Title: Competitive binding between Id1 and E2F1 to Cdc20 regulates E2F1 degradation and thymidylate synthase expression to promote esophageal cancer chemoresistance

Running Title: Id1 regulates E2F1 degradation and cancer chemoresistance

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Translational relevance

Esophageal cancer ranks as the 6th most frequent cause of cancer death in the world. Neoadjuvant or adjuvant chemotherapy is widely used in treatment of esophageal cancer but development of chemoresistance can compromise treatment efficacy or even result in recurrence. A better understanding of the molecular mechanisms and development of novel strategies to improve treatment outcome is urgently needed. This study provides the first evidence that Id1 confers 5-fluorouracil (5-FU) chemoresistance through E2F1-dependent induction of IGF2 and thymidylate synthase, a critical target of anti-cancer drugs especially 5-FU. Analysis of gene expressions, clinical data and multiple GEO datasets reveals that concurrent high expression of Id1 and IGF2 is associated with poor survival in esophageal, colon, liver, lung, and breast cancers. By providing solid evidence on the importance of the Id1-E2F1-IGF2 regulatory axis in promoting chemoresistance, our study offers new insights into developing novel therapeutic interventions and prognostic strategies for esophageal cancer.

Abstract

Purpose: Chemoresistance is a major obstacle in cancer therapy. We found that fluorouracil (5-FU)-resistant esophageal squamous cell carcinoma cell lines, established through exposure to increasing concentrations of 5-FU, showed upregulation of Id1, IGF2, and E2F1. We hypothesized that these genes may play an important role in cancer chemoresistance.

Experimental Design: *In vitro* and *in vivo* functional assays were performed to study the effects of Id1-E2F1-IGF2 signaling in chemoresistance. Quantitative real-time PCR, Western blot, immunoprecipitation, chromatin immunoprecipitation, and dual-luciferase reporter assays were used to investigate the molecular mechanisms by which Id1 regulates E2F1 and by which E2F1 regulates IGF2. Clinical specimens, tumor tissue microarray and Gene Expression Omnibus datasets were used to analyze the correlations between gene expressions, and the relationships between expression profiles and patient survival outcomes.

Results: Id1 conferred 5-FU chemoresistance through E2F1-dependent induction of thymidylate synthase expression in esophageal cancer cells and tumor xenografts. Mechanistically, Id1 protects E2F1 protein from degradation and increases its expression by binding competitively to Cdc20, whereas E2F1 mediates Id1-induced upregulation of IGF2 by binding directly to the IGF2 promoter and activating its transcription. The expression level of E2F1 was positively correlated with that of Id1 and IGF2 in human cancers. More importantly, concurrent high expression of Id1 and IGF2 was associated with unfavorable patient survival in multiple cancer types.

Conclusions: Our findings define an intricate E2F1-dependent mechanism by which Id1 increases thymidylate synthase and IGF2 expressions to promote cancer chemoresistance. The Id1-E2F1-IGF2 regulatory axis has important implications for cancer prognosis and treatment.

Introduction

Chemotherapy, alone or in combination with other treatment modalities, is widely used in cancer treatment. However, development of resistance to chemotherapeutic drugs remains a serious challenge in the management of human cancer because this may result in disease recurrence and more aggressive tumor phenotypes. A better understanding of the genetic alterations and molecular mechanisms responsible for cancer chemoresistance, as well as novel strategies to improve treatment outcome are urgently needed.

We recently succeeded in establishing cell line models of acquired chemoresistance by treating esophageal cancer cells with increasing concentrations of 5-fluorouracil (5-FU) up to 80 μM for 18 months. Besides upregulation of thymidylate synthase (TS) (1) , which is an essential enzyme for *de novo* synthesis of thymidylates and a critical target of 5-FU (2, 3) , and activation of AKT (4), we have obtained novel evidence in the present study that there was significant increase in the expression of E2F1, inhibitor of DNA binding 1 (Id1), and insulin-like growth factor 2 (IGF2) proteins in these 5-FU-resistant (FR) cell lines. The increase of E2F1 in the FR cell lines was not surprising because E2F1 has been reported to increase the resistance of cancer cells to 5-FU, and to directly induce the transcription and expression of TS (5, 6). However, the functions of Id1 and IGF2 in 5-FU resistance have not been reported. Our previous study showed that Id1 overexpression upregulates IGF2 in a variety of cancer cells, and that blockade of insulin-like growth factor type 1 receptor (IGF1R), which is the main receptor that mediates the biological functions of IGF2, can inhibit the PI3K/AKT pathway and sensitize esophageal cancer cells to 5-FU treatment (1). Whether there is a causal link between increased Id1/IGF2 and E2F1 upregulation in 5-FU chemoresistance warrants investigation.

