ECRG2 Disruption Leads to Centrosome Amplification and Spindle Checkpoint Defects Contributing Chromosome Instability

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Cancer cells contain an abnormal number of chromosomes (aneuploidy), which is a prevalent form of genetic instability in human cancers. Abnormal amplification of centrosomes and defects of spindle assembly checkpoint are the major causes of chromosome instability in cancer cells. Here we present biochemical evidence to suggest a role of ECRG2, a novel tumor suppressor gene, in maintaining chromosome stability. ECRG2 localized to centrosomes during interphase and kinetochores during mitosis. Further analysis revealed that ECRG2 participates in centrosome amplification in a p53-dependent manner. Depletion of ECRG2 not only destabilized p53, down-regulated p21, and increased the cyclin E/CDK2 activity, thus initiating centrosome amplification, but also abolished the ability of p53 to localize to centrosomes. Overexpression of ECRG2 restored the p53-dependent suppression of centrosome duplication. Furthermore, ECRG2-depleted cells show severely disrupted spindle phenotype but fail to maintain the mitotic arrest due to minimal BUBR1 protein levels. Taken together, our results indicate that ECRG2 is important for ensuring centrosome duplication, spindle assembly checkpoint, and accurate chromosome segregation, and its depletion may contribute to chromosome instability and aneuploidy in human cancers.

A critical component of normal cell division is the accurate distribution of chromosomes and other cellular components during mitosis (1). Abnormal cell division and chromosome content are hallmarks of cancer (2). The mechanisms, by which cells acquire chromosome changes, have not been fully identified, but alterations of centrosomes, the mitotic spindle apparatus, and the spindle assembly checkpoint appear to play important roles (3).

In normal cell division, centrosomes undergo one round of duplication in a manner analogous to the replication of chromosomal DNA during S phase (4). During mitosis, centrosomes direct the formation of bipolar mitotic spindles that ensure equal segregation of chromosomes between daughter cells (5). Therefore, at any given time point, a homeostasis of centrosomes must be maintained (6). The presence of more than two centrosomes increases the frequency of mitotic defect and unbalanced chromosome segregation (7). Indeed, centrosome amplification is frequently observed in cancer cells (8–10). This is thought to result in distortion of the mitotic apparatus and abnormal sorting of chromosomes during cell division. Although recent studies have shown a handful of protein kinase families, including p53 (11), POLO (12), PIK4 (13–14), AURORA (15), and MPS1 (16–17), regulate centrosome duplication and mitotic progression, they provide no insight into the genetic instability and aneuploidy, the identity and function of centrosome components, and their potential derangement in cancer, remain to be fully characterized.

Another mechanism by which chromosome segregation is mediated is called the spindle assembly checkpoint (SAC) (18). The spindle assembly checkpoint delays anaphase onset when any kinetochores in the cell is not properly bound with the spindle microtubules or when kinetochores are not under tension normally produced by bipolar attachment (19, 20). Activation of the SAC involves kinetochore localization of several spindle checkpoint proteins, including MAD1, MAD2 (21, 22), BUB1, BUB3 (23), BUBR1, and MPS1 (24–26). Mutations in any of these genes or changes of protein kinase activity result in failure to arrest the cell cycle at G2/M, and cells exit mitosis prematurely (27). Thus, the loss of the mitotic checkpoint machinery may cause aneuploidy. Despite extensive studies on the order of kinetochore association of the checkpoint proteins, not much is known about the regulation and function of each checkpoint protein at kinetochores.

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Here we report that ECRG2 depletion triggered accumulation of supernumerary centrosomes, abnormalities of the mitotic spindle, and marked chromosome changes. This was associated with disregulated of checkpoint genes p53 and p21 as well as cyclin E/CDK2. Furthermore, ECRG2-depleted cells exit from mitotic arrest in the presence of spindle damage due to low levels of BUBR1. Together our data suggest that ECRG2 contributes to chromosome instability via regulating centrosome amplification and spindle assembly checkpoint.

**EXPERIMENTAL PROCEDURES**

**Generation of ECRG2 Antibody**—We generated anti-ECRG2 monoclonal antibodies by using the C-terminal of ECRG2 peptide (KSNGRVQFLHDGSC) as immunogen (28). 217–258 encoding portions of human ECRG2 were amplified using Pfu polymerase (Stratagen), then cloned into pGEX-4T-3 (GE Healthcare) as BamHI/NotI restriction fragments. Glutathione S-transferase fusion proteins were expressed in BL21 Escherichia coli cells by induction with 1 mM isopropyl β-D-galactopyranoside at 37 °C then purified using glutathione-Sepharose beads (GE Healthcare) according to the manufacturer’s instructions. The anti-ECRG2 monoclonal antibody was generated following procedures described previously (28). Briefly, mice were immunized with glutathione S-transferase-ECRG2. Splenocytes from mice with positive immune responses were isolated, fused with myeloma cells, and the resulting hybridomas screened by enzyme-linked immunosorbent assay for reactivity against recombinant ECRG2. One positive hybridoma, 4E8, was expanded and used for all subsequent experiments.

