient features of LT and 17k. The K1 mutation abolishes pRB binding. (C) Graph depicting the relative frequency of cells with foci. Error bars indicate standard deviations. The following numbers of nuclei were counted: 1,443 for vector, 1,344 for LT, 1,178 for dl89-97, 218 for 17k K1, and 286 for 17k K1/dl89-97.

cific mechanism underlying the formation of micronuclei is unknown, it has often been associated with genotoxic damage or chromosome missegregation events. Collectively, our data indicate that LT induces characteristic features associated with a DDR via Bub1 binding.

We investigated the genetics of the ability of LT to induce markers associated with a DDR. We especially wished to determine if pRB binding was required, since other viral oncoproteins have been shown to induce the DDR via this pathway (45). We recently demonstrated that the expression of the 17k gene product, which encodes residues 1 to 131 of LT plus four unique residues at its C terminus (schematically depicted in Fig. 1B), can induce premature senescence in BJ/tert cells when made pRB binding deficient (via a K1 mutation) (28). Recent reports have demonstrated that both replicative and oncogene-induced senescences reflect an underlying persistent activation of DDRs that are likely triggered by aberrant cellular DNA replication (4, 17, 21). Therefore, we examined if the expression of 17k K1 can induce markers of the DDR. As shown in Fig. 1A, we found that, indeed, 17k K1 elicits the formation of γ-H2AX/53BP1 foci and micronuclei. Once again, cells expressing the Bub1 binding mutant were mostly negative for these foci (17k K1/dl89-97) (Fig. 1A). We carefully quantitated and compared the frequencies of γ-H2AX and 53BP1 foci for BJ/tert cells expressing vector, LT, dl89-97, 17k K1, or 17k K1/dl89/97 (Fig. 1C). Strikingly, both LT and the 17k K1 construct induced efficient γ-H2AX/53BP1 focus formation in a Bub1 binding-dependent manner. Although the frequencies of focus formation were similar for LT and 17k K1, a slightly greater number of foci per cell were often induced by LT. Thus, we cannot exclude the possibility that other activities of LT (including the pRB binding site) might contribute to the overall response observed with full-length LT.

LT activates ATM/ATR-mediated DNA damage signaling. Since we detected foci of γ-H2AX and 53BP1 in LT-expressing cells, we examined additional aspects of ATM/ATR signaling by immunoblotting analysis. Signaling pathways involving ATM and ATR are depicted in Fig. 2A. Initially, we analyzed signaling via the ATR branch. Using the phosphorylation of chk1 at the ATR site on Ser317 as a readout, we found that the ATR signaling branch was activated in LT-expressing BJ/tert cells. Lower levels of phospho-Ser317 were seen in cells expressing the dl89-97 mutant, with none at all in the control cells (Fig. 2B). This is consistent with a previous report demonstrating chk1 activation following SV40 infection of permissive CV1 cells (42). The amount of total chk1 was also upregulated by LT, potentially via a transcriptional effect through E2F sites in the promoter (63). Moreover, we found that the level of Claspin, the mediator protein that facilitates the ATR-driven phosphorylation of chk1, was dramatically increased in LT-expressing cells compared to that in dl89-97-expressing and control cells. Levels of the ATRIP and TopBP1 proteins, critical ATR binding partners and regulators, were also upregulated by LT but not in a Bub1 binding-dependent manner. Additionally, the ATR target Rad17 was phosphorylated at Ser645 when LT, but not dl89-97, was expressed.