As a transcription factor, E2F1 is capable of directly binding to DNA consensus sequences to exert transcriptional effects. Recently, the anaphase promoting complex/cyclosome (APC/C)-associated protein Cdc20 (cell division cycle protein 20) , which is an interaction partner of Id1 (7), was found to target E2F1 for degradation (8), but the significance and regulation of this mechanism in cancer are yet unknown. We therefore hypothesize that there is competitive binding between Id1 and E2F1 to Cdc20 in cancer cells, so that increased Id1 in FR cells may stabilize E2F1 protein and protect it from degradation. To test this hypothesis, we investigated whether Id1 modulates E2F1 protein stability, and whether this mechanism regulates TS expression and 5-FU chemoresistance. In addition, gain- and loss-of function experiments were carried out to demonstrate the effect of IGF2 on TS expression and the significance of IGF2 in acquired chemoresistance in esophageal squamous cell carcinoma (ESCC) cells. We also aim to decipher the mechanism by which Id1 regulates IGF2, and to determine if E2F1 mediates the regulation of IGF2 by Id1.

Materials and Methods

Cell lines

Human ESCC cell lines KYSE150, KYSE270, KYSE410 (DSMZ, Braunschweig, Germany) (9), T.Tn (JCRB Cell Bank, Osaka, Japan) (10), human colon carcinoma cell line Caco-2 (ATCC, Rockville, MD) and human hepatocarcinoma cell line SMMC-7721 (CAMS, Beijing, China) were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37ºC in 5% CO_2. The 293 phoenix cells (ATCC) were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum. All cell lines were authenticated by short tandem repeat profiling.

Primary tumor tissues and tissue microarray

Human ESCC tumors and the corresponding adjacent normal esophageal tissues were collected with informed consent and Institutional Review Board approval from 50 patients undergoing surgical resection of primary esophageal tumor at Queen Mary Hospital in Hong Kong from 2011 to 2014, and at the First Affiliated Hospital, Zhengzhou University in Zhengzhou, China, from 2008 to 2010. All specimens were snap-frozen in liquid nitrogen and stored at -80ºC. Total RNA isolated from another cohort of human ESCC tumors with complete patient clinical data, collected from 35 patients at Queen Mary Hospital from 2003 to 2007, was used for survival correlation analysis. A tissue microarray (TMA) containing 35 cases of human ESCC in duplicated cores (Catalogue no. ES802, Biomax, Rockville, MD) was also used to evaluate the correlation between E2F1 and IGF2.

In vitro BrdU cell proliferation, migration, Western blot, ELISA, quantitative real-time PCR, ChIP, immunoprecipitation, and luciferase reporter assays

Cell proliferation was determined based on BrdU incoporation. Transwell chambers (Millipore, Billerica, MA) were used to examine cell migration (11). Preparation of cell and tumor lysates, and details of immunoblotting were described previously (12). More detailed experimental procedures can be found in the Supplementary Materials and Methods.

In vivo tumorigenicity in nude mice

Female BALB/c nude mice aged 6-8 weeks were maintained under standard conditions according to the institutional guidelines for animal care. All the animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. The tumorigenicity experiments were performed as described previously (4).

Immunohistochemistry and evaluation of staining

After antigen retrieval and blocking with normal serum, the slides were incubated overnight at 4 ºC with the primary antibody against E2F1 (#SC-251, Santa Cruz Biotechnology, Santa Cruz, CA) followed by biotinylated secondary antibodies and peroxidase-conjugated avidin-biotin complex. Immunostaining was visualized using 3, 3'-diaminobenzidine (DAKO) as chromogen, and then the sections were counterstained with hematoxylin. The E2F1 immunostaining in the TMA was assessed using a grading system based on the percentage of positive nuclei (13): 0, no nuclear staining; 1, < 10% positive staining; 2, 10-50%; 3, > 50%. Immunostaining of IGF2 was performed with an anti-human IGF2 antibody (#AF-292-NA)

from R&D Systems (Minneapolis, MN;) and evaluated as described previously (1). Specimens assigned scores of 0 to 1 were considered weak, whereas scores 2 to 3 were considered strong.

Analysis of gene expression and survival data from cancer patient datasets

Microarray gene expression and survival data of cohorts of ESCC (14), EAC (15, 16), colon cancer (17, 18), hepatocellular carcinoma (HCC) patients (19), lung cancer (20), and breast cancer (21, 22), were downloaded from the