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RAS promotes tumorigenesis through genomic instability induced by imbalanced expression of Aurora-A and BRCA2 in midbody during cytokinesis

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The oncogene *RAS* is known to induce genomic instability, leading to cancer development; the underlying mechanism, however, remains poorly understood. To better understand how RAS functions, we measured the activity of the functionally related genes Aurora-A and BRCA2 in ovarian cancer cell lines and tumor samples containing RAS mutations. We found that Aurora-A and BRCA2 inversely controlled RAS-associated genomic instability and ovarian tumorigenesis through regulation of cytokinesis and polyploidization. Overexpression of mutated RAS ablated BRCA2 expression but induced Aurora-A accumulation at the midbody, leading to abnormal cytokinesis and ultimately chromosomal instability via polyploidy in cancer cells. RAS regulates the expression of Aurora-A and BRCA2 through dysregulated protein expression of farnesyl protein transferase β and insulin-like growth factor binding protein 3. Our results suggest that the imbalance in expression of Aurora-A and BRCA2 regulates RAS-induced genomic instability and tumorigenesis.

Key words: RAS, Aurora-A, BRCA2, polyploid cancer cells, cytokinesis, genomic instability
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RAS signaling induces genomic instability,[1] which provokes cancer development in many organs; however, the underlying mechanism remains elusive. Activation of RAS largely depends on its active form without CAAX at the C-terminus (C, Cys; A, usually aliphatic amino acid; X, another amino acid) that is processed by farnesyl transferase (FT) during posttranslational modification of RAS proteins.[2] Thus, various inhibitors of farnesyl protein transferase activity, designed to prevent the farnesylation of RAS, have been developed to treat RAS-associated cancers.[3] The insulin-like growth factor binding protein 3 (IGFBP-3) was shown to block RAS cleavage and thus to inhibit farnesyl protein transferase in lung carcinoma and head and neck squamous cell carcinoma.[4] These reports suggest that FT and IGFBP-3 may be involved in regulating RAS-induced genomic instability and tumor development.

Genomic instability is largely classified into two types: microsatellite instability, which is associated with a mutator phenotype, and chromosome instability, which is associated with gross chromosomal abnormalities.[5] The centrosome is believed to play an important role in maintaining chromosome stability by aiding in the formation of bipolar spindles during cell division,[6] thereby ensuring equal segregation of duplicated chromosomes into two daughter cells, whereas multipolar mitotic spindles are usually resulted from various centrosome abnormalities such as amplification in cancer

What's new?

The mechanism underlying *RAS*-induced genomic instability, an event associated with cancer development, has remained poorly understood. Here, in an attempt to better understand the phenomenon, *RAS* transformation was found to be associated with ablated expression of the tumor suppressor *BRCA-2* and accumulation of Aurora-A during cytokinesis. The tumorigenicity of cancer cells with RAS mutation is determined by the expression ratio of BRCA2 and Aurora-A. The regulation of Aurora-A and BRCA-2 was further discovered to be mediated through insulin-like growth factor binding protein 3 and farnesyl protein transferase beta in the presence of mutated RAS. These findings suggest that BRCA2 and Aurora-A signaling loop plays a critical role and may provide novel targets for therapeutic intervention in RAS-mutated cancers.

cells, which leads to unequal distribution of chromosomes and results in aneuploidy or polyploidy of daughter cells.[7] The serine/threonine kinase Aurora-A (*AURKA*) plays a critical role in maintenance of genetic stability through regulation of centrosome separation, bipolar spindle assembly and chromosome segregation;[8,9] at the same time, however, amplification of Aurora-A increases the number of centrosomes and multipolar spindles, which have been observed in numerous human cancers.[10,11] Recent studies have shown that Aurora-A is required for RAS-mediated oncogenic transformation of oral cancer[12] and bladder cancer.[13] Thus, Aurora-A may be associated with genomic instability in RAS-induced tumorigenesis.

The breast cancer susceptibility gene 2 (*BRCA2)* is a tumor suppressor gene that is known to be involved in maintaining genomic stability in different cancers.[14] Although *BRCA2* is rarely mutated in sporadic cancers such as ovarian and breast cancers, the transcription or expression of BRCA2 is repressed in these tumor tissues.[15] Loss of BRCA2 either by mutation or transcriptional and post-transcriptional aberrations is associated with cancer genomic instability.[16] Recently, a study revealed that a heterozygous germline mutation of *BRCA2* can promote pancreatic ductal adenocarcinomas driven by Kras (G12D) mutation,[17] whereas another report showed that BRCA2 in HCT116 (a colon cancer cell line) can be suppressed by activated KRAS in 3D culture.[18] In addition, studies have shown that BRCA2 mutation is associated with Aurora-A amplification in breast cancer,[19] and that BRCA2 may suppress polyploidy by stabilizing Aurora-A.[20] We have shown recently that Aurora-A can suppress BRCA2 expression in ovarian cancer.[21] The above evidence suggests that Aurora-A and BRCA2 likely function to synergistically regulate RAS-induced genomic instability and tumorigenesis, although the underlying mechanism remains unclear.

To improve our understanding how RAS regulates the genomic instability, we designed a study to investigate the function of Aurora-A and BRCA2 in relation to RAS activation. Because the RAS/RAF mutation accounts for 30–40% of low-grade serous and borderline ovarian cancer cases,[22] we mainly conducted the study in ovarian cancer cell lines and human ovarian tumor tissues with RAS mutations. Our results provide insight into how RAS/RAF mutations induce genomic instability and tumorigenesis.

Material and Methods

Plasmids and siRNAs

We used pBabe/Aurora-A/puromycin[23] and pBabe/U6/Aurora-A shRNA (targeting 5′-GUCUUGUGUCCUUCAAA UU-3′ of Aurora-A mRNA) (puromycin or neomycin)[21] to deliver Aurora-A into immortalized ovarian epithelial cell lines T29 and T80 and Aurora-A shRNA into RAS-transformed cell lines T29H, T80H and ovarian cancer cell line HEY. A plasmid (PCINBRCA2) containing a full-length BRCA2 cDNA was used to deliver BRCA2 into RAS-transformed cells and Capan-1 cells (a pancreatic cancer cell line) using a previously described method.[24] Clones were selected after confirmation of BRCA2 expression by Western blotting. The retroviral expression plasmid IGFBP-3 (pBabe/IGFBP-3/puromycin) was generated with a pair of primers (sense: 5′-AT**GGATCC**atgcagcgggcgcgacccacgctc-3′, bold cases are *Bam*HI site, and antisense: 5′- CA*GAATTC*ctacttgctctgcatgctgtagc-3′, italic cases are *Eco*RI site) using a template of an adenoviral expression vector containing IGFBP-3 cDNA (a kind gift from Dr. Ho-Young Lee). pBabe/U6/IGFBP-3 shRNA/puromycin was generated to target IGFBP-3 mRNA at 403–422nt (5′-ggaaatgctagtgagtcgga-3′) using the protocols described in our previous publication.[25] The control vectors were empty plasmids (pBabe/puromycin or PCIN) or constructed by directly inserting GFP shRNA into pBabe/U6/puromycin or neomycin vectors.[26] Retrovirus production and target cell infection were performed with our well-established method.[25] FTβ siRNA (#sc-35417) and control siRNA (#sc-37007) were purchased from Santa Cruz Biotech (Santa Cruz, CA). FTI-276 (#F9553) was purchased from Sigma Aldrich (St. Louis, MO).