**siRNA Constructs**—The human ECRG2 small interfering RNA (siRNA) expression plasmids were created by inserting two sets of siRNA oligonucleotides (5′-AAAGTAAATGGAAGAGTTCGATT-3′ and 5′-AATGATATTCCTACATCATC-3′, targeting open reading frames and 3′-UTR of ECRG2 mRNA, respectively) into the pSUPER.Retro.puro vectors (Oligoengine), generating the pSUPER.Retro.puro-ECRG2 siRNA and pSUPER.Retro.puro-ECRG2–3′UTR siRNA plasmids using BglII and HindIII restriction enzymes, respectively. All constructs were verified by sequencing.

**Generation of Retrovirus**—The packaging cell line GP2–293T (Clontech) was grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 10 mM Hepes, 2 mM l-glutamine, 1 mM minimal essential medium and sodium pyruvate. Cells in 100-mm dishes were transfected by calcium phosphate precipitation with 15 μg of pSUPER.Retro.puro-ECRG2 siRNA, pSUPER.Retro.puro-ECRG2–3′UTR siRNA plasmid, or pSUPER.Retro.puro vector. The medium was replaced 12 h post-transfection. Forty-eight hours post-transfection, the supernatant was collected and filtered through a 0.45-μm syringe filter.

**Infection of Target Cells and Establishment of Stable Knockdown Cells (ECRG2KD and ECRG23′UTR-KD)**—HCT 116 cells were purchased from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 10 mM Hepes, 2 mM l-glutamine, 1 mM minimal essential medium, and sodium pyruvate. The p53-null HCT 116 and p21-null HCT 116 cells were kindly provided by Bert Vogelstein (Howard Hughes Medical Institute). The stable ECRG2 knockdown cells were generated by infection of the HCT 116 or p53/p21-null cells with pSUPER.Retro.puro-ECRG2 siRNA (ECRG2KD), pSUPER.Retro.puro-ECRG2–3′UTR siRNA (ECRG23′UTR-KD), or the empty pSUPER.Retro.puro retrovirus. Briefly, the day before infection, 1 × 10⁵ cells were plated in a 60-mm dish. The next day, virus supernatant was added with Polybrene (5 μg/ml final concentration), and the cells were incubated overnight. The next day, the medium was replaced and the incubation continued until analysis. Colonies showing resistance to puromycin (5 μg/ml) were clonally isolated, and immunoblotting assay was used to screen for the suppression of ECRG2 expression.

**Generation of Inducible ECRG23′UTR-KD-Tet-on Cell Clones**—We used a doxycycline-inducible expression system to obtain conditional expression of the ECRG2 in HCT 116 cells as described previously. Briefly, we subcloned ECRG2 cDNA into pTRE-2hyg vector. This construct was transfected into HCT 116 cells stably expressing the reverse tetracycline transactivation using Lipofectamine 2000 (Invitrogen) and clones were selected based on absence of expression under basal conditions and strong induction by doxycycline. The positive clones were then infected with pSUPER.Retro.puro-ECRG2–3′UTR siRNA retrovirus to knockdown endogenous ECRG2 protein (ECRG23′UTR-KD-Tet-on) as described above.

**Pulse-Chase Experiment**—ECRG2 plasmid was transiently transfected into HCT 116 cells and incubated with methionine-free medium for 4 h. The cells were then pulse-labeled with fresh medium containing 100 μCi of [35S]methionine (PerkinElmer Life Sciences) per ml for 5 min and chased for 10, 30, 60, 120, 180, and 240 min. After each chase period the supernatants were harvested and the cells were lysed. Both the supernatants and cell lysates were incubated with antibodies against ECRG2 or normal IgG (control) for 2 h at 4 °C, followed by incubation with protein A-agarose beads for an additional 2 h at 4 °C. The beads were pelleted, washed extensively with immunoprecipitation buffer, boiled in SDS sample buffer, fractionated by SDS-PAGE, and analyzed by autoradiography.

**Mitotic Spread**—Exponentially growing ECRG2KD or control cells were treated with colcemid (Invitrogen) at a final concentration of 100 ng/ml for 2 h. Mitotic cells were collected and resuspended in 75 mM KCl and incubated at 37 °C for 25 min. Cells then were fixed in methanol/acetic acid (3:1) three times for a total of 30 min. Fixed cells were dropped on a glass slide and mounted with 4',6-diamidino-2-phenylindole.

**Immunofluorescence**—HCT 116 vector control or ECRG2KD cells grown on glass coverslips were fixed at 4 °C in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked in 2% BSA. Alternatively, cytoplasmic proteins were extracted using 1% CHAPS in PHEM buffer containing protease and phosphatase inhibitors (60 mM Pipes, 25 mM Hepes pH 6.9, 10 mM EGTA, 4 mM MgSO₄, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 50 mM β-glycerophosphate, 200 μM Na₃VO₄) for 60 s at room temperature, fixed at 4 °C in 4% paraformaldehyde, and blocked in 2% BSA. Cells were incubated in 2% BSA for 1 h with primary antibodies against ECRG2 (4E8), α-tubulin, γ-tubulin (Sigma), MAD1, MAD2, BUB1, BUB3, BUB1, and CREST (Santa Cruz), washed 3 times in...
phosphate-buffered saline, incubated in 2% BSA for 1 h with 488 or 594 Alexa Fluor secondary antibodies (Molecular Probes), and washed 3 times in phosphate-buffered saline. Cells were mounted with Prolong Gold containing 4’,6-diamidino-2-phenylindole (Molecular Probes). Fluorescence images were viewed with a Nikon E800 epifluorescence microscope with a ×60/1.40NA plan apo or ×100/1.3NA plan fluor oil immersion objective. Images were captured with a Roper CoollSnap HQ CCD camera and processed with Metamorph 5.0 and Adobe Photoshop 6.0 software.

