Supplemental Figures

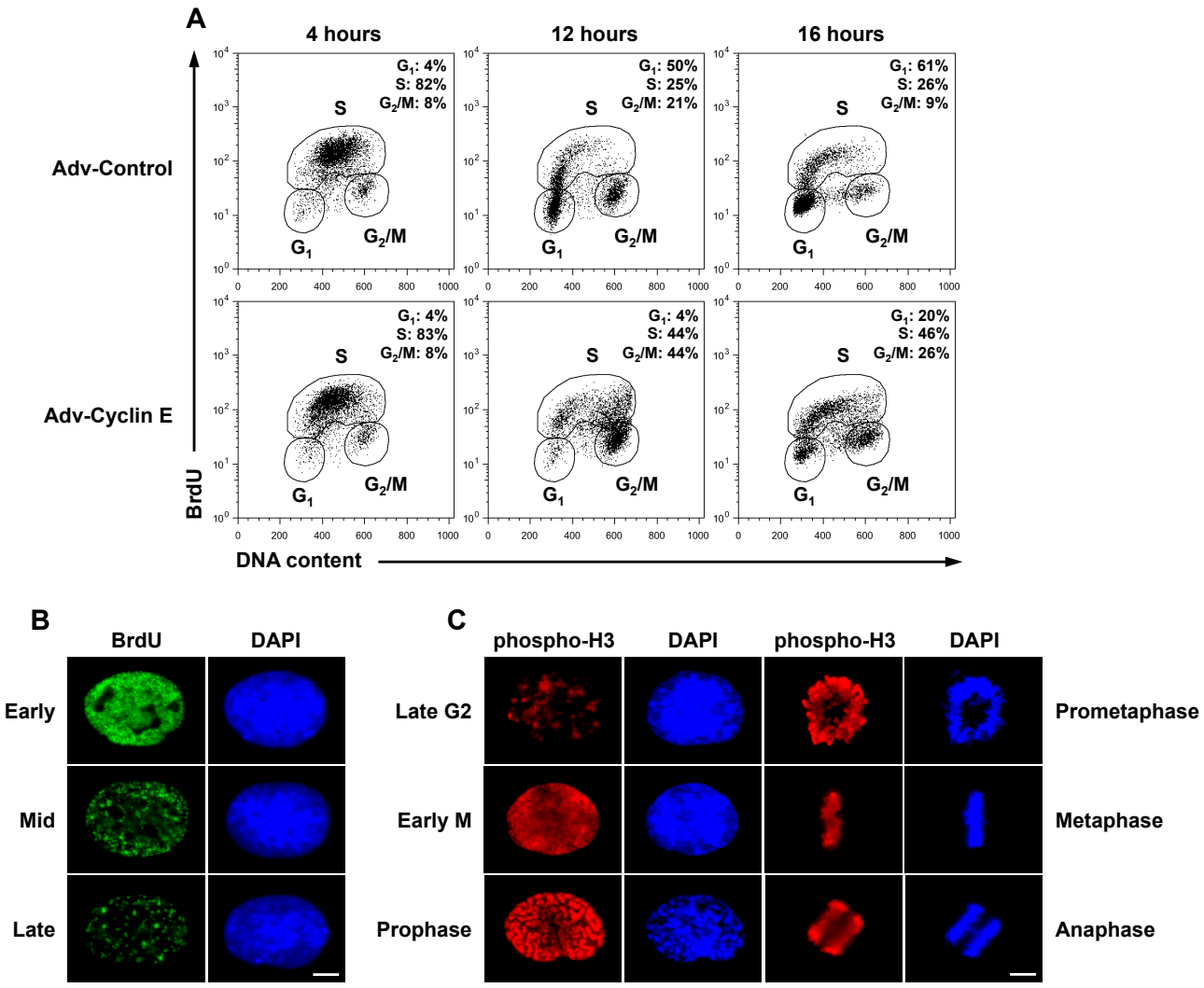


Figure S1: Reduced progression through S phase upon cyclin E deregulation. Related to Figure 1.

(A) Flow cytometry analysis of HME1 cells transduced with adenovirus control or cyclin E, synchronized with a double-thymidine block protocol, and released for the indicated time points. Results are representative of three independent experiments.

(B and C) BrdU and phospho-H3 as markers of S and M phase progression. Immunofluorescence of HME1 cells pulsed with BrdU for 10 min and stained with anti-BrdU to visualize cells in S phase (B) or stained with phospho-H3 to visualize cells in M phase (C). Cells in different stages of S phase show specific BrdU staining according to size, number, and nuclear location of foci. Cells in different stages of M phase show correlation of phospho-H3 staining with chromatin condensation as visualized by DAPI staining. Scale bars, 5 μm.