Cell culture and tumor formation

T29, T29H, T80 and T80H cells have been described previously.[27] Ovarian cancer cell lines HOC-7, SKOV3 and HEY and pancreatic cancer cell line Capan-1 were cultured with EMEM or DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. T29 cells transformed by $KRAS^{V12}$ (T29K) was described previously.[27] To generate tumor growth *in vivo*, we subcutaneously injected 5×10^6 T29H/BRCA2, T80H/BRCA2, T29/Aurora-A, T80/Aurora-A, T29H/Aurora-A shRNA (Aurora-Ai), T80H/Aurora-Ai cells or control cells expressing empty vectors or

GFP shRNA (GFPi) into 4- to 6-week-old BALB/c nu/nu mice (U.S. National Cancer Institute's Frederick Cancer Research Facility) following protocols approved by the institutional committee of MD Anderson Cancer Center for animal experiments. For T29H and T80H cells transfected with BRCA2, one of three clones from each cell line with high BRCA2 expression was used to conduct tumor formation assays. Each cell line was injected into two sites in eight mice, for a total of 16 injections. Tumor burden was assessed and recorded using methods described previously.[28]

Western blotting

For all Western blots, we analyzed samples with a total of 40-μg proteins from whole-cell lysates using the protocol described in our previous publication.[21] The primary antibody used to detect Aurora-A (cat. #GTX13824) was obtained from GeneTex (Irvine, CA), while the antibody used to detect BRCA2 (cat. #MAB2476) was from R&D Systems (Minneapolis, MN). Antibodies against RAS either targeting N-terminus (#sc-166691) or C-terminus (#sc-521, KRAS; #sc-520, HRAS) and antibodies against IGFBP-3 (#sc-9028), FTα (#sc-487) and FTβ (# sc-137) were purchased from Santa Cruz Biotech (Santa Cruz, CA).β-Actin (cat. #A2228, Sigma Aldrich, St. Louis, MO) was used as a loading control. T29/Vector and T29/Aurora-A cells were treated with proteasome inhibitor MG-132 (Sigma) at the concentration of 10 μM and analyzed for the expression of Aurora-A and BRCA2 by Western blotting. The intensity of protein bands was quantified with ImageJ software downloaded from NIH website (http://imagej.nih.gov/ij/).

Immunofluorescence

Immunofluorescence staining was performed according to a published protocol.[21] Primary antibodies against Aurora-A and BRCA2 were obtained from GeneTex and R&D Systems, respectively. DNA dye To-Pro-3 was obtained from Molecular Probes (Carlsbad, CA). In brief, cells were cultured in chamber slides (Nalge Nunc International, Rochester, NY) for 24 hr, fed with fresh medium to increase the number of mitotic cells for 8–16 hr and then fixed (with PBS-buffered paraformaldehyde solution: 3% paraformaldehyde, PBS, pH 7.4, 2% sucrose) and permeabilized (with a buffer containing 0.5% Triton X-100, 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose). Slides were blocked by a 2-hr incubation with 20% FBS and 2% goat serum in PBS, and then the slides were incubated with primary antibody at 4°C overnight. Afterward, the cells were incubated with fluorescein isothiocyanate (FITC)–conjugated secondary antibody against mouse IgG or Texas red–conjugated antibody against rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) for 30 min. Stained cells were examined and photographed with an Olympus FV500 confocal fluorescence microscope.

Cell cycle and cytogenetic analysis

Cells were applied for cell cycle analysis by flow cytometry according to our previously published method.[21] T29/vector, T29/Aurora-A, T80/vector, T80/Aurora-A, T29H/GFPi, T29H/Aurora-Ai, T80H/vector and T80H/BRCA2 cells were cultured for 24 hr and collected for chromosome preparation using standard procedures.[29] Briefly, cells were exposed to Colcemid (0.04 μg/mL) for 1 hr, subjected to hypotonic treatment (0.075 M KCl for 20–25 min at room temperature) and fixed in a mixture of methanol and acetic acid. Slides were stained with Giemsa stain and examined for structural and numerical abnormalities in the chromosomes. A minimum of 30 metaphase spreads were analyzed for each cell line, and representative spreads were captured using a Genetiscan imaging system. The proportions were compared using chi-squared analysis of Fisher's exact test. The assay was repeatedly performed by Molecular Cytogenetics Core Facility personnel in the Department of Genetics at The University of Texas MD Anderson Cancer Center.

Immunostaining of Aurora-A and BRCA2

Ovarian tumor tissues from 22 patients diagnosed with low-grade serous carcinoma or borderline tumor were analyzed by immunohistochemical staining for expression of Aurora-A and BRCA2. KRAS/BRAF mutations with either G12V or V600E were identified from tissue genomic DNA of all cases by PCR amplification with specific primers targeting the coding regions of RAS-G12V and RAF-V600E, followed by sequencing of the DNA fragment. Ten cases were confirmed with RAS/RAF mutations, while the remaining cases lacked KRAS/BRAF mutations. The use of tissue blocks and chart reviews were approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. Slides were treated and stained using the method published before.[21] The primary antibody against Aurora-A (GTX13824, monoclonal antibody, Genetax) or BRCA2 (MAB2476, monoclonal antibody, R&D Systems) was applied at a dilution of 1:200 or 1:100 at 4°C in a humidified chamber.

Evaluation of staining intensity and expression percentage for BRCA2 and Aurora-A was scored. using the following criteria: tissues with <5% of cells positive for BRCA2 or Aurora-A were given a score of 0, those with 5–20% positive cells were scored as 1, those with 20–40% positive cells were scored as 2, those with 50–70% positive cells were scored as 3 and those with 70–100% were scored as 4. The expression correlation of BRCA2 and Aurora-A was analyzed by Pearson's correlation using SPSS16.0 software.