Preparation of Cell Lysates and Immunoblotting Analysis—Adherent cells were washed in ice-cold phosphate-buffered saline, scraped, and resuspended into cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 0.5% Nonidet P-40) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM NaF, 1 mM sodium vanadate), and centrifuged at 14,000 × g for 15 min at 4 °C to pellet insoluble cell debris. Protein concentrations of supernatants were determined using a Protein Assay Kit (Bio-Rad). Equal amounts of protein were resolved by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corporation), and subjected to immunoblot analysis using the following primary antibodies: anti-ECRG2 monoclonal antibody (4E8), MAD1, MAD2, BUB1, BUB3, BUBR1 monoclonal antibodies, and rabbit anti-p53 (DO-1) antibody (Santa Cruz), phospho-H3 (Ser10) antibody (Cell Signaling), mouse anti-p27 and anti-human CREST (BD Pharmingen). Secondary antibodies, Alkapan phosphatase-conjugated AffiniPure goat anti-mouse IgG or anti-rabbit IgG-Alkapan phosphatase antibody (Sigma), were incubated for 1 h at room temperature and then processed for chemiluminescence detection using the CDP-Star reagent (Roche).

Immunoprecipitation-Western Blot Assay—Cells were lysed in an immunoprecipitation buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM NaF, 1 mM sodium vanadate), centrifuged for 5 min at 10,000 × g, and the insoluble debris was discarded. Cell lysate (200–500 μg of protein) was incubated with antibodies against ECRG2 or p53, or normal IgG (control) for 2 h at 4 °C, followed by incubation with protein A-agarose beads for an additional 2 h at 4 °C. The beads were pelleted, washed extensively with immunoprecipitation buffer, boiled in SDS sample buffer, fractionated by SDS-PAGE, and analyzed by anti-ECRG2 or p53 antibody.

Synchronization and Kinase Assay—Synchronized populations were generated by treating the vector control or ECRG2KD cells with thymidine-double block, and then releasing them into nocodazole. The cells were harvested at various times after nocodazole treatment, and processed for CDC2 kinase assay. ECRG2KD-Tet-on cell lysates were used for CDK2 kinase assay. Briefly, for isolation of CDC2 or CDK2 complexes, immunoprecipitation assay were performed by incubating 500 μg of total proteins from cell lysates described above or ECRG2KD-Tet-on cell lysates with rabbit polyclonal anti-CDC2 or anti-CDK2 antibodies (Santa Cruz) for 2 h at 4 °C, respectively. The immunocomplexes binding to Protein A-Sepharose beads were resuspended in 30 μl of kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 0.1 mg/ml BSA) and used directly in a kinase activity assay with histone-1 (H1) as a substrate. The kinase reaction mixture contained 1 μg of H1, 30 μM ATP, and 5 μCi of [γ-32P]ATP. The kinase assay was carried out at 37 °C for 30 min and terminated by adding sample buffer.

Purification of ECRG2-associated Proteins—ECRG2 cDNA expression plasmid was transfected into 293T cells. At 2 days after transfection, cells (~2 × 106) were treated with 30 μM MG132 for 4 h, lysed in a total of 10 ml of immunoprecipitation buffer, centrifuged for 5 min at 10,000 × g, and insoluble debris was discarded. The lysate was precleared with protein A-Sepharose beads for 30 min, and then incubated with a 40-μl bed volume of protein A-Sepharose beads and ECRG2 antibody for 4 h at 4 °C. The beads were washed with SNNTBE buffer (5% sucrose, 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, 500 mM NaCl, 1% Nonidet P-40) and boiled in SDS sample buffer. The eluted proteins were fractionated on SDS-PAGE and silver staining. Proteins copurified with ECRG2 were excised from the gel and subjected to protease digestion and peptide sequencing by mass spectrometry.

In Vitro Ubiquitination Assay—ECRG2UTR-KD or ECRG2UTR-KD-Tet-on cells in 100-mm plates were transfected with combinations of 5 μg of His6 ubiquitin expression plasmid and 5 μg of p53 expression plasmid. Adding or removing doxycycline induced or silenced ECRG2. Cells were lysed in buffer A (6 M guanidinium HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-HCl, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) and incubated with Ni2+-NTA beads (Qiagen) for 4 h at room temperature. The beads were washed with buffer A (8 μM urea, 0.1 M Na2PO4/NaH2PO4, 0.01 M Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol), buffer B (8 μM urea, 0.1 M Na2PO4/NaH2PO4, 0.01 M Tris-HCl, pH 6.3, 10 mM β-mercaptoethanol), and bound proteins were eluted with buffer C (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS). The eluted proteins were analyzed by Western blot for the presence of conjugated p53 by DO-1 antibody.