Subsequently, we analyzed DDR signaling mediated by ATM. Notably, we observed the activation of ATM, as detected by phosphorylation at Ser1981, and of its downstream effector kinase chk2. As before, these events were dependent largely upon Bub1 binding. However, the level of Nbs1, a member of the Mre11-Rad50-Nbs1 complex which is a proposed sensor of the DDR, was not significantly altered by LT. Taken together, we noted an ability of LT to induce or activate elements from both the ATM and ATR pathways.
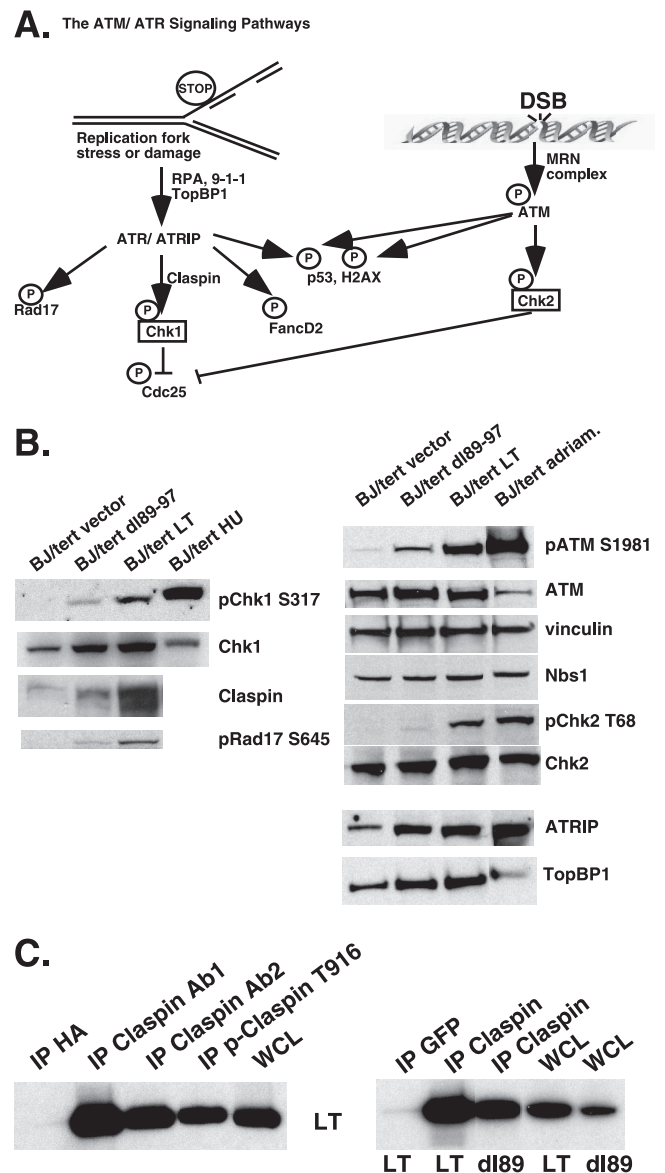

FIG. 2. LT activates ATM/ATR-mediated DDR signaling. (A) Simplified diagram of the ATM/ATR signaling pathways including some of the substrates and regulators. Cross talk between ATM and ATR has not been depicted due to complexity. (B) Stable BJ/tert cell lines expressing empty vector, dl89-97, or LT were monitored by Western blotting for expression or the activation state of several signaling components from the ATM/ATR pathways. Hydroxyurea (HU) or adriamycin (adriam.) treatment was used as a positive control when relevant. (C) Interaction of LT with Claspin was examined by immunoprecipitation (IP) with either of two total Claspin antibodies, an antibody toward phospho-Claspin T916, or a negative control antibody (hemagglutinin [HA]), followed by Western blotting for LT. Whole-cell lysate (WCL) (2% of the total) was used as a positive control. This experiment was repeated three times, with identical outcomes. The interaction of dl89-97 with Claspin was determined by a similar coimmunoprecipitation assay but using green fluorescent protein antibody as a control.

Since some of the effects of LT on DDR components could be mediated by a direct interaction, we carried out coimmunoprecipitation assays. As shown in Fig. 2C, we found that LT consistently coprecipitated with Claspin using either of two

different Claspin antibodies or a phospho-Claspin Thr916 antibody (12, 36). Moreover, the dl89-97 mutant retained its ability to bind Claspin. We failed to detect any interaction with ATM, ATR, or ATRIP (data not shown). Further investigations will be required to determine the significance of the Claspin interaction.

Binding of LT to Bub1 is linked to p53 stabilization and site-specific phosphorylation. Since p53 is stabilized both during a DDR and when LT is stably expressed, we wondered if LT-mediated p53 stabilization is driven by DDR elements. First, we examined the level of p53 protein in stable U2OS cell lines expressing various alleles of LT by Western blotting. We found that LT expression, as previously reported, could dramatically increase p53 levels, but the dl89-97 mutant was substantially defective in this regard (Fig. 3A). We also examined BJ/tert fibroblasts stably expressing either LT or the dl89-97 mutant. Here, wt LT also increased the p53 level, whereas dl89-97 caused only a minor increase (Fig. 3B). We examined the stability of p53 in each case by a cycloheximide chase experiment. Cells were treated with cycloheximide for 0, 4, 8, and 24 h, and the p53 levels at each time point were determined by Western blotting. Consistent with early reports, LT efficiently stabilized p53 (43) (Fig. 3C). Furthermore, the defect in p53 accumulation for the dl89-97 mutant resulted from a failure to stabilize the protein.