Cell treatment with FTβ siRNA or FTI-276

To transfect HEY and T29K cells with FTβ siRNA, 5×10^5 cells per well in 6-well plates were used for FTβ siRNA and control siRNA transfection using the manufacturer's protocol from Santa Cruz Biotech Inc. (Santa Cruz, CA). The transfection medium was replaced with fresh growth medium 12 hr later, and the cells were kept in culture for additional 24, 48 and 72 hr and harvested to detect FTβ, Aurora-A and BRCA2 expression. A similar cell number was used for

treatment either with FTI-276 for 24 hr, and the cells were analyzed for expression of the above-listed proteins.

Results

RAS-induced transformation enhances Aurora-A expression but represses BRCA2 expression

To better understand how RAS promotes genomic instability, we measured the expression of BRCA2 and Aurora-A in RAS-transformed human ovarian surface epithelial (OSE) cell lines previously developed in our laboratory.[27] While the expression of BRCA2 was markedly lower in RAS-transformed cells than in control cells (Fig. 1*a*), the expression of Aurora-A was dramatically increased in these cells, suggesting that RAS suppresses the expression of BRCA2 but increases the expression of Aurora-A. Next, we determined whether BRCA2 can regulate the expression of Aurora-A or RAS by transfecting RAS-transformed ovarian epithelial cell lines (T29H and T80H) with a vector expressing BRCA2.[24] Selected stable clones with ectopic expression of BRCA2 showed a marked decrease of Aurora-A and RAS (Fig. 1*b*), indicating that BRCA2 suppresses Aurora-A and RAS expression. To determine whether Aurora-A can suppress the expression of BRCA2, we delivered Aurora-A cDNA into immortalized nontumorigenic T29 and T80 cells (Aurora-A; Figs. 1*c* and 1*d*) or silenced Aurora-A expression in T29H and T80H cells with Aurora-A-specific short hairpin RNA (shRNA) (Aurora-Ai; Figs. 1*c* and 1*d*). Ectopic expression of Aurora-A suppressed BRCA2 expression, but did not stimulate RAS expression in T29 and T80 cells compared with in vector-transfected control cells, and knockdown of Aurora-A restored the BRCA2 level and reduced RAS expression in T29H and T80H cells, suggesting that Aurora-A also negatively regulates the expression of BRCA2. We infer from these results that RAS-driven malignancy is modulated by Aurora-A and BRCA2.

Aurora-A and BRCA2 regulates cell cycle progression and tumor growth of RAS-transformed cells

As Aurora-A and BRCA2 participate in cell cycle regulation which controls ovarian tumorigenesis, we detected the cell cycle distribution by flow cytometry (Fig. 1*e*). Introduction of RAS or Aurora-A in immortalized OSE cells promoted cell cycle progression by increasing cell population in S phase and downregulating cell population in G_0/G_1 phase as compared to these in control cells. In contrast, overexpression of BRCA2 or knockdown of Aurora-A in RAS-transformed cells promoted cell arrest at G_0/G_1 phase and reduced cells in S phase as compared to these in control cells.

To test whether Aurora-A and BRCA2 affect ovarian tumor growth, we injected RAS-transformed T29H or T80H cells overexpressing BRCA2 into nude mice and compared tumor growth to that in mice receiving vector control cells. No tumors were observed in any of the mice injected with BRCA2-transfected T29H and T80H cells, while all the mice injected with vector control cells experienced rapid tumor growth within 4–7 weeks (Figs. 1*f* and 1*g*), indicating that the expression of BRCA2 completely blocked tumor formation of the RAS-transformed cells. In addition, when ectopic Aurora-A expression was induced in immortalized nontumorigenic T29 and T80 cells, subcutaneous tumor growth resulted (Figs. 1*h* and 1*i*), whereas shRNA-induced knockdown of Aurora-A in RAS-transformed cells reduced or delayed tumor growth, compared with tumor growth in control cells expressing GFPi (Figs. 1*j* and 1*k*). Taken together, the above data demonstrated that Aurora-A and BRCA2 play opposite roles in RAS-associated tumor formation *in vivo*.

Unbalanced expression of Aurora-A and BRCA2 in cancer cells and tissues with RAS/RAF mutations

As the above results were derived from RAS-transformed OSE cells, we set out to confirm the results in a panel of cells including normal OSE cells, ovarian cancer cells and pancreatic cancer cells harboring KRAS mutations. We detected higher expression of BRCA2 and lower expression of Aurora-A in OSE 151 cells (Fig. 2*a*), a normal OSE cell line described in our previous report,[25] but lower BRCA2 and higher Aurora-A in the ovarian cancer cell lines HOC-7 and HEY with confirmed mutations in *KRAS* (Supporting Information Fig. 1) and in the pancreatic cancer cell line CAPAN-1, which has a reported KRAS mutation and a truncated BRCA2 mutation (Fig. 2*a*). Furthermore, knockdown of Aurora-A by shRNA in HEY cells and introduction of BRCA2 in CAPAN-1 cells resulted in decreased Aurora-A expression and increased BRCA2 expression (Fig. 2*a*).

The above results also suggested the possibility that Aurora-A and BRCA2 are negatively regulated in ovarian cancer, particularly in low-grade serous ovarian carcinomas and ovarian borderline tumors with KRAS/BRAF mutations. Thus, we selected tumor tissue samples from 22 cases diagnosed with low-grade serous ovarian carcinoma and borderline tumor with or without identified KRAS/BRAF mutations and detected Aurora-A and BRCA2 expression by immunostaining. We measured high expression of Aurora-A and low expression of BRCA2 in six of ten (60%) samples with *RAS/RAF* mutations ($p = 0.018$, two-tailed Pearson's correlation), but high expression of BRCA2 and low expression of Aurora-A in eight of 12 (66.7%) samples without *KRAS/BRAF* mutations ($p = 0.023$, two-tailed Pearson's correlation) (Table 1). No statistical differences in Aurora-A and BRCA2 expression were found in samples with low-grade serous carcinoma and in those with borderline tumor or between samples with KRAS mutation and those with BRAF mutation. Representative images are shown in Figure 2*b*.