RESULTS

Expression and Localization of ECRG2—The expression of ECRG2 protein was analyzed in different cell lines using 4E8 monoclonal anti-ECRG2 antibody. As shown in supplemental Fig. S1a, ECRG2 was expressed in HCT 116, HeLa, and HFF cells but absent in esophageal cancer cells (NEC and EC109). Although the first 18 residues of the ECRG2 protein are a signal peptide to mediate targeting to the endoplasmic reticulum for secretion, pulse-chase experiments showed that ECRG2 was transferred to both intracellular and extracellular cells (supplemental Fig. S1, b and c). Subcellular localizations of endogenous ECRG2 in growing HCT 116, HeLa, and HFF cells were determined by immunofluorescence microscopy. In interphase cells, ECRG2 localized to centrosomes. ECRG2 was readily detected at both unduplicated (Fig. 1A and supplemental Fig. S2a, upper panel) and duplicated centrosomes (Fig. 1a and supplemental Fig. S2a, middle panel). During mitosis, ECRG2 accumulated around kinetochores (Fig. 1A and supplemental Fig. S2a, lower panel).
Further analysis confirmed that the C-terminal GFP tag ECRG2 localized to centrosomes (supplemental Fig. S2b). The deletion mutants study showed that 18–55 was a centrosome binding domain (Fig. 1C).

ECRG2 Knockdown Causes Chromosome Instability—When ECRG2 was partially down-regulated by transient RNA interference, we observed an increase in aneuploid cells (data not shown), suggesting a potential role of ECRG2 in chromosomal instability. To investigate this, we established cell lines with severe ECRG2 knockdown (>95% depletion) in HCT 116 cells by using the pSUPER.Retro virus infection approach (ECRG2KD, Fig. 2A). We chose the HCT 116 human colon carcinoma cell line because it has a normal centrosome duplication and mitotic checkpoint and is amenable to gene targeting. We performed chromosome counts on metaphase spreads to test the importance of ECRG2 on chromosome instability. As shown in Fig. 2, B–D, the ECRG2KD cells displayed a significant increase in the frequency (97%) of aneuploid metaphases (more than or less than 46 chromosomes) relative to vector control cells, which have a relatively stable karyotype (46 chromosomes). The chromosome morphology strongly suggests chromosome instability in cells with severely down-regulated ECRG2.

Loss of ECRG2 Induces the Formation of Supernumerary Centrosomes—To test directly whether the centrosomally localized ECRG2 participates in the regulation of centrosome amplification, we examined the status of the centrosomes by immunofluorescence microscopy in ECRG2KD cells. Cells were fixed and stained for γ-tubulin or centrin after shutoff of ECRG2. Cells with multiple centrosomes were observed both in interphase and in mitosis (Figs. 3A and Fig. 5A). After 24 h of repression, the cells began to accumulate supernumerary centrosomes, suggesting that the centrosome amplification is an early effect of ECRG2 down-regulation (Fig. 3B). The percentage of ECRG2KD cells with aberrant centrosome numbers increased with time. By 96 h, 53% of the cells had more than two centrosomes and cells with up to eight γ-tubulin or centrin spots could be observed (Fig. 3C). Among the cells with more than two centrosomes, about 28% have three or five centrosomes, and 72% have four or six centrosomes, suggesting that the centrosome amplification may also be caused by unbalanced mitotic division.

Centrosome Amplification Caused by ECRG2 Knockdown Can Be Prevented with Expression of Exogenous ECRG2—To further support the role of ECRG2 in centrosome amplification, and to rule out the possibility that centrosome amplification in ECRG2 knockdown cells are caused by off-target effects, we set up the ECRG2 Tet-On inducible system in HCT 116 cells.
ECRG2 Contributes Chromosome Instability

FIGURE 2. ECRG2 knockdown by siRNA induces aneuploidy in HCT 116 cells. A, knockdown of ECRG2 by pSUPER.retro virus infection. The lysates prepared from HCT 116 cells stably infected with either pSUPER.retro vector or a pSUPER.retro plasmid containing small interfering RNA specific for ECRG2 (ECRG2KD) were immuno-blotted using anti-ECRG2 (top panel) as well as β-actin (loading control, bottom panel) antibodies. B, chromosome number abnormality in ECRG2KD cells. Representatives of metaphase spreads from HCT 116 vector control or ECRG2KD cells (top panel) as well as normal (46 chromosomes) and cells with abnormal karyotype (aneuploidy) (ID). The percentages with the indicated number of chromosomes are shown. More than 300 cells were scored for each group.

The expression of endogenous ECRG2 was down-regulated by siRNA targeted at the 3′-untranslated regions (3′-UTR) of ECRG2 mRNA (ECRG23′-UTR-KD, Tet-on). Expression of the exogenous ECRG2 was not affected by the 3′-ECRG2 siRNA because the exogenous pTet-TRE-ECRG2 contains no 3′-UTR (Fig. 4A). We found the infection of pSUPER.Retro.puro-ECRG2–3′-UTR depleted by more than 95% endogenous ECRG2 protein. Adding doxycycline significantly restored exogenous ECRG2 protein (Figs. 4A and 7A, top panel). As shown in Fig. 4B, targeting ECRG2 3′-UTR alone increased the percentage of cells with centrosome amplification. However, when turned on the expression of ECRG2 by adding doxycycline in these cells significantly reduced centrosome amplification. And centrosome amplification increased again when turning off the expression of ECRG2 by removing the doxycycline. These data further support the role of ECRG2 in centrosome amplification and ruled out the off-target effect of ECRG2 RNA interference on centrosome amplification.