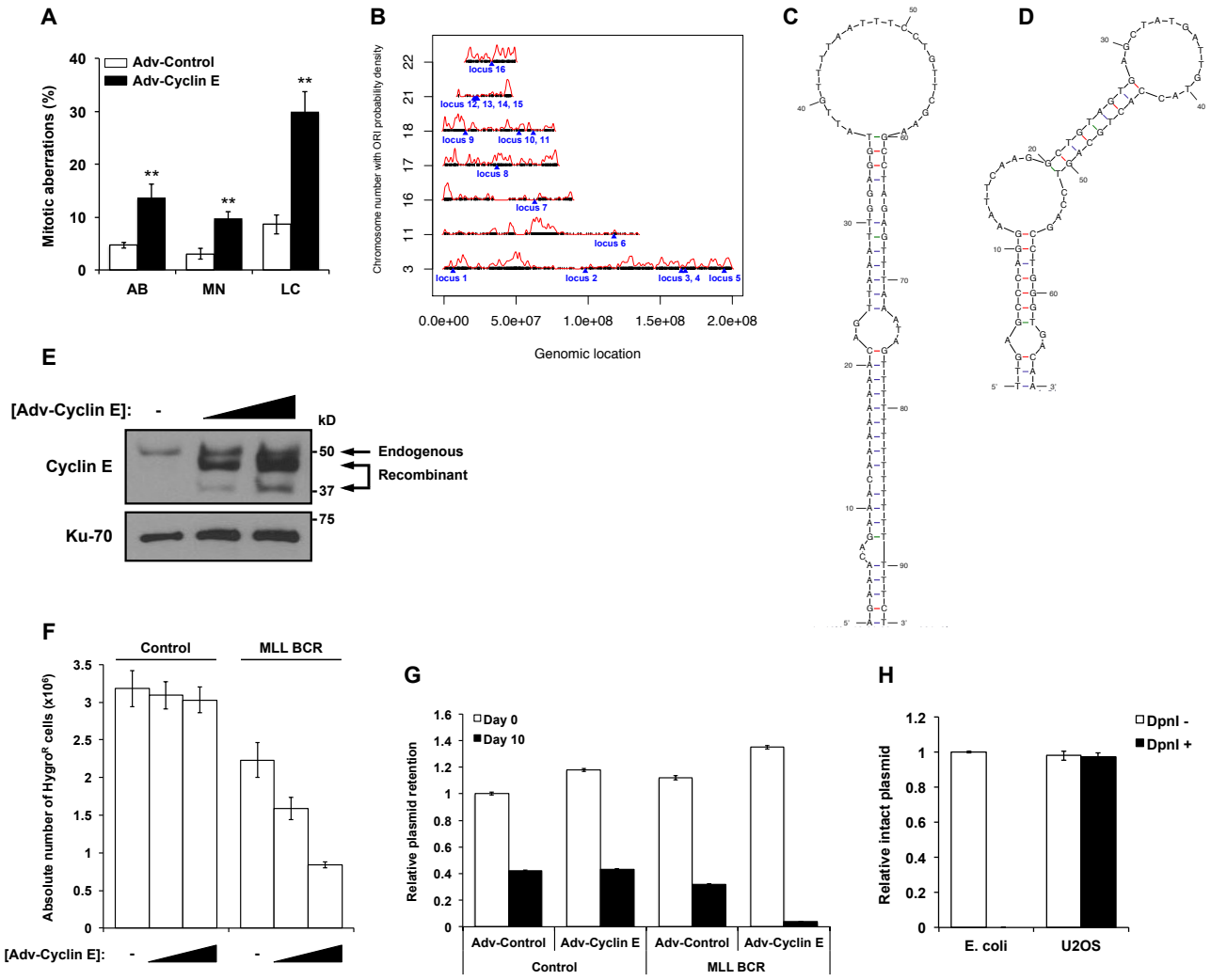


Figure S2: Origin paucity and unusual DNA structures contribute to cyclin E-mediated genomic loss. Related to Figures 2 and 3.

(A) Frequency of anaphase bridges (AB), micronuclei (MN), and lagging chromosomes (LC) in HME1 cells transduced with control (white bars) or cyclin E adenovirus (black bars).