Since the stabilization of p53 can be mediated by p53 N-terminal phosphorylation (46), we explored this possibility. To examine p53 phosphorylation states, we immunoprecipitated total p53 and immunoblotted for the phosphoforms using a panel of phosphospecific antibodies (Fig. 3D). We adjusted the amount of lysate from each cell line that was immunoprecipitated to equalize the total amount of p53 brought down. Treatment with adriamycin was used as a positive control, since this DNA-damaging agent induces phosphorylation at almost all p53 sites examined. Parental U2OS cells did not show significant phosphorylation at any of the sites (data not shown). However, consistent with recent observations, LT induced the specific phosphorylation of Ser15, Ser20, and Ser37 (7). Strikingly, the dl89-97 mutant was largely defective in this respect. Phosphorylation at these sites is considered to be one of the hallmarks of a DNA damage response (3). Conversely, phosphorylation at other sites like Ser33, Ser46, or Ser392 was not affected by the LT deletion. Thus, LT induces the phosphorylation of p53 at a subset of sites dependent on Bub1 binding.

LT stabilization of p53 is dependent on Ser15 phosphorylation. Next, we wanted to assess if the LT-mediated induction of p53 phosphorylation at specific sites was a prerequisite for its stabilization. We focused on Ser15 and Ser37, which have been implicated in stability control (5, 55) and are directly targeted by ATM/ATR kinases (1). We utilized a cell system previously developed to decipher the functional role of these phosphorylation sites in response to UV damage in which p53-deficient cells were engineered to inducibly express wt p53, the Ser15Ala and Ser37Ala phosphorylation site mutants, or an allele mutant at both sites (5). Upon doxycycline removal, either wt p53 or a given p53 mutant is expressed. Each of these cell lines was infected with a retrovirus encoding LT, followed by drug selection to generate a stable line. The expression of p53 was repressed until the initiation of the experiment, at which point p53 was induced for 24 h. As expected, LT expression significantly stabilized wt p53, causing its accumulation (Fig. 4). Strikingly, the Ser15Ala mutant could not be stabilized by LT, and neither could the mutant at both sites. Conversely, the Ser37Ala mutant was stabilized by LT. Thus, the phosphory-
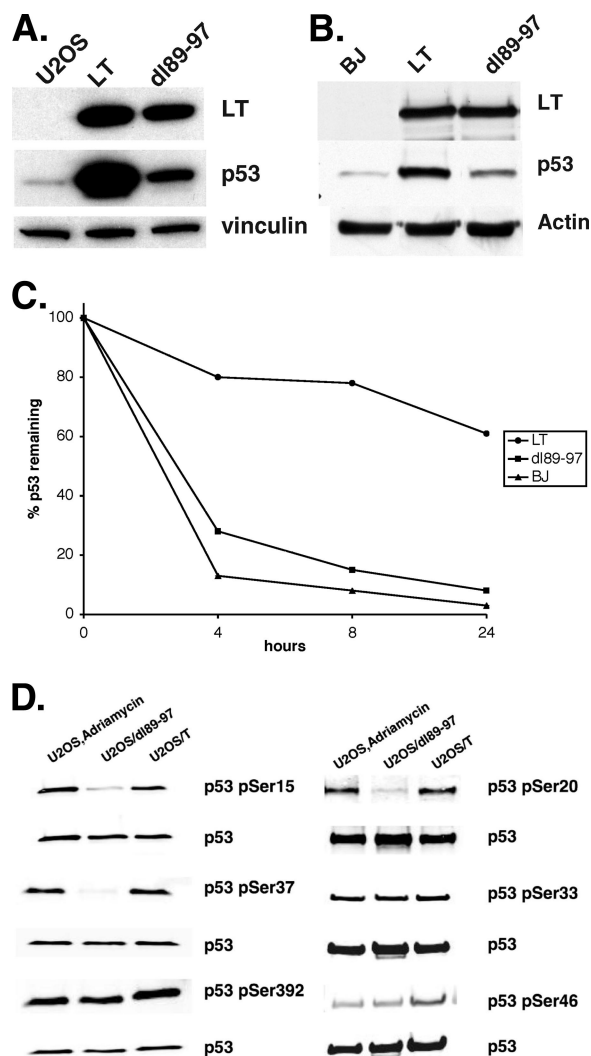