Farnesyl protein transferase β and insulin-like growth factor binding protein 3 mediate the regulation of Aurora-A and BRCA2 in RAS-associated cancer cells

As IGFBP-3 was reported to inhibit farnesyl protein transferase, which thereby blocks RAS cleavage.[4] We measured the expression of IGFBP-3 and farnesyl protein transferase in a

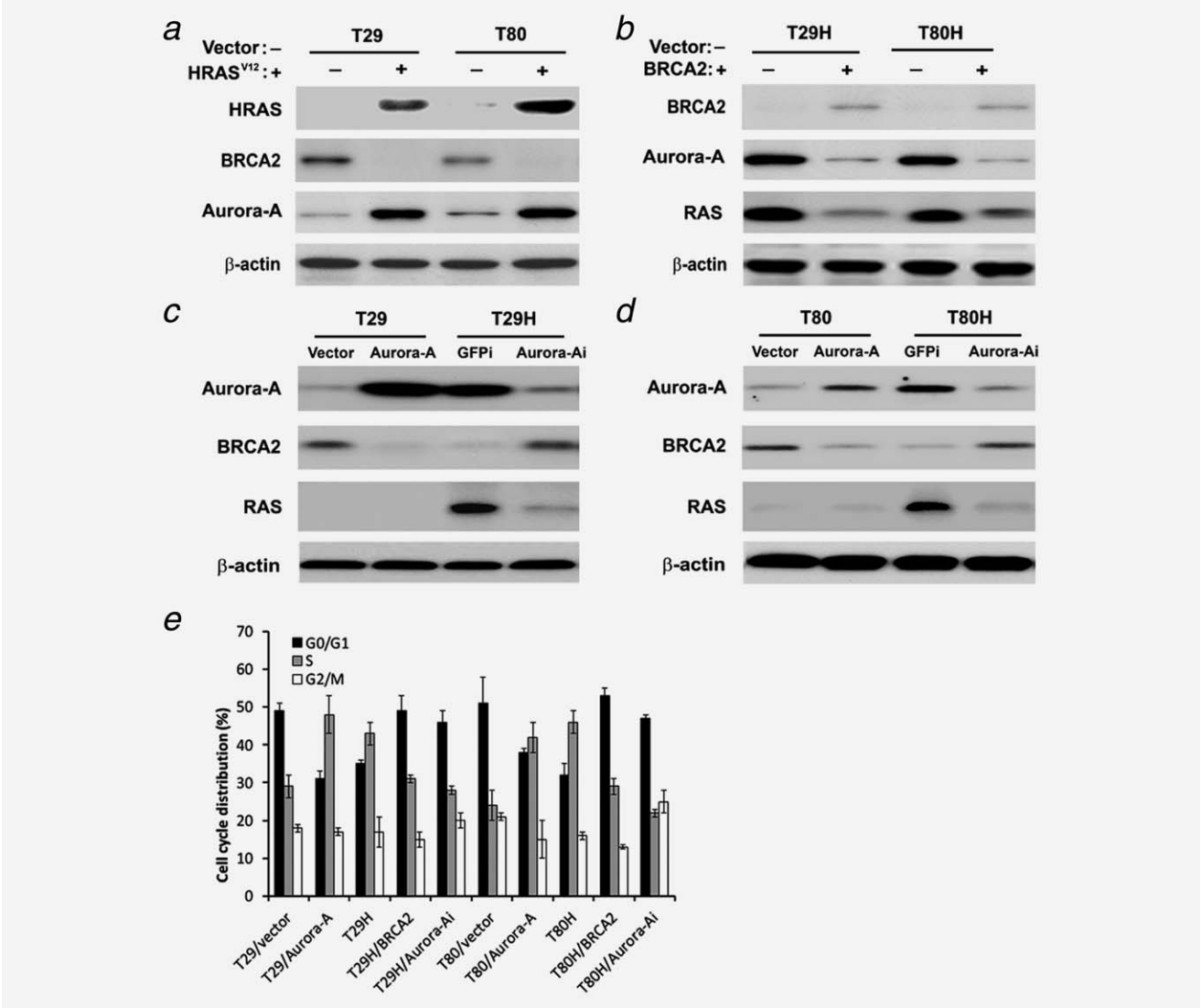

Figure 1. Protein expression status detected by immunoblotting and tumor growth curve in nude mice. (*a*) RAS transformation (+) induced concurrent suppression of BRCA2 and amplification of Aurora-A in immortalized cells (T29 and T80). HRAS was detected by antibody against C-terminus (#sc-520). (*b*) Ectopic expression of BRCA2 (+) inhibited Aurora-A and RAS expression in RAS-transformed cells. (*c* and *d*) Overexpression of Aurora-A decreased BRCA2 expression in immortalized cells (T29 and T80), and knockdown of Aurora-A by specific shRNA (Aurora-Ai) increased BRCA2 expression and decreased RAS level in RAS-transformed cells (T29H and T80H). Vector- or GFPi-treated cells were used as relative controls. (*e*) Cell cycle distribution detected by flow cytometry. (*f* and *g*) The mean tumor sizes in mice receiving vector control cells (T29H/vector and T80H/vector) or BRCA2-transfected cells transformed with HRAS^{V12} (T29H/BRCA2 or T80H/BRCA2) are shown. The data suggest that tumor formation was completely blocked by the introduction of wild-type BRCA2 in RAS-transformed cells. (*h* and *i*) Tumor formation in mice was induced by the introduction of Aurora-A in immortalized cells (T29/Aurora-A and T80/Aurora-A) compared to vector controls (T29/vector and T80/vector). (*j* and *k*) Knockdown of Aurora-A in RAS-transformed cells (T29H and T80H) cells markedly hampered or delayed tumor growth. Data were collected in three independent experiments. Error bars = 95% confidence intervals.

panel of ovarian cell lines. We found that the expression of IGFBP-3 was decreased in T29H and T29/Aurora-A cells compared with vector control cells, but was increased by ectopic introduction of BRCA2 in T29H and Capan-1 cells, and by disruption of Aurora-A in HEY cells compared with in their corresponding control cells (Fig. 3*a*). In contrast, the expression of farnesyl protein transferase β(FTβ) (but not FTα, data not shown) was increased in RAS- and Aurora-A-transformed cells (T29H and T29/Aurora-A), but decreased in BRCA2-transfected cells (T29H/BRCA2, Capan-1/BRCA2) and Aurora-A shRNA-treated cells (HEY/Aurora-Ai) compared with in their controls. These changes led to corresponding increases or decreases in RAS farnesylation (Fig. 3*a*). These results suggest that transformation of ovarian epithelial cells by RAS or Aurora-A can inhibit IGFBP-3, leading to an increased expression of FTβ, which may in turn promote RAS farnesylation and ovarian tumorigenesis. They also suggest that the restoration of BRCA2 expression