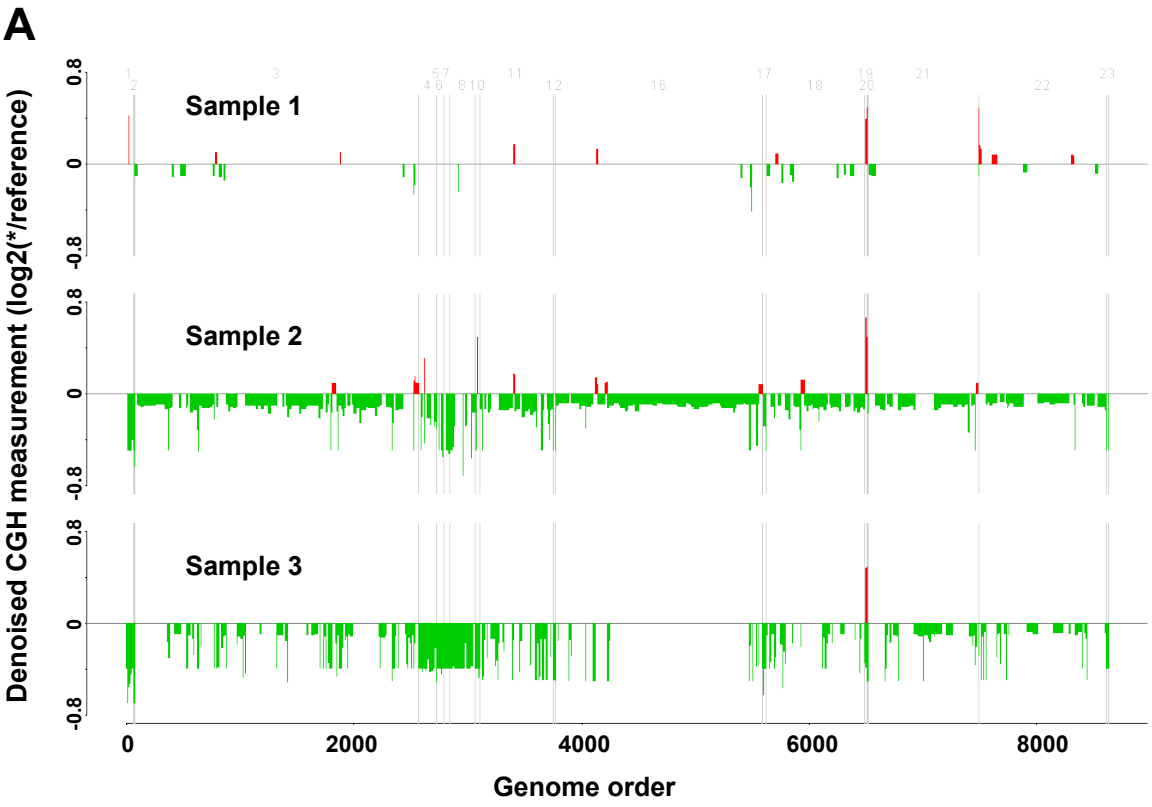
(B) Schematic representation of the 16 CNV loci identified in this study along with the ORIs recently identified on human chromosomes [S1].

(C and D) Schematic representation of the putative stemloop structures located in intron 7 (C) and intron 8 (D) of the MLL BCR N terminus. Stemloop structures have ΔG = -7.15 kcal and - 9.69 kcal, respectively [S2].

(E) Immunoblot analysis of U2OS cells transduced with control adenovirus (-) or increasing amounts of cyclin E adenovirus.

(F) Absolute numbers of hygromycin-resistant cells presented in Figure 3C. Cell survival analysis in U2OS cells transfected with pCEP4 plasmid encoding a fragment of the MLL BCR N terminus (MLL BCR) or a control fragment lacking the stemloop structures

(Control), transduced with control adenovirus (-) or increasing amounts of cyclin E adenovirus, and selected with hygromycin (see Supplemental Experimental Procedures).
(G) Relative plasmid levels presented in Figure 3D. Real-time PCR analysis of *Dp*nI-treated pCEP4 plasmids on day 0 (white bars) and day 10 (black bars) containing part of the MLL BCR sequence (MLL BCR) or a control sequence lacking the two stemloop-forming regions (Control). Numbers represent the ratio of Ct values obtained for pCEP4 plasmid to Ct values obtained for human beta-actin gene. Data are normalized to cells transfected with pCEP4 plasmid encoding the MLL BCR control sequence (Control), transduced with control adenovirus (Adv-Control), and analyzed at Day 0.
(H) Real-time PCR analysis of pCEP4 plasmids containing a control sequence from the MLL BCR locus extracted from *E. coli* or U2OS human cells, and treated (black bars) or not (white bars) with *Dp*nI.