FIG. 3. LT-induced accumulation and specific phosphorylation of p53 correlate with Bub1 binding. (A) U2OS or stable derivatives expressing wt LT or dl89-97 were processed for Western blotting of whole-cell lysates using p53-, LT-, and vinculin-specific antibodies. The total amount of protein was normalized between samples. Vinculin serves as a control of equal loading. (B) BJ/tert cells or stable derivatives expressing LT or dl89-97 were examined by Western blotting for LT, p53, and actin expression. Actin served as a marker of equal loading. (C) BJ/tert cells, or derivatives expressing LT or dl89-97, were studied in a cycloheximide chase experiment. The time points of the chase were 0, 4, 8, and 24 h. The p53 level at different time points of the cycloheximide chase was quantitated using a FluorImager. This graph depicts average values derived from three independent experiments. (D) Lysates from U2OS cells expressing wt LT or dl89-97 were immunoprecipitated for total p53 followed by Western blotting with a panel of phospho-p53 antibodies. Lysate amounts were adjusted to achieve equal amounts of total p53, which was confirmed by reprobing the blots for total p53 after phosphodetection. U2OS cells treated with adriamycin were used as positive controls for phosphoantibody reactivity.
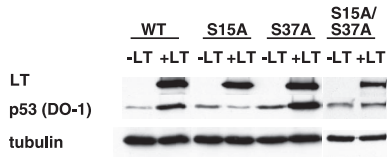
FIG. 4. Ser15 phosphorylation is required for LT to stabilize p53. Cell lines inducibly expressing wt p53 or the Ser15Ala, the Ser37Ala, or the Ser15Ala/Ser37Ala p53 mutant were infected with an LT retrovirus. After LT was stably expressed, p53 expression was turned on for 24 h, and the levels of total p53 and LT were examined by Western blotting. Tubulin levels were used to monitor equal loading.

lation of p53 at Ser15 induced by LT binding to Bub1 is instrumental for p53 stabilization.

Efficient LT induction of p53 Ser15 phosphorylation involves ATR. After elucidating the functional consequence of p53 phosphorylation, we investigated the kinases involved. The most straightforward scenario might be that LT acts as a molecular scaffold to connect Bub1 with its substrate p53. However, as we recently showed, purified recombinant Bub1 phosphorylates only p53 on Ser37 in vitro but not on the other sites

(65). Moreover, Bub1 RNA interference could block only Ser37 phosphorylation. Since the p53 phosphorylation sites induced via LT binding to Bub1 are commonly associated with the DDR (3), we explored the potential involvement of ATM or ATR in the LT-induced phosphorylation of p53. As reported previously (7), we did see a partial inhibition of p53 Ser15 phosphorylation in U2OS/T cells when the cells were treated for 2 h with caffeine (Fig. 5A), a pharmacological inhibitor whose targets include ATM and ATR (51). However, in contrast to a previously reported study (7), we never saw greater than 50% inhibition. Treatment of cells with caffeine for 24 h did not exert a greater impact on Ser15 phosphorylation (data not shown). Caffeine is reported to be more potent at inhibiting ATM than ATR. Treatment for 2 h with the inhibitors wortmannin (10 μM) and LY294002 (20 μM) at concentrations that inhibit ATM/DNA-PK but not ATR (52) had no effect on the phosphorylation of Ser15 in U2OS/T cells. However, a potential caveat with inhibitor experiments is the fact that we do not know the rate at which the p53 Ser15 phosphorylation site is dephosphorylated.