Depletion of ECRG2 Leads to Aberrant Spindles—To determine whether supernumerary centrosomes are able to mature and to become functional, we examined the mitotic spindle structure in ECRG2KD cells by double-labeling using antibodies to γ- and α-tubulin. In vector control HCT 116 cells, we observed bipolar mitotic spindles with two centrosomes (Fig. 5A, top panel). In contrast, ECRG2-depleted cells uniformly demonstrated irregular multipolar mitotic spindles with supernumerary centrosomes (Fig. 5A, 2nd, 3rd, and 4th panels). ECRG2-depleted mitotic cells with multiple spindle poles increased with the length of time following the shutoff of ECRG2 transcription. A significant increase in multipolar mitotic spindle formation was detected from 4 to 68% in ECRG2-depleted populations (p < 0.05) by 96 h (Fig. 5B). These data indicated that the supernumerary centrosomes detected by γ-tubulin staining were functional and contribute to mitotic spindle pole formation.

ECRG2 Interacts with p53 in Vivo—To elucidate how ECRG2 monitors centrosome amplification, we used a complex proteomics approach to screen for novel ECRG2-interacting proteins. Briefly, we transiently transfected human ECRG2 expression plasmid into 293T cells, immunoprecipitated these proteins with 4E8 monoclonal anti-ECRG2 antibody, and analyzed the resulting immunocomplexes by SDS-PAGE gel and subjecting to silver staining (supplemental Fig. S3). Mass spectrometric sequencing of multiple ECRG2-copurifying bands led to the identification of several ECRG2-interacting molecules, including p53. To confirm the interaction between ECRG2 and p53, co-immunoprecipitation was performed in 293T cells. Immunoprecipitation with anti-ECRG2 antibody and subsequent immunoblotting with an anti-p53 antibody (or the reverse experiment) showed that ECRG2 and p53 form a complex in vivo (Fig. 6A). Mapping of p53 binding sites on ECRG2 revealed that region 1–20 was required for p53-p21-cyclin E/CDK2 pathway has

Loss of ECRG2 Promotes p53 Ubiquination and Degradation—To address the correlation between ECRG2 and p53, we use ECRG23′-UTR-KD, Tet-on inducible cells as described above to test the effect of ECRG2 on the stability and activity of p53. Interestingly, p53 and p21 protein levels significantly decreased when endogenous ECRG2 protein was depleted and restored when exogenous ECRG2 protein was expressed (Fig. 7A, 2nd and 3rd panels). Loss of ECRG2 leads to ~6-fold increased protein levels of cyclin E and decreased to normal when ECRG2 was re-expressed as shown in Fig. 7A, 4th panel. To confirm this, we measured the cyclin E-associated CDK2 kinase activity. As shown in Fig. 7B, the CDK2 kinase activity increased dramatically when ECRG2 was depleted and decreased when ECRG2 was restored. The p53–p21–cyclin E/CDK2 pathway has
ECRG2 Participates in the Regulation of Centrosome Amplification through the p53-p21-Cyclin E/CDK2 Transduction Pathway—p53 controls centrosome duplication via transactivation-dependent and transactivation-independent mechanisms. In its transactivation-dependent control, p21 acts as a major effector, likely guarding against untimely activation of cyclin E/CDK2 kinase, a key initiator of centrosome duplication. Our results indicate that the signal in response to ECRG2 deficiency is achieved through a p53-dependent decrease in the p21 protein. We hypothesize that ECRG2 regulates centrosome amplification through the p53-p21-cyclin E/CDK2 pathway. To test this possibility, we set up an ECRG2 Tet-On inducible system in HCT 116 p53−/−, p53+/+ cells and HCT 116 p21−/−, p21+/+ cells (Fig. 8, A and C) to determine their ability to amplify centrosome. In both p53- and p21-null cells, there was around 40% increase in the number of cells with ≥3 centrosomes after HU treatment whatever ECRG2 was expressed.
or shut off. In contrast, there was a marked decrease in the number of cells with \( \geq 3 \) centrosomes when ECRG2 protein was expressed in both \( p53^{-/-} \) and \( p21^{-/-} \) cell lines (\( p \leq 0.05 \)) and increased again when ECRG2 was silenced (Fig. 8, B and D), suggesting that ECRG2 regulates centrosome amplification through the p53-p21-cyclin E/CDK2 pathway.

**ECRG2 Is Required for p53 to Localize to Centrosomes**—Recent studies have shown that both transactivation function and centrosome binding activity are required for p53 to control centrosome duplication at a maximal level, it is necessary to identify whether ECRG2 abolishes the ability of p53 to localize to centrosomes besides affecting its transactivation function. We transfected vector control or ECRG2\(^{KD} \) cells with FLAG-p53 and then subjected the cells to the established immunohistochemical techniques. In control cells, p53 localized at centrosomes (Fig. 9A, top panels). In contrast, FLAG-p53 failed to localize to centrosomes in ECRG2-depleted cells (Fig. 9A, bottom panels). Thus, we successfully identified that ECRG2 not only affects p53 transactivation function, but also abrogates the ability to localize to centrosomes to the level undetectable by immunocytochemistry.