A
Sample 1
Sample 2
Sample 3
Denoised CGH measurement (log2(*/reference)
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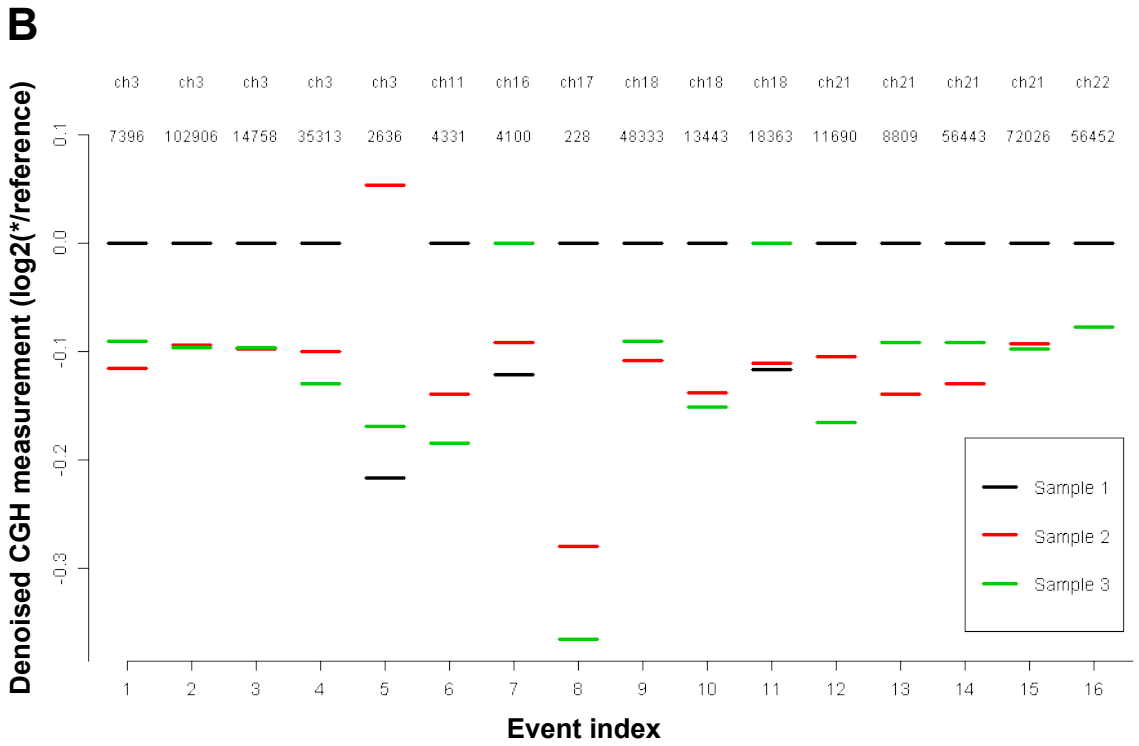
0
-0.8
0
2000
4000
6000
8000
Genome order


B
ch3 ch3 ch3 ch3 ch3 ch11 ch16 ch17 ch18 ch18 ch18 ch21 ch21 ch21 ch21 ch22
7396 102906 14758 35313 2636 4331 4100 228 48333 13443 18363 11690 8809 56443 72026 56452
Sample 1
Sample 2
Sample 3
Denoised CGH measurement (log2(*/reference)
0.1
0.0
-0.1
-0.2
-0.3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Event index

C

Locus	Chrom	CCNE+ CNV-	CCNE+ CNV+	CCNE- CNV-	CCNE- CNV+	*P* value
1	3	1	93	31	1,837	0.794833899
2	3	8	86	16	1,852	8.06E-06
3	3	5	89	14	1,854	0.001545781
4	3	5	89	13	1,855	0.001183403
5	3	0	94	6	1,862	1
6	11	8	86	318	1,550	0.993347365
7	16	10	84	524	1,344	0.999984597
8	17	4	90	100	1,768	0.74694646
9	18	3	91	63	1,805	0.622302983
10	18	5	89	83	1,785	0.415056828
11	18	9	85	84	1,784	0.030828652
12	21	8	86	66	1,802	0.022741209
13	21	6	88	73	1,795	0.173868041
14	21	8	86	71	1,797	0.032344755
15	21	7	87	69	1,799	0.067892434
16	22	8	86	220	1,648	0.873667314

Figure S3: Cyclin E deregulation promotes copy number loss in specific genomic regions. Related to Table 1 and Figure 2.

(A) Estimated copy number profiles of three samples with cyclin E deregulation based on CGH arrays. X-axis represents the genome order of the 8,627 probes, which showed copy number alterations in at least one of the three samples. Estimated DNA copy number of each probe was represented with a vertical bar, which was colored in red for amplification and in green for deletion. Probes from different chromosomes were separated by gray verticle lines, and chromosome numbers (in gray) were labelled in the top of the figure. CGH array experiments were performed using Genome-Wide Human SNP Array 6.0 (Affymetrix). The raw data were processed using R package *Aroma* [S3-S5], and the copy number gains/losses were inferred using R package *cghFLasso* [S6].