To further examine the contributions of ATM and ATR to the induction of p53 Ser15 phosphorylation in U2OS/T cells,
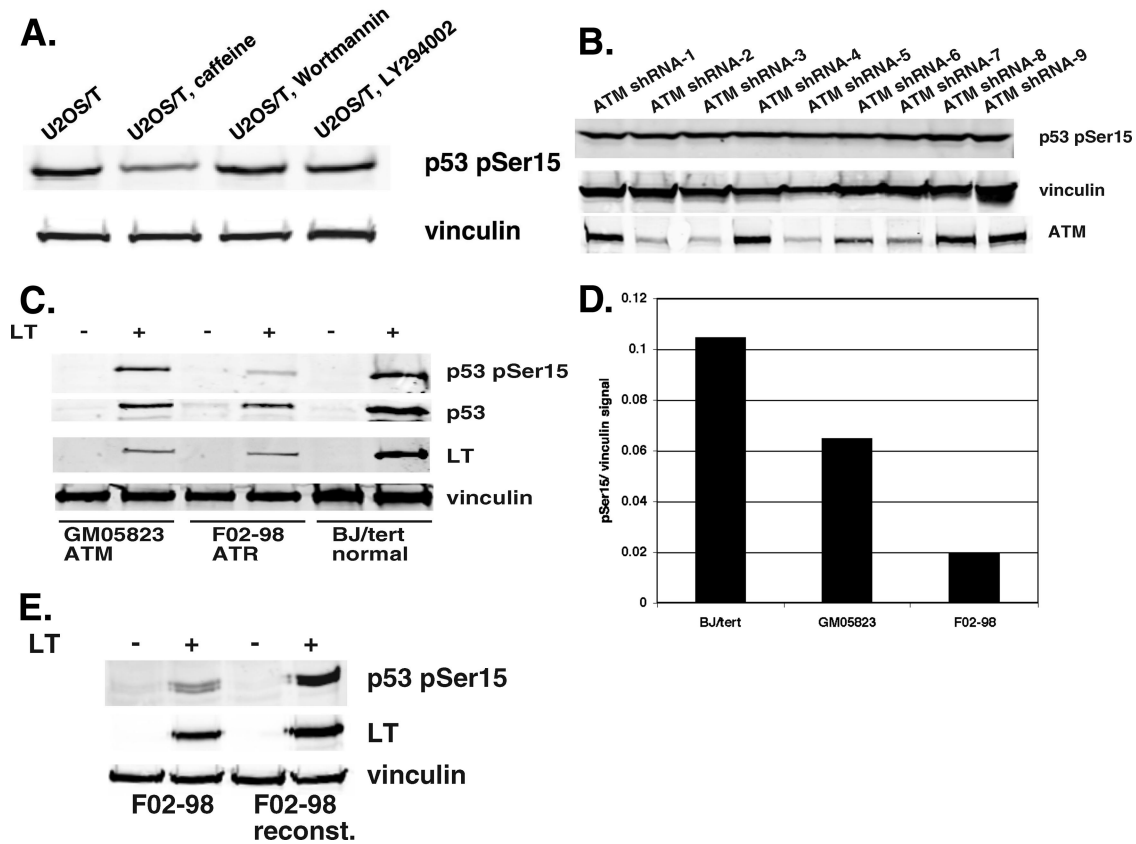

FIG. 5. Efficient LT induction of p53 Ser15 phosphorylation involves ATR. (A) U2OS/T cells were exposed to 5 mM caffeine, 10 μM wortmannin, or 20 μM LY294002 for 2 h. Levels of pSer15 were assessed by immunoblotting. (B) U2OS/T cells were infected with one of nine different lentiviruses expressing distinct ATM shRNA target sequences. Stable cell lines were generated following puromycin selection. Levels of ATM, pSer15, and vinculin were monitored by immunoblotting. (C) Normal BJ/tert human fibroblasts or fibroblasts deficient in ATM (GM05823) or ATR (F02-98) were infected with an LT retroviral vector to establish stable cell line pools. Levels of LT, p53 pSer15, and total p53 were determined by Western blotting. Vinculin levels were used to monitor equal loading. (D) The amount of pSer15 signal relative to that of vinculin was quantitated and is depicted. (E) LT was expressed in either F02-98 Seckel cells or the equivalent ATR-reconstituted (reconst.) cells, and levels of pSer15, LT, and vinculin were assessed by Western blotting.

we attempted a stable knockdown of these kinases using lentiviral delivery of shRNAs, followed by puromycin selection. We used nine distinct ATM shRNA target sequences, several of which resulted in a substantial reduction of ATM levels (Fig. 5B). Strikingly, none of these shRNAs had any significant impact on the accumulation of p53 phosphorylated on Ser15. However, when we attempted to silence ATR expression in a similar manner using eight distinct shRNAs, we found that none of these yielded any significant reduction in ATR or pSer15 levels, suggesting that shRNAs to ATR may be lethal in U2OS/T cells (data not shown).

To help resolve the roles of ATM/ATR in pSer15 accumulation, we used cells from ataxia telangiectasia patients (GM05823 cells, which are ATM deficient) or Seckel syndrome patients (F02-98 cells, which are ATR hypomorphic due to a splice site mutation) (41). For comparison, we used normal human fibroblasts (BJ/tert). All the cell types were infected with a recombinant amphotropic retrovirus that directs the expression of LT (7). After infection and selection with blasticidin, cells were demonstrated to stably express LT by Western blotting (Fig. 5C). Interestingly, LT retained the ability to induce significant p53 Ser15 phosphorylation and stabilization in ATM-deficient cells. However, LT, even when expressed at levels comparable to those in ATM-deficient cells, induced only a modest increase in Ser15 phosphorylation when ATR was severely reduced. The quantitated and normalized pSer15 signal (relative to vinculin) from the Western blot analysis is depicted in Fig. 5D. The expression of LT in Seckel cells resulted in the lowest overall pSer15 signal. It is important to consider that Seckel cells still have a small amount of ATR activity, since a complete loss of ATR is lethal (8). The defect that we observed is of the same magnitude as that previously reported for Seckel cells (41, 58). Moreover, reconstitution of the Seckel cells with full-length ATR led to a significant increase in Ser15 phosphorylation only when LT was expressed (Fig. 5E). Thus, our results suggest that ATR contributes to the induction of Ser15 phosphorylation, and ATM is not absolutely required. However, our data leave open the possibility that other kinases participate in the LT-induced phosphorylation of Ser15.