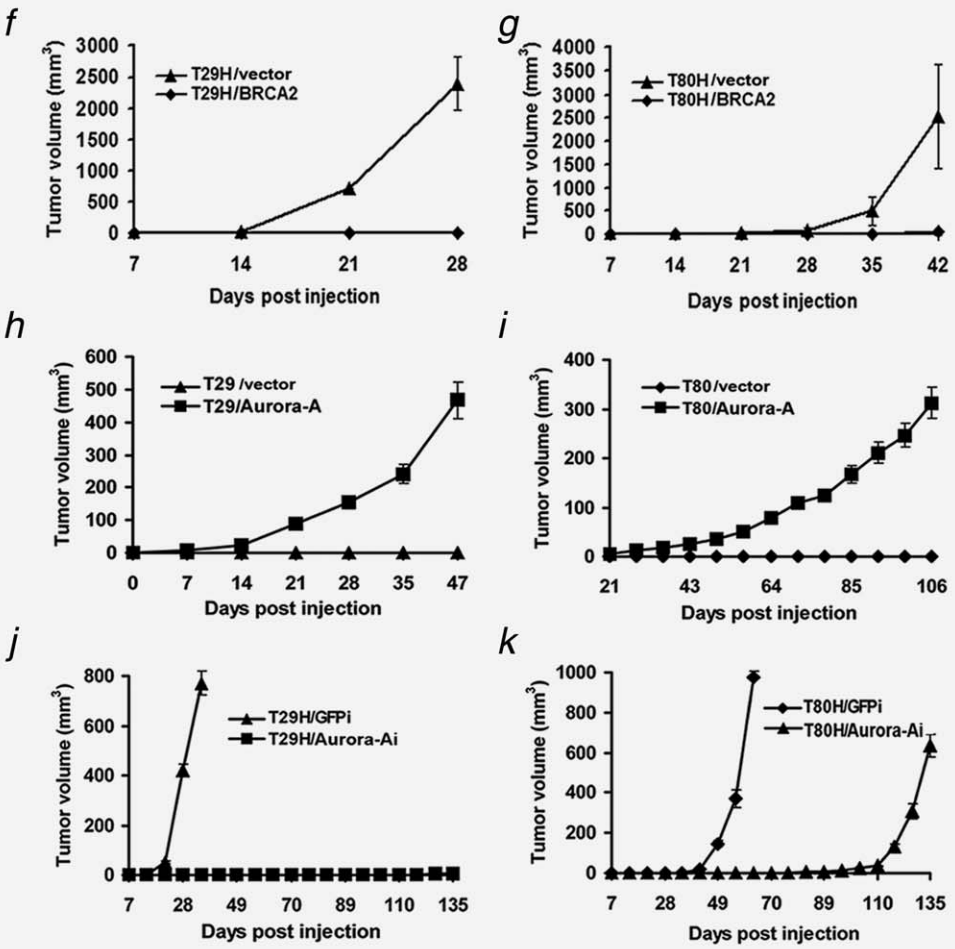

Figure 1. Continued

by silencing Aurora-A or introducing BRCA2 induces IGFBP-3 overexpression, which inhibits the activity of FTβ, leading to reduced farnesylation of RAS, which may in turn decrease ovarian tumor formation.

To confirm that IGFBP-3 is able to suppress FTβ expression, we transfected T29 and SKOV3 (an ovarian cancer cell line) cells with either IGFBP-3 cDNA or IGFBP-3 shRNA. As shown in Figure 3*b*, overexpression of IGFBP-3 reduced FTβ in T29 cells, whereas silencing of IGFBP-3 increased FTβ in T29 and SKOV3 cells compared with in their control cells. Quantification data of FTβ and IGFBP-3 expression with ImageJ software was shown in Supporting Information Figure 2. These results suggest that IGFBP-3 is involved in regulation of Aurora-A and BRCA2 through FTβ in terms of farnesylation of RAS. To strengthen evidence for this notion, we treated HEY and T29K ($KRAS^{V12}$-transformed T29 cells) with FTβ-specific siRNA or with FTI-276, which specifically inhibits farnesyl protein transferases activity.[30] As shown in Figure 3*c*, treatment with FTβ-specific siRNA reduced the farnesylation of KRAS and Aurora-A expression, which is consistent with a recent report,[31] but increased BRCA2 expression compared with control siRNA-treated cells at the same time point. Moreover, treatment of HEY and T29K cells with FTI-276 yielded the same results as those from treatment with FTβ siRNA (Fig. 3*d*). These data suggest that FTβ not only regulates RAS by farnesylation but also controls the expression of Aurora-A and BRCA2 through a mechanism that may be associated with IGFBP-3.

Aurora-A and BRCA2 regulate chromosomal instability through dysregulated cytokinesis

Amplification of Aurora-A and inactivation of BRCA2 are known to be closely associated with chromosomal instability. By analyzing chromosomal aberrations, as expected, the proportion of polyploid cells was markedly higher in RAS- and Aurora-A-transformed cells than in control cells, and the knockdown of Aurora-A or introduction of BRCA2 in RAS-transformed cells resulted in less polyploidy in the experimental cell lines than in the control lines (Table 2, Fig. 3*e*). In addition, the overall chromosome aberration was increased in cells overexpressing RAS or Aurora-A compared with in control cells; however, the ectopic expression of BRCA2 or silencing of Aurora-A in the transformed cells decreased the overall chromosome aberration. These results demonstrate

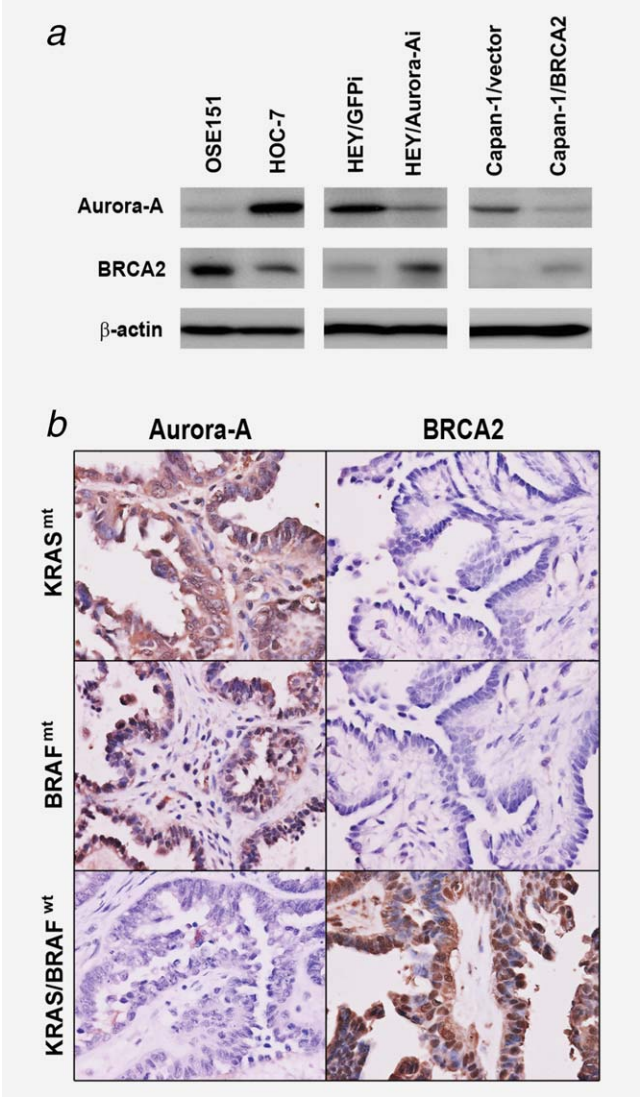

Figure 2. Inverse expression of Aurora-A and BRCA2 in normal and cancer cells and ovarian tumor tissues with KRAS/BRAF mutations. (*a*) Aurora-A and BRCA2 expression in normal ovarian surface epithelial cells, ovarian cancer cells and pancreatic cancer cells treated with Aurora-A shRNA or BRCA2 cDNA. (*b*) Representative images from cancer tissues with or without KRAS/BRAF mutations from patients diagnosed with low-grade serous ovarian carcinoma. High Aurora-A expression was correlated with negative expression of BRCA2 (×400) (upper and middle panels). High expression of BRCA2 was correlated with negative detection of Aurora-A in another case (×400) (bottom panel).