(B) Inferred DNA level variation for the 16 genomic regions shown in Table 1. These regions were choosen with the criteria of being underreplicated in at least two of the three samples and with at least 5 probes. The Y-axis of each small bar in the figure represents the inferred relative DNA level of the corresponding genomic region in one sample. Chromosome numbers and sizes (in bp) of the 16 regions were annotated on the top of the figure.

(C) Correlation of cyclin E amplification and loss of genomic regions in breast cancer. CNV analysis from 1,962 breast cancer patients [S7]. CCNE+, cyclin E1 amplification; CCNE-, cyclin E1 neutral or loss; CNV-, copy number variation loss; CNV+, copy number variation neutral or gain (see Supplemental Experimental Procedures).

Table S1: Cyclin E deregulation promotes copy number loss in specific genomic regions. Related to Table 1.

(A) Inferred DNA copy numbers for the 8,627 probes with copy number alterations in at least one sample.

(B) Information for the 372 probes with deletion in at least two samples. The 16 loci in Table 1 are also annotated here.

(C) Summary numbers for the inferred copy numbers.

Table S2: DNA replication origin distribution along the 16 genomic regions. Related to Figure 3.

Summary results from the analysis of the ORI data from two different studies [S8, S1] for the 16 loci in this study. Columns “Locus”, “Chromosome”, “Start position” and “End position” contain the locus and chromosome number, and starting and ending position on the chromosome for each of the 16 CNV regions, respectively. Columns “Start of closest ORI” and “End of closest ORI” provide the starting and ending position of the closest ORI to each CNV region, respectively. The column “Distance to locus” indicates the distance between each CNV region and its closest ORI, and the column “Number of ORIs” indicates the number of ORIs in the 1 Mb neighborhoods of each locus.

Supplemental Experimental Procedures

Cell lines. hTERT-HME1 (ATCC, CRL-4010), an immortalized human mammary epithelial cell derived from the mammary glands of a healthy donor, was grown in MCDB 131 medium (Gibco) supplemented with 70 µg/mL bovine pituitary extract (Hammond Cell Tech), 1% newborn calf serum (Gemini Bio-Products), 5 µg/mL holo-transferrin (Sigma), 10 ng/mL human epidermal growth factor (Invitrogen), 0.5 µg/mL hydrocortisone (Sigma), 5 µg/mL insulin (Sigma), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). MDA-MB-157 (ATCC, HTB-24) was grown in Leibovitz's L-15 medium (Gibco) supplemented with 10% newborn calf serum (Gemini Bio-Products), 100 U/mL penicillin, and 100 µg/mL streptomycin. SUM149PT (Asterand) was grown in Ham's F-12 medium (Gibco) supplemented with 5% newborn calf serum (Gemini Bio-Products), 5 µg/mL insulin (Sigma), 1 µg/mL hydrocortisone (Sigma), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). HEK-293A and Phoenix amphotropic packaging cell lines and U2OS cells were grown in DMEM medium (Gibco) supplemented with 10% newborn calf serum (Gemini Bio-Products), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). Cells were maintained in a humidified 37^{o}C incubator with 5% CO_2, except for MDA-MB-157 (maintained in air atmosphere).

Cell synchronization. For the double-tymidine block-release synchronization protocol, HME1 cells were treated with 2 mM thymidine (Calbiochem) for 18 hours. Then, cells were washed 3 times with medium and released in fresh medium for 8 hours. Adenoviral transductions were carried out in the first 2 hours of release (see below). Cells were treated again with 2 mM thymidine for 16 hours, washed 3 times with medium, and released in fresh medium for the indicated time points. For the mitotic shake-off synchronization protocol, cells were treated for a double-thymidine protocol as above and released in fresh medium for 4 hours, when 25 ng/mL nocodazole (Calbiochem) was added for 12 hours. Rounded-up cells were detached from petri dishes by gently rinsing and pipetting off the medium, and resuspended in sorting buffer (1x PBS, 1 mM EDTA, 25 mM Hepes pH 7.0, 1% newborn calf serum, 1 µg/mL propidium iodide). Live cells were separated from dead cells on a FACSAria (BD Biosciences) as a propidium iodide-negative population.