LT induces tetraploid DNA content dependent on Bub1 binding. Genotoxic damage can promote changes in ploidy (29, 67). Given the ability of LT to stimulate the DDR via its Bub1 binding site, we determined if LT binding to Bub1 might also underlie its ability to cause chromosome instability. We examined the ability of LT to cause alterations in chromosome numbers by investigating the BJ/tert cell lines stably expressing LT or dl89-97. Metaphase chromosome spreads were prepared 1 week after infection to ensure that the effects of LT on chromosome integrity are direct and not a consequence of changes upon culturing. Consistent with the literature, we found that LT causes significant tetraploidy (11, 27). Representative chromosomes are depicted in Fig. 6A, and quantitative results of counted chromosomes are shown in Fig. 6B. Strikingly, the dl89-97 mutant was deficient in inducing tetraploidy. When metaphase spreads were prepared 2 weeks postinfection, the outcome was similar except that LT tended to generate aneuploidy, rather than tetraploidy, at this later time point (data not shown).

To confirm our results with chromosome spreads, we also performed FISH analysis. While chromosome spreads allow one to look at chromosome number only in mitotic cells, FISH is useful for assessing chromosome copy numbers at all stages of the cell cycle including interphase. We used centromeric probes for chromosomes 6 and 17. As shown in Fig. 6C, BJ/tert cells expressing LT had a significant population of cells with four copies of chromosomes 6 and 17, suggesting tetraploidy, whereas the cells expressing dl89-97 more rarely showed this configuration. In fact, after counting a significant number of nuclei, we detected a significant difference in the proportions of cells with four copies of chromosomes 6 and 17 when LT and dl89-97 were compared (Fig. 6D). A caveat in the FISH-based analysis of tetraploids is that diploid cells in G_2 phase also have four dots, which we believe accounts for most of this population in the dl89-97 mutant. In summary, the FISH analysis is consistent with the counting of mitotic figures from chromosome spreads, thus demonstrating that LT causes tetraploidy largely via Bub1 binding.

DISCUSSION

This study investigated LT's ability to induce, via Bub1 binding, DDR signaling as well as tetraploidy, key phenomena thought to play a role in oncogenic transformation in some cases. We show for the first time that LT expression alone is sufficient to induce a DDR, resulting in hallmark changes including the development of nuclear γ-H2AX/53BP1 foci; p53 phosphorylation/stabilization; the activation of ATM/ATR, chk1/chk2, and Rad17; and the accumulation of the Claspin mediator. Surprisingly, while Bub1 binding plays a pivotal role in the activation of the DDR, the inactivation of the pRB pathway was not required here even though it is involved in DDRs in other contexts (45).

Our findings resonate with recent demonstrations that SV40 infection elicits a bona fide DDR. In contrast to most studies with intact SV40 virus that focused on the ATM response (54, 70), our studies have emphasized links between LT and the ATR pathway. Notably, one previous study did observe the activation of the ATR/chk1 pathway in SV40-infected monkey cells (42). Hence, we believe that an ATR-dependent DDR could be important during a productive SV40 infection. The potential differences between DDR pathways invoked by whole virus versus those seen when only LT is expressed probably reflect variations in the "lesion" that is sensed. With whole virus, there is ongoing viral replication, which includes some aberrant replication structures that may be sensed by the host. In contrast, we expressed LT only without a functional origin, precluding viral replication but still causing a DDR response. During viral replication, additional DDR pathways are likely triggered.