**Loss of ECRG2 Disrupts the Mitotic Spindle Checkpoint Function**—To examine whether the function of spindle checkpoint is activated when ECRG2 was knockdown, we exposed asynchronous vector control or ECRG2\(^{KD} \) HCT 116 cells to nocodazole (200 ng/ml) to be blocked at mitosis and then measured the mitotic index. After 12 h, most of the vector control cells have rounded up and detached from the culture dish, whereas the ECRG2\(^{KD} \) cells remained adherent as they continue to cycle (Fig. 10A). 98% of control cells arrest by 18 h, whereas only 39% of ECRG2\(^{KD} \) cells do so (Fig. 10B). To determine whether the ECRG2-depleted cells escape the mitotic block induced by nocodazole and enter a G1-like state, cell cycle markers (p27, phospho-Ser10-H3, and cyclin B) were probed in these cells. In the ECRG2\(^{KD} \) cells, the markers of G1/M arrest (phospho-Ser10-H3 and cyclin B) were suppressed, whereas the marker of G1 arrest (p27) were overexpressed (Fig. 10C), suggesting that ECRG2-depleted cells treated with nocodazole enter and

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**FIGURE 5.** Multipolar spindle formations in ECRG2\(^{KD} \) cells. A, representatives of normal spindle in control and multiple polar spindles in ECRG2\(^{KD} \) cells. Centrosomes were stained with anti-\( \gamma \)-tubulin (red), mitotic spindle with anti-\( \alpha \)-tubulin (green), and DNA with 4',6-diamidino-2-phenylindole (blue). Scale bar, 5 \( \mu \)m. B, quantification of mitotic abnormal spindles at various times after ECRG2 knockdown. Only metaphase cells were evaluated. Each bar represents mean \( \pm \) S.D. of three independent experiments with at least 200 mitotic cells counted per experiment.

**FIGURE 6.** ECRG2 physically interacts with p53. A, physical association of ECRG2 and p53 in 293T cells. Cell lysates were immunoprecipitated with normal IgG (control), or anti-p53 (left panel) or anti-ECRG2 (right panel) antibody, and immunoprecipitates were immunoblotted with anti-ECRG2 (left panel) or anti-p53 (right panel) antibody. B, the region of ECRG2 required for p53 binding was determined by expression of ECRG2-GFP deletion mutants in 293T cells followed by GFP immunoprecipitation (IP) and p53 Western blot. C and D, the region of p53 required for ECRG2 binding was determined by expression of FLAG-p53 deletion mutants in 293T cells followed by FLAG IP and Western blot for endogenous ECRG2. 293T cell lysates were incubated with beads bound with FLAG alone or with a series of p53 deletion mutants. Bound proteins were resolved and immunoblotted with anti-ECRG2 antibody. Expression of p53 mutants were confirmed by Western blot using anti-FLAG antibody. * indicates the expected positions of mutant proteins. E, structural schematic of p53 (left panel) or ECRG2 (right panel). The binding domain is shown with the black bar.
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The ECRG2 gene was a novel candidate tumor suppressor gene cloned in our laboratory (GenBank™ accession AF268198). Reverse transcriptase-PCR and Northern blot results showed that the ECRG2 gene was expressed in normal esophageus, liver, colon, and lung tissues, but was down-regulated in the adjacent and cancerous tissues, especially with low frequency in esophageal cancer (29, 30). Identifying the function of the ECRG2 gene may provide opportunities to elucidate the cancer mechanisms and its role in tumor development and progression. In this study, we have examined the localization and function of ECRG2. All our data concur to demonstrate that ECRG2 is essential for the centrosome duplication and spindle assembly checkpoint. Loss of ECRG2 contributes to centrosome amplification and thus disrupted spindle phenotype via a p53-dependent manner, and this effect can be recovered by the introduction of exogenous ECRG2 protein. Furthermore, ECRG2-depleted cells failed to arrest at mitotic in response to spindle damage. These results collectively suggest a mechanism by which loss of the ECRG2 gene may promote malignant progression.

Our investigation into the effect of ECRG2 on the frequency of aneuploidy revealed that cells depleted of ECRG2 showed significantly more aneuploidy than control cells. The assay of mitotic spreads showed that ECRG2 depletion leads to

**FIGURE 7.** ECRG2 depletion does affect the transactivation function of p53 in HCT 116 cells. A, the cell lysates prepared from ECRG23UTR-KD-Tet-on cells as described in the legend to Fig. 4 were subjected to immunoblot analysis using 4E8 anti-ECRG2 (top panel), anti-p53 (DO-1) (second panel), anti-p21 (third panel), anti-cyclin E (fourth panel), and anti-β-actin (loading control). The immunoprecipitated proteins in protein A-Sepharose beads were used for kinase activity analysis with histone H1 as a substrate. C, depletion of ECRG2 promotes p53 ubiquitination and degradation. The level of p53 ubiquitination was determined by Ni-NTA purification and p53 Western blot. Dox, doxycycline.