Viral transductions. Recombinant adenovirus expressing part of the beta-globin cDNA (Adv-Control) or recombinant adenovirus expressing the cyclin E cDNA (Adv-Cyclin E) [S9] were amplified in HEK-293A cells and purified by ultracentrifugation on a CsCl gradient. HME1 cells were transduced for 2 hours with 1-10 x 10^3 viral particles/cell in a low volume of complete MCDB 131 medium. Recombinant retrovirus expressing the histone H2B cDNA fused to GFP was produced in Phoenix cells and harvested from cell supernatants as previously described [S10]. HME1 cells were transduced with retrovirus-containing supernatant for 16 hours, and transduced cells were selected after 48 hours in 300 µg/mL neomycin (Gibco).

Western blotting. HME1 cells were transduced with Adv-Control or increasing amounts of Adv-Cyclin E and cells were harvested after 24 hours. Total cell extracts were prepared in cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, 2 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin), sonicated, and cleared by centrifugation for 10 min at 10,000 *g* at 4°C. Protein concentration was determined by Bradford protein assay (Bio-Rad), sample buffer was added (50 mM Tris pH 6.8, 2% SDS, 0.01% bromophenol blue, 2.5% beta-mercaptoethanol and 10% glycerol), and extracts were incubated for 5 min at 100°C. Samples were separated in 4-12% SDS-PAGE gels (Invitrogen) and transferred onto PVDF membranes (Immobilon-P, Millipore). Membranes were blocked for 1 hour at room temperature in blocking buffer (20 mM Tris pH 7.6, 500 mM NaCl, 0.02% Triton X-100, 5% nonfat milk), incubated overnight at 4°C with primary antibody in blocking buffer, and then incubated for 1 hour at room temperature with secondary antibody in blocking buffer. Antibodies used were mouse monoclonal anti-cyclin E (HE12, Santa Cruz Biotechnology), mouse monoclonal anti-Cdc2 (POH1, Cell Signaling Technology), mouse monoclonal anti-Ku-70 (A-9, Santa Cruz), and HRP-conjugated goat anti-mouse IgG (Pierce Biotechnology).

Flow cytometry. At indicated time points, cells were pulsed with 20 µM BrdU (Sigma) for 30 minutes at 37°C. Cells were harvested, washed in 1x PBS, and immediately fixed in 70% ice-cold ethanol overnight. Cells were then washed in 1x PBS, 1% BSA for 5 minutes, treated with 2 N HCl, 0.5% Triton X-100 for 30 minutes at room temperature with agitation, neutralized with 0.1 M Borax (Sigma), and washed again in 1x PBS, 1% BSA for 5 minutes. Cells were labeled with fluorescein-conjugated anti-BrdU antibody (ABFM-18, Phoenix Flow Systems) in staining buffer (1x PBS, 1% BSA, 0.5% Tween-20) for 1 hour at room temperature, washed in staining buffer, and labeled with propidium iodide in staining buffer (1x PBS, 0.1% Triton X-100, 20 µg/mL propidium iodide, 1 mg/mL RNase A prepared in 50 mM Tris pH 8.0, 15 mM NaCl) overnight at 4°C. Cells were run on a FACSCalibur flow cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star).

Immunofluorescence. Cells were grown on poly-L-lysine-coated (P9155, Sigma), 18 mm circular cover glasses N°.1 (12-545-100, Fisher Scientific), synchronized with a double-tymidine protocol, transduced with Adv-Control or Adv-Cyclin E, and released into cycle for 6, 8 or 10 hours (peak of mitotic cells). Cells were shortly pulsed with 20 µM BrdU for 10 minutes at 37°C, and then immediately fixed in fresh 2% paraformaldehyde for 10 minutes, 100% methanol for 10 minutes, and treated in 1x PBS, 0.1% Triton X-100 for 10 minutes, all steps at room temperature. Cells were stored in 70% ice-cold ethanol overnight at 4°C. For phosphorylated histone H3 staining, cells were washed in wash buffer (20 mM Tris pH 7.6, 500 mM NaCl, 0.02% Tween-20) for 15 minutes at room temperature, blocked in block buffer (1x PBS, 1% BSA, 0.5% Tween-20) for 15 minutes at room temperature, and labeled with a rabbit polyclonal anti-phospho-histone H3 antibody (06-570, Millipore) in block buffer overnight at 4°C. Cells were then washed three times for 20 minutes each, labeled with

rhodamine red-conjugated donkey anti-rabbit IgG (711-295-152, Jackson ImmunoResearch Laboratories) for 1 hour, washed three times again, fixed in fresh 2% paraformaldehyde for 5 minutes, and rinsed once with wash buffer, all steps at room temperature. For BrdU staining, cells were treated with 2 N HCl for 15 minutes, washed twice in 1x PBS for 15 minutes each, washed in wash buffer for 15 minutes, treated with block buffer for 15 minutes (all steps at room temperature), and labeled with a sheep polyclonal anti-BrdU (NB500-235, Novus Biologicals) in block buffer overnight at 4^{o}C. Cells were then washed three times for 20 minutes each, labeled with fluorescein-conjugated donkey anti-sheep IgG (713-095-003, Jackson ImmunoResearch Laboratories) for 1 hour, and washed three times again, all steps at room temperature. Cover glasses were mounted onto slides with ProLong Gold antifade reagent with DAPI (P36931, Invitrogen). Cells were visualized with an AxioScope 2 microscope attached to an AxioCam MRm monochrome CCD camera (Carl Zeiss), and analyzed using AxioVision software (Zeiss).