We have systematically examined the involvement of ATM/ATR kinases in the LT-induced DDR using hypomorph or deficient cells derived from patients, pharmacological inhibitors, stable shRNA suppression, and conditionally ATR-deficient HCT116 cells (15) (Fig. 5 and data not shown). Based on experiments with Seckel cells and their reconstituted equivalents, we demonstrate that the LT-induced phosphorylation of p53 on Ser15 in normal human fibroblasts involves ATR. This is consistent with the LT induction of pChk1 Ser317, pRad17 Ser645, and Claspin accumulation (Fig. 2), events that are
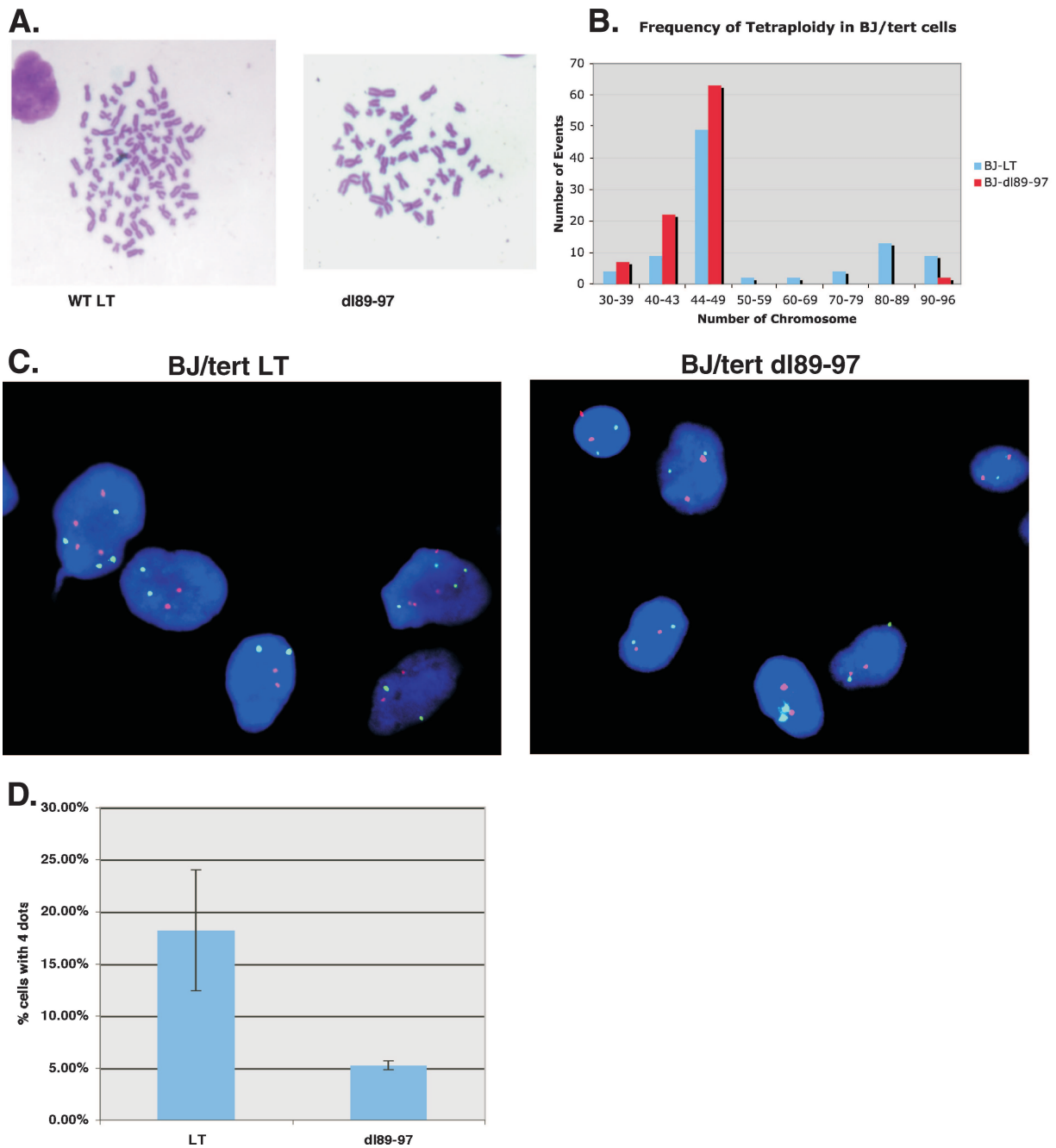
FIG. 6. LT induces tetraploidy via Bub1 binding. (A) Representative chromosome spreads from BJ/tert cells stably expressing LT (near tetraploid) or dl89-97 (diploid) prepared at 1 week postinfection. (B) Chromosome spreads from BJ/tert cells expressing either LT or dl89-97 were counted, and their distributions of chromosome numbers are shown. Chromosome numbers were determined by microscopic examination of 92 metaphase figures for LT and 94 metaphase figures for dl89-97. (C) Chromosome spreads from BJ/tert cells expressing LT or the dl89-97 mutant were processed for FISH analysis with Vysis centromeric probes for chromosome 6 (green) and chromosome 17 (red). (D) The relative frequencies of cells with four copies of chromosomes 6 and 17 were calculated based on 255 (LT) and 231 (dl89-97) cells, respectively.