Table 1. Immunohistochemical analysis of low-grade serous (LGS) and serous borderline tumor (SBT) with or without RAS/RAF mutations

Case no.	Diagnosis	*KRAS*	*BRAF*	Aurora-A expression (%)	BRCA2 expression (%)
1	LGS	wt	wt	10	90[1]
2	LGS	wt	wt	30	55[1]
3	LGS	wt	wt	0	20
4	LGS	wt	wt	20	70[1]
5	LGS	wt	wt	40	50
6	LGS	wt	wt	10	80[1]
7	LGS	wt	wt	0	90[1]
8	LGS	**Mt**	wt	80[1]	20
9	LGS	**Mt**	wt	70[1]	10
10	LGS	wt	**Mt**	90[1]	5
11	LGS	wt	**Mt**	40	50
12	SBT	wt	wt	0	65[1]
13	SBT	wt	wt	15	75[1]
14	SBT	wt	wt	5	85[1]
15	SBT	wt	wt	30	40
16	SBT	wt	wt	40	20
17	SBT	**Mt**	wt	75[1]	10
18	SBT	**Mt**	wt	80[1]	30
19	SBT	**Mt**	wt	50	40
20	SBT	wt	**Mt**	55	60
21	SBT	wt	**Mt**	30	50
22	SBT	wt	**Mt**	65[1]	10

[1]Cases with statistical significance ($p < 0.05$).

that dysregulation of Aurora-A and BRCA2 led to chromosomal instability in RAS-transformed cells.

Cytokinesis occurs during the last step of mitosis at which point a cell divides into two daughter cells. Abnormal cytokinesis usually results in cell multinuclearity and eventually induces chromosomal instability. Since Aurora-A and BRCA2 are involved in regulating cytokinesis,[32,33] we examined the expression of both Aurora-A and BRCA2 in the midbody of late mitotic T29, T29H and T29/Aurora-A cells. In immortalized T29/vector cells, BRCA2 and Aurora-A colocalized at the midbody during the late stage of mitosis (Fig. 3*f*). The transformation of T29 cells by RAS or Aurora-A diminished the localization of BRCA2 and increased the accumulation of Aurora-A in the midbody as compared with T29/vector cells. Counts of cells with two or more nuclei showed that the transformation by RAS or Aurora-A induced at least four times as many as were induced in their control cells (Fig. 3*g*). In normal ovarian epithelial cells (OSE151), Aurora-A and BRCA2 were colocalized at the midbody, while BRCA2 was undetectable in the midbody of mitotic HOC-7, HEY and Capan-1 cells with KRAS mutations (Figs. 4*a*, 4*b* and 4*c*). However, knockdown of Aurora-A or introduction of BRCA2 in HEY or Capan-1 cells restored BRCA2 accumulation in the midbody (Figs. 4*b* and 4*c*). Consistent with the results from T29, T29H and T29/Aurora-A cells, there were fewer OSE151 cells with multinuclearity than HOC-7 cells, and the number of HEY/GFPi and Capan-1/vector cells with multinuclearity was greater than HEY/Aurora-Ai and Capan-1/BRCA2 cells (Fig. 4*d*). These results suggest that RAS mutations can diminish BRCA2 and enhance Aurora-A expression