Time-lapse microscopy. HME1 cells expressing GFP-H2B to visualize chromosomes in real time were grown in µ-Dish$^{35mm,\ high}$ petri dishes (81156, ibidi), transduced with Adv-Control or Adv-Cyclin E, and analyzed 48 hours later in a LSM 710 laser scanning confocal microscope attached to an Observer Z1 microscope (Carl Zeiss). Cells were maintained in a Live Cell chamber (Pathology Devices) at 37^{o}C, 66% humidity, and 5% CO_2, and analyzed with a 63x oil-immersed PlanApo objetive with a 1.4 NA (Carl Zeiss) attached to an objetive heater system (Bioptechs). Metaphase cells were recorded once every minute at a step-size of 0.7 µm z-section slices (stack of ~ 15-20 focal planes) until they reached telophase. Images were collected using Zen software (Carl Zeiss) and analyzed using Imaris software (Bitplane Scientific Software).

Comparative genomic hybridization. Mitotic cells were prepared and isolated as described in section 2 (*Cell synchronization*). Genomic DNA from cells transduced with Adv-Control or Adv-Cyclin E was extracted using QIAmp® DNA Mini Kit (Qiagen) according to manufacturer's instructions, and submitted to the Vanderbilt Microarray Shared Resource (Vanderbilt University, TN) for CGH analysis with the Genome-Wide Human SNP Array 6.0 (Affymetrix). DNA samples were processed, hybridized to arrays, scanned, and passed QC tests. All these steps were repeated for a total of three independent experiments. The raw array data were first normalized using R package *Aroma* [S3-S5]. Then, we used the data of the three control samples as reference arrays, and for each of the three cyclin E samples, log2 ratio of the intensities in the target array versus that in the reference arrays were calculated. In the end, DNA copy number alterations were inferred using R package *cghFLasso* [S6] using a stringent false discovery rate controlled at 0.001. The probability of false convergence on any of the 16 genomic loci in two of three experiments due to background noise is 5.17×10^{-6}.

Real-time PCR. HME1 cells were transduced with adenovirus control or cyclin E twice a week for three weeks. Total DNA was extracted using QIAmp® DNA Mini Kit (Qiagen) and real-time PCR reactions were done with iQ™ SYBR® Green Supermix (Bio-Rad)

according to manufacturer's instructions. Each sample was run in quadruplicate on a Chromo4 real time PCR instrument (MJ Research) and analyzed using Opticon Monitor software (Bio-Rad). Primer sequences were designed as previously described [S11] and can be provided upon request. Copy number (CN) of specific genomic regions were normalized to control genomic regions, usually located at the other arm of the same chromosome, and were determined using the formula: $CN = 2^{Ct(\text{control region}) - Ct(\text{specific region})}$, where Ct is the cycle threshold. Relative CNs were dertermined using the formula: cyclin E CN/control CN.

Whole Genome Amplification. HME1 cells were transduced twice with adenovirus control or cyclin E within a 72-hour interval and harvested 24 hours after the second transduction. Single cells were isolated using a MoFlo Astrios EQ (Beckman Coulter) and genomic DNA from individual cells was homogeneously amplified using the REPLI-g Single Cell Kit (Qiagen) according to manufacturer's instructions. Each sample was run and analyzed as described in the previous section (*Real-time PCR*). Copy number (CN) of specific genomic regions were normalized to the control genomic region of chromosome 3, and were determined using the formula: $CN = 2^{Ct(\text{control region}) - Ct(\text{specific region})}$, where Ct is the cycle threshold.