**FIGURE 8.** Restoration of the normal centrosome profile in p53+/+ and p21+/+ HCT 116 cells by induced expression of ECRG2, but not p53−/− and p21−/− cells. A and C, the lysates were prepared from exponen- ially ECRG23UTR-KD-Tet-on p53+/+ , p53−/− , p21+/+ , and p21−/− HCT 116 cells in the absence and presence of doxycycline (Dox) for 96 h. The lysates were subjected to immunoblot analysis using anti-ECRG2 and anti-p53 or anti-p21 antibodies. β-Actin was used as loading control. B and D, the cells described in A and C were subjected to immunostaining with anti-γ-tubulin antibody, and the number of centrosome per cell was scored under a fluorescence microscope. For each cell line, >200 cells were examined. The data represent the mean ± S.D. determined from three separate experiments.
numerical chromosome abnormalities (Fig. 2). We then further elucidated the molecular mechanisms leading to chromosome instability following ECRG2 depletion.

By immunofluorescence microscopy, ECRG2 was localized to both unduplicated and duplicated centrosome of interphase. During mitosis, however, pronounced labeling of kinetochores could be seen (Fig. 1A and supplemental Fig. S2a). The localization of ECRG2 to centrosomes and/or kinetochores indicates that ECRG2 may participate in centrosome duplication and/or mitotic spindle checkpoint. Interestingly, we found that ECRG2 depletion caused severe abnormalities during cell cycle. ECRG2-depleted cells often had more than two γ-tubulin foci (Fig. 3) and microtubules emanated from every γ-tubulin focus resulting in abnormal spindles with diffuse or multiple poles (Fig. 5). More than 60% of ECRG2-depleted mitotic cells exhibited these severely disrupted spindle phenotypes, which were found in fewer than 4% of control cells (Fig. 5B). The centrosomes play a key role in the establishment of bipolar spindles during mitosis as core components of spindle poles (31). As chromosomes are pulled toward each spindle pole, the formation of bipolar spindles is essential for accurate chromosome segregation (32). Because a diploid complement of chromosomes is segregated to more than two poles in ECRG2-depleted cells, this increase in centrosome number therefore represents a potential mechanism by which ECRG2 depletion may promote chromosome instability.

Despite the fact that ECRG2 binds the centrosomes and that the lack of ECRG2 results in centrosome amplification, it remains to be illustrated how ECRG2 regulates centrosome amplification. Through a comparative mass spectrometric analysis, we identified ECRG2 associated with p53 in vivo (Fig. 6). p53 has been known to negatively regulate centrosome duplication in a

FIGURE 9. ECRG2 affects the centrosome localization of p53 in HCT 116 cells. A, HCT 116 vector control or ECRG2KD cells were transiently transfected with FLAG-p53 and then subjected to co-immunostaining with anti-FLAG (red) and anti-γ-tubulin (green) antibodies. Cells were also stained for DNA with 4',6-diamidino-2-phenylindole. Scale bar, 5 μm. The right panels show 4-fold magnified images of centrosomes. Scale bar, 2 μm. B, the expression level of FLAG-p53 is shown using anti-FLAG antibody. C, quantitative analysis of localization of p53 at centrosomes. More than 200 cells were examined.

FIGURE 10. ECRG2KD cells escape mitotic arrest in the presence of nocodazole. A, HCT 116 vector control or ECRG2KD cells were exposed to nocodazole (200 ng/ml) for 12 h before cells were analyzed. Representatives showed the phase-contrast pictures. Bar, 1 μm. B, accumulation of the G2/M population in HCT 116-vec control and ECRG2KD cells in response to nocodazole treatment. C, cell extracts, prepared from the samples described in A, were probed by Western blot with anti-p27 (top panel), anti-phospho-H3 (second panel), and anti-cyclin B (third panel) antibodies. β-Actin (bottom panel) was used as loading control. D, cyclin B-associated Cdc2 kinase activity. HCT 116 vector control (top panel) or ECRG2KD cells (lower panel) were synchronized with thymidine-double block, and then the cells were released into nocodazole for various times. The resultant cell lysates were then subjected to Cdc2 kinase assay using histone H1 as substrate.
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The function of ECRG2 affecting the spindle assembly checkpoint is dependent on BubR1. (A) up-regulation of BubR1 by expression of ECRG2 in ECRG23-UTR-KD-Tet-on HCT 116 cells. The cell lysates prepared from ECRG23-UTR-KD-Tet-on cells as described in the legend to Fig. 4 were subjected to immunoblot analysis using anti-BubR1, anti-MAD1, anti-MAD2, anti-Bub1, and anti-Bub3 antibodies. β-Actin is used as loading control. BubR1 is dependent on ECRG2 for its kinetochore localization. HCT 116 vector control or ECRG2KD cells were analyzed by IIF to determine the localization of spindle checkpoint proteins using anti-BubR1, anti-MAD1, anti-MAD2, anti-Bub1, and anti-Bub3 antibodies. Representative showing kinetochores (red), spindle checkpoint protein (green), and DNA (blue) are shown. Scale bar, 5 μm. C, p53 physically associates with both BubR1 and ECRG2. 293T cells were transiently transfected with p53 plasmid. Cell lysates were immunoprecipitated with normal IgG (control) or anti-p53 antibody, and immunoprecipitates were immunoblotted with anti-BubR1 (top panel) or anti-ECRG2 (bottom panel) antibody.