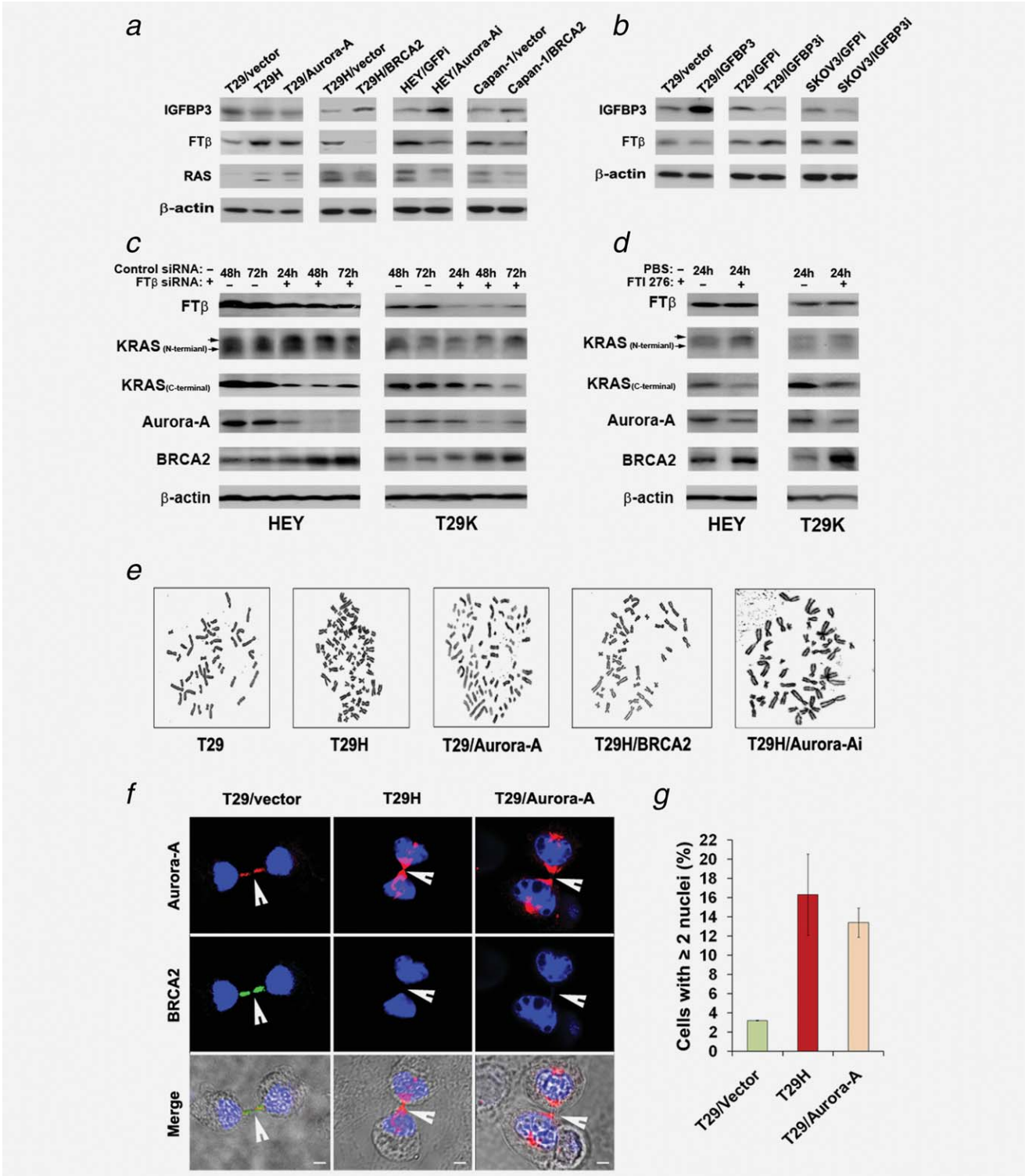

Figure 3. Alteration of signal molecules and detection of chromosomal abnormality and abnormal cytokinesis in RAS-associated cancer cells. (*a*) Transformation of ovarian epithelial cells (T29) by RAS or Aurora-A represses IGFBP-3 but induces FTβ over expression in T29H and T29/Aurora-A cells compared with in control cells. However, introduction of BRCA2 cDNA or Aurora-A shRNA into T29H, Capan-1 or HEY cells resulted in increased IGFBP-3 and decreased FTβ, which in turn reduced the farnesylation of RAS. (*b*). Introduction of IGFBP-3 cDNA or IGFBP-3 shRNA suppressed or increased FTβ expression. (*c*) Treatment of cells with FTβ siRNA reduced the expression of FTβ, RAS farnesylation and Aurora-A expression but elevated BRCA2 protein level. (*d*) Treatment of cells with farnesyl protein transferase inhibitor FTI-276 suppressed KRAS farnesylation and Aurora-A expression but simultaneously restored BRCA2 level. (*e*) The selected images show that RAS (T29H) or Aurora-A (T29/Aurora-A) transformation led to more polyploid cells than were observed in parental cell lines (T29), but transfection of T29H with BRCA2 or Aurora-A shRNA (Aurora-Ai) reduced cell polyploidy. (*f*) Colocalization of Aurora-A and BRCA2 was detected in the midbody of T29/vector cells during late mitosis, but overexpression of Aurora-A in RAS- or Aurora-A-transformed cells (T29H, T29/Aurora-A) diminished the localization of BRCA2 in the midbody. Blue dye To-Pro-3 indicates nucleus. Scale bars, 5 μm. (*g*) Quantification of cells with multiple nuclei in RAS- or Aurora-A-transformed cells. Introduction of RAS or Aurora-A resulted in more cells with multiple nuclei. Error bars = 95% confidence intervals from three independently repeated experiments.

Table 2. Cytogenetic analysis of chromosome abnormalities in immortalized ovarian epithelial cells after overexpression of HRAS or Aurora-A and in HRAS-transformed cells after knockdown of Aurora-A or overexpression of BRCA2

ID	Cell line[1]	Cells with chromosome aberrations (%)	Cells with DNA breaks (%)	Diploid cells (%)	Polyploid cells (%)
1682	T29/vector	6.4[2]	6.4	81.8[3]	6.1[2]
1683	T29/Aurora-A	12.1[2](↑)	6.1	64.5[3](↓)	32.3[2](↑)
1684	T80/vector	2.9[3]	2.9	93.7[3]	0[2]
1685	T80/Aurora-A	6.3[3](↑)	6.3	73.5[3](↓)	23.5[2](↑)
1688	T29H/GFPi	8.3[2]	0	75[3]	16.7[2]
1689	T29H/Aurora-Ai	2.9[2](↓)	2.9	85.3[3](↑)	8.8[2](↓)
1686	T80H/vector	11.4[2]	11.4[2]	57.1[3]	25.7[3]
1687	T80H/BRCA2	3[2](↓)	3[2](↓)	75.7[3](↑)	18.2[3](↓)

[1]For each cell line, 30–36 cells in metaphase were examined. Increase or decrease in chromosomal aberrance in terms of DNA breaks, diploidy and polyploidy was indicated as ↑ or ↓, respectively.
[2]$p < 0.01$.
[3]$p < 0.05$.

in the midbody during cytokinesis, which results in cell multinuclearity and genomic instability in human ovarian cancer cells as well as pancreatic cancer cells.

Discussion

Using RAS-transformed OSE cells as a model system in this study, we have identified a negative regulatory loop between Aurora-A and BRCA2, which are downstream targets of RAS. We showed that RAS transformation of ovarian epithelial cells can induce amplification of Aurora-A and simultaneously repress BRCA2 expression, which was also observed in ovarian cancer cell lines and ovarian cancer tissues with RAS/RAF mutations. Aurora-A and BRCA2 oppositely regulated RAS-induced genomic instability in RAS-mutated cells through abnormal cytokinesis. In normal or immortalized ovarian epithelial cells, Aurora-A and BRCA2 are colocalized at the midbody during late mitosis, in which BRCA2 and Aurora-A may control the segregation of two daughter cells through regulation of cytokinesis and prevent the generation of polyploid cells. A model on how Aurora-A and BRCA2 function in RAS mutated cancer is illustrated in Figure 4*e*. Upon activation by the RAS oncogene, which tips the balance toward Aurora-A, the diminished expression of BRCA2 and the accumulation of Aurora-A in the midbody may hamper the abscission of cleavage furrow to induce polyploidy or aneuploidy, which ultimately results in cellular genomic instability and tumorigenesis.