Fluorescence *in situ* hybridization. HME1 cells were transduced with adenovirus control or cyclin E and expanded for 1 week. Cells were then harvested, treated with warm 0.075 M KCl for 30 minutes at 37^{o}C, fixed in ice-cold 75% methanol, 25% acetic acid, and stored at 4^{o}C overnight. Fixed cells were spread dropwise onto ice-cold water-wetted slides, heated for 1 minute at 70^{o}C on a humidified heating block, and stored for 24 hours at room temperature. Slides were pre-treated with 2x SSC for 30 minutes at 37^{o}C, dehydrated for 1 minute in 70% ethanol, followed by 1 minute in 85% ethanol, and 1 minute in 100% ethanol. Slides were then processed for hybridization with Vysis LSI MLL Dual Color Break Apart Rearrangement probe (05J90-001, Abbott Molecular) according to manufacturer's instructions. Cover glasses were mounted onto slides with ProLong Gold antifade reagent with DAPI (P36931, Invitrogen). Cells were visualized with an AxioScope 2 microscope attached to an AxioCam MRm monochrome CCD camera (Carl Zeiss), and analyzed using AxioVision software (Zeiss).

Plasmid retention. DNA folding of the MLL BCR N terminus was analyzed through the mFold web server [S2]. A DNA sequence of 721 bp containing one putative stemloop structure at each end (MLL BCR) or the 505 bp DNA core sequence lacking the two stemloop-forming structures (Control) were amplified from the BAC clone RP11-770J1 (BACPAC Resource Center, Children's Hospital Oakland Research Institute). DNA fragments were inserted into the non-integrating mammalian episomal plasmid pCEP4 at *No*tI/*Ba*mHI sites (Life Technologies). pCEP4 vector contains oriP and EBNA1 sequences, which allow extrachromosomal replication monitoring, and a hygromycin resistance gene for selection. U2OS cells were transiently transfected with pCEP4-MLL BCR or -Control plasmids, selected with hygromycin for 8 days, and allowed to grow in hygromycin-free complete media for 24 hours. Cells were then transduced with Adv-

Control or different amounts of Adv-Cyclin E, and were transduced again 72 hours later. Another 4 days later, cells were seeded onto 10 cm plates at a density of 8 x 10^4 cells/plate (day 0). Hygromycin was reintroduced on day 1 to select against cells that have lost the plasmid during replication, with control cells still cultured in hygromycin-free media. Cells were transduced again on day 2 and day 6. On day 8, cells were harvested and subjected to cell number count. Total DNA from cells cultured in hygromycin-free media were extracted on day 0 and day 10 using the Blood and Tissue Kit (Qiagen), treated with *Dp*nI for 2 hours at 37°C to remove input bacterial DNA and submitted to real-time PCR. Primers on pCEP4 plasmid (pCEP4-*Dp*nI-F: CATGGCGGTCATATTGGACATG, and pCEP4-*Dp*nI-R: CCAAGCTAGTCGACCAATTCTC) were used to amplify a region containing two *Dp*nI sites, and primers on human beta-actin gene (Actin-F: AAGGAGAAGCTGTGCTACGTC, and Actin-R: CGGATGTCCACGTCACACTTC) were used as normalization control. In Figure S2H, plasmid DNA (pCEP4-Control) was extracted from *E. coli* and U2OS cells using the Qiagen Plasmid Kit and the Hirt extraction method, respectively, as described [S12]. Plasmid DNA was treated with *Dp*nI and submitted to real-time PCR as described above.

Replication origin analysis. The spatial relationship between the 16 CNV regions identified in this study and the DNA replication origins (ORIs) recently identified on human chromosomes by two different studies [S8, S1] was investigated through download from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE28911, and GSE37583, respectively. We tested whether there were significantly less ORIs in a +/- 1 Mb neighborhoods around the 16 CNV regions compared to the rest of the genome. Specifically, we derived the p-value by comparing the total number of ORIs falling into the neighborhoods of the 16 CNV regions with that in the neighborhoods of randomly chosen 16 locations on the genome and repeating for 1,000,000 times. The ORI probability density was estimated by the density function in the statistical R package *stats* with the kernel parameter at "gaussian" and the bandwidth at 0.5 Mb. Note that the ORI probability density pattern does not change significantly with different bandwidths.

Breast cancer sample analysis. Array CGH data and estimated DNA copy number profiles of 1,992 breast cancer patients were retrieved from the European Genome-phenome Archive (EGA; http://www.ebi.ac.uk/ega/) under accession number EGAS00000000083 [S7]. The 1,962 breast cancer patients who had DNA copy number measurements on gene *CCNE1* were used for the statistical analysis. For each sample, we labelled as CCNE1+, if the four probes within *CCNE1* gene fell into a segment with estimated copy number greater than 2, and CCNE1- otherwise. Similarly, for each of the 16 candidate regions, we labelled as CNV-, if all probes within that region had estimated copy number smaller than 2, and CNV+ otherwise. Fisher exact test was then performed to test the dependency between the copy number gain of *CCNE1* (CCNE1+ versus CCNE1-) and the copy number loss of each of the 16 candidate regions (CNV- versus CNV+).

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