manner dependent or independent of its transactivation function (33, 34). p53 binds to both duplicated and unduplicated centrosomes (35). p21Waf1/Cip1, a potent inhibitor of CDK and a major transactivation target of p53, is expressed constitutively at a basal level throughout the cell cycle in a p53-dependent manner, which restrains untimely activation of CDK2/cyclin E, a key initiator of DNA replication, triggers centrosome duplication, and thus prevents untimely initiation of centrosome duplication (36–38). Our data showed that when ECRG2 was lost, p53 becomes unstable, leading to down-regulation of p21, which in turn lose the ability to suppress the CDK2 activity, and thus initiating centrosome amplification, leading to 70% of ECRG2-depleted cells with multipolar spindles. On the other hand, depletion of ECRG2 disrupted the localization of p53 to centrosomes (Fig. 9), and thus failed to suppress centrosome amplification. These results indicate that the signal in response to being ECRG2 deficient is achieved through a p53-dependent manner. But further studies will be required to unravel the mechanism of how ECRG2 regulated p53 stability and activity.

The SAC is another important surveillance mechanism for maintaining chromosome stability (39). Several lines of evidence in this study indicate that ECRG2 contributes to SAC. First, ECRG2, like other checkpoint components, localize to kinetochores during early mitosis (Fig. 1A and supplemental Fig. S2a). They are thus thought to cooperate in a signaling pathway that prevents anaphase onset until all kinetochores have undergone correct bipolar attachment. Second, ECRG2-depleted cells gradually escape the mitotic block induced by spindle inhibitors, passed through mitosis and entered a G1-like state (Fig. 10). Damage to mitotic spindle fibers can activate mitotic checkpoint genes, whose products arrest mitosis and repair the spindles (40). Our data show that ECRG2 depletion leads to abnormal spindle apparatus but failed to arrest in mitosis, indicating that disruption of ECRG2 leads to being mitotic checkpoint-defective, which initiate premature anaphase. Despite its severely disrupted spindle phenotype, most ECRG2-depleted cells seem to exit mitosis.

Interestingly, a significant reduction of BUBR1 was seen after depletion of ECRG2, although MAD1, MAD2, BUB1, and BUB3 remained at the constant level (Fig. 11A). Immunofluorescence further confirmed that the kinetochore association of BUBR1 was abolished in the ECRG2-depleted cells, whereas MAD1, MAD2, BUB1, and BUB3 still localized to kinetochores efficiently (Fig. 11B). Interestingly, 39% of ECRG2-depleted cells, in which the kinetochore association of BUBR1 was abolished, showed lagging chromosome (Fig. 11B, 2nd panels, yellow arrow). Several studies have shown that disruption of BUBR1 activity results in a loss of checkpoint control, chromosomal instability (41). Thus, it seems like ECRG2 contributes to the spindle assembly checkpoint dependent on BUBR1. Our co-immunoprecipitation data showed that p53 associated with both ECRG2 and BUBR1. But we have not been able to map the binding region between ECRG2 and BUBR1, suggesting that in vivo interaction between ECRG2 and BUBR1 require additional components or modification. Oikawa et al. (42) reports that p53 and BUBR1 associated with each other and in the absence of p53, the BUBR1 expression level is minimal (43). It is possible that ECRG2 depletion caused reduction of p53, which in turn interfered with BUBR1, which results in failure to arrest the cell cycle at G2/M. But other molecular explanations are possible. In future studies, it will be interesting to explore the mechanism how ECRG2 regulates BubR1 function.

Taken together, we propose a model in which ECRG2 contributes to chromosome stability (Fig. 12). The mechanism of chromosome instability in ECRG2-depleted cells may be due to abnormal spindle structure or and be checkpoint-defective. The centrosome duplication is under two major controls. 1) Establishment of the correct timing of initiation of centrosome duplication, which normally occurs at late G1. This in turn ensures the coordinated initiation of centrosome and DNA duplication and 2) suppression of re-duplication of centrosomes within a single cell cycle (44). ECRG2 is directly involved in both regulations by interacting with p53. Loss of ECRG2, through a p53-dependent manner, not only leads to promiscu-
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![Diagram](image)

**FIGURE 12.** A, involvement of ECRG2 in the major regulatory mechanisms imposed on centrosome duplication and spindle assembly checkpoint. B, a working model regarding functions of ECRG2 in centrosome duplication and spindle assembly checkpoint.

The understanding of the mechanism of this model, in particular, chromosome segregation errors and aneuploidy. A better event will ultimately disrupt normal progression of mitosis leading to or defects in the spindle assembly checkpoint, which can disasters in the genome.

Therefore, there are two points to consider: p53-null HCT 116 cells, but also disrupts the ability of p53 to localize to centrosomes. On the other hand, ECRG2 depletion abolishes the function of BUBR1, weakening the checkpoint response to mitotic defects associated with amplified centrosome and multipolar spindles. Therefore, there are two disasters in ECRG2-depleted cells: centrosome amplification and/or defects in the spindle assembly checkpoint, which can eventually disrupt normal progression of mitosis leading to chromosome segregation errors and aneuploidy. A better understanding of the mechanism of this model, in particular, how ECRG2 affect the functions of p53 and BUBR1, will eventually lead to new strategies to combat cancer.

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