connected primarily with the ATR pathway. Pharmacological inhibitors as well as stable shRNA depletion indicate that ATM is not involved in Ser15 phosphorylation in U2OS/T cells. Furthermore, ATM is not absolutely required for Ser15 phosphorylation in human fibroblasts based on our experiments with ATM-deficient cells. Despite these observations, we paradoxically detected ATM and chk2 activation in BJ/tert

cells expressing LT. There may be cell type-dependent effects of LT on ATM/ATR that may reconcile this, or alternatively, compensation may occur through ATR when ATM is depleted or vice versa. There are intrinsic technical limitations in assigning a specific phosphorylation event to ATM or ATR because substrates are often redundant (13), there is cross talk at many levels (30), total ATR loss is lethal (8, 15), and ATR depletion

is known to induce collapsed replication forks and double-strand breaks leading to ATM activation (14).

The most straightforward explanation for our DDR data, given the induction of pATM Ser1981, is that LT causes double-strand breaks. However, it is also possible that LT simply mimics DNA damage or that ATR acts upstream to phosphorylate ATM on Ser1981 (58). One possibility is that LT causes a deregulation of normal cellular DNA replication dynamics, for example, rereplication or increased replication fork stalling, and this is sensed as DNA damage (71). Another possibility would be for LT to directly interact with DDR pathway components to elicit activation as in, for example, the binding of LT to Claspin that we demonstrated. It also remains uncertain exactly how Bub1 is connected to the DDR response. The involvement of Bub1 could be indirect either via its role in checkpoints and mitotic progression or via a direct signaling effect, for example, mediated by the phosphorylation of a DDR target. A more detailed understanding of this interplay may illuminate new connections between the spindle checkpoint and DDR pathways. A number of connections have already been made between these two key mechanisms that are critical for genome integrity; for example, chk1, a key element of the DDR, is also required for the spindle checkpoint (25, 32, 68).

Viral deregulation of DDRs is multifaceted (34). While some viruses inactivate the response, others actively exploit it. Mouse polyomavirus and SV40 strains appear to benefit from their activation of the DDR (18, 54, 70) since the inhibition of ATM blocks viral replication. The exact underlying mechanisms whereby DDR activation promotes the viral life cycle are not clear and are likely to differ between viruses. For SV40, it might involve either the ATM/ATR-mediated phosphorylation of LT at the Ser120 regulatory site (54), the degradation of the Mre11-Rad50-Nbs1 complex, or proper localization at viral replication centers, which colocalize with γ-H2AX during productive infection (70). It is not entirely clear why LT targets the ATR pathway, but since ATR/chk1 monitors and restarts replication forks, it seems likely to benefit continuing viral replication (61). Perhaps it prevents fork stalling during high-level viral replication. Alternatively, it may prevent excessive damage during replication, thus allowing high-fidelity replication while preserving viability.

Considerable interest has arisen concerning the functions of LT that are required to destabilize the host genome, since these functions may contribute to long-term effects of LT that are important for tumor induction. One relevant aspect is numerical chromosome instability, specifically tetraploidy, which LT was previously shown to promote. Here, we demonstrate that Bub1 binding is critical for the induction of tetraploidy in normal human fibroblasts. This makes sense when considering Bub1's role in safeguarding chromosomal stability (9, 31, 38). We speculate that p53 binding is not required per se for tetraploidy induction but is required for its maintenance (22). Interestingly, tetraploidy occurs concomitant with DDR induction and with a similar dependence on Bub1. It is unclear if the convergent genetic requirements for the activation of the DDR and tetraploidy mean that they are causally linked and, if so, which is causal to the other. The exact mechanism whereby LT via Bub1 induces tetraploidy is not clear. Future studies will address if the mechanism involves rereplication (67) or abortive mitosis/cytokinesis (59).

The literature also suggests that p53 stabilization may play a role in naturally occurring p53 gain-of-function mutants that exhibit an enhanced transformation potential (62), and it is tantalizing to think that LT might make use of a p53 gain-of-function in transformation (6, 19, 20). Thus, the interaction of LT with Bub1 might contribute to transformation both in an acute fashion via p53 stabilization and via a long-term effect on chromosome stability.

Collectively, our studies have uncovered an intriguing requirement for Bub1 binding in DDR and tetraploidy induction by LT. While the evidence indicates Bub1 binding is important, it is also likely that other functions of LT also contribute. Thus, we believe that p53 inactivation is critical for the survival of cells with induced DDR and tetraploid DNA content. Furthermore, we do not yet comprehend the molecular mechanisms whereby Bub1 contributes to the generation of a DDR and tetraploidy. Future studies will be required to analyze the full rationale, underlying mechanisms, and consequences associated with DDR activation.

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