Cytokinesis is the last important step of cell division where identical eukaryotic daughter cells finally separate. The association of cancer with abnormal cytokinesis has been frequently reported over the past 20 years. The proteins that regulate or participate in abnormal cytokinesis in cancer cells

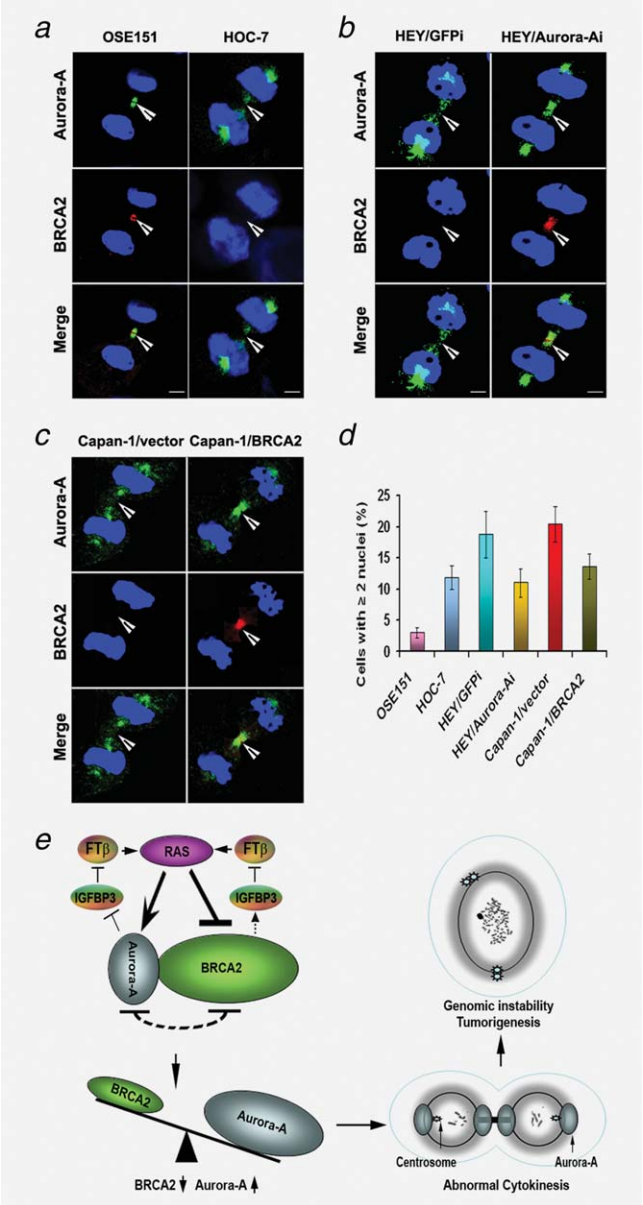

Figure 4. Analysis of Aurora-A and BRCA2 during cytokinesis. (*a–c*) Colocalization of Aurora-A and BRCA2 in the midbody of normal ovarian surface epithelial cells (OSE151), ovarian cancer (HOC-7 and HEY) and pancreatic cancer cells (Capan-1) with KRAS mutations. KRAS mutation results in Aurora-A increase and BRCA2 depletion in midbody during cytokinesis, whereas knockdown of Aurora-A in HEY cells or introduction of BRCA2 in Capan-1 cells restored the appearance of BRCA2 in the midbody although the reduced level of Aurora-A was still detectable in HEY/Aurora-A and Capan-1/BRCA2 cells. (*d*) The number of cells with multinuclearity was higher in HOC-7, HEY and Capan-1 cells than in OSE151 cells, but the decreased multinuclearity was observed in HEY and Capan-1 cells after transfection with Aurora-A shRNA or BRCA2 cDNA. (*e*) A schematic model illustrating that RAS induces unbalanced expression of Aurora-A and BRCA2, which are in turn to regulate IGFBP-3 and FTβ to activate RAS signaling. The accumulation of Aurora-A and the depletion of BRCA2 result in abnormal cytokinesis and cell multinuclearity, which eventually induce genomic instability and tumorigenesis.

include kinases (such as Aurora-A, Aurora-B and PLK1), mitotic checkpoint proteins (such as ATM, CHK1 and CHK2) and mitotic regulators (such as BRCA1 and centrobin).[34] One of the phenomena induced by abnormal cytokinesis is multinuclearity leading to chromosomal polyploidy or aneuploidy,[35] which largely contributes to genomic instability and tumorigenesis.[36] Both Aurora-A and BRCA2 are cell cycle regulatory proteins participating in cellular mitosis.[37,38] We have identified in this study that Aurora-A and BRCA2 are two mediators that colocalize at the midbody of late mitotic cells to control the genomic instability of cells which is regulated by mutated RAS oncogene. We and other research groups have found that BRCA2 is involved in regulation of cytokinesis,[32,39] although a recent study reported that BRCA2 may not regulate cytokinesis in Hela cells,[40] indicating that the role of BRCA2 in regulation of cytokinesis is sophisticated. It is interesting to note that no RAS or BRCA2 mutations in Hela cells were reported, but the amplification of RAS or Aurora-A has been observed in some literatures.[41,42] Therefore, it is possible that mutated RAS may be essential to defective cytokinesis through altering the expression ratio of Aurora-A and BRCA2.

Our results demonstrated that FTβ and IGFBP-3 plays an important role in mediating the effect of RAS on Aurora-A and BRCA2. The two proteins appear to form a negative regulatory loop to repress the expression of each other; such negative loop plays an important role in regulating the expression of Aurora-A and BRCA2 and the chromosomal instability induced by RAS. However, whether the interaction of FTβ and IGFBP-3 could regulate chromosomal instability without directly involving RAS, Aurora-A and BRCA2 is unknown, although it has been reported that FTβ-stimulated farnesylation can increase RAS activity, and RAS-induced MAPK activation can lead to resistance of breast cancer cells to IGFBP-3.[43] It is known that RAS mutations or Aurora-A amplification can activate the NF-κB which is involved in regulating the expression of FTβ and IGFBP-3,[44–46] therefore, it will be very interesting to examine the role of NF-κB in the regulation of FTβ and IGFBP-3 and RAS-mediated transformation.

Currently, there are no reports to show that Aurora-A can regulate BRCA2; however, as a kinase in cancer cells, over expression of Aurora-A may phosphorylate BRCA2 and result in proteasome-mediated degradation during the late stage of mitosis, leading to abnormal cytokinesis. Moreover, emerging evidences suggest that both Aurora-A and BRCA2 can be regulated by various factors during cell cycle by proteolysis-mediated degradation. Studies have demonstrated that BRCA2 can interact with multiple gene products such as USP11 (a deubiquitinating enzyme),[47] Skp2 (a subunit of the Skp1-Cul1-F-box protein ubiquitin complex),[48] and cancer-associated BRAD1 beta,[49] leading to its proteasome-mediated ubiquitination and degradation in different cancer cells. Polyubiquitination of Aurora-A by anaphase-promoting complex, or Cdh1 (a WD40 repeat protein) can promote the proteasome-mediated degradation of Aurora-A.[50] Thus, we treated T29 and T29/Aurora-A cells with proteasome inhibitor MG-132 at the concentration of 10 μM and found that the increased full length of Aurora-A and BRCA2 was accompanied with the decreased degradation of Aurora-A and BRCA2 over a time course of 1, 4 and 8 hr (Supporting Information Fig. 3). However, the detailed regulation of Aurora-A and BRCA2 by proteolysis mediated ubiquitination and degradation in cells with RAS mutations will require additional studies. As RAS/Aurora-A is amplified in multiple epithelial cancers, the molecules we identified in this study should have a general implication in clinical treatment of those cancers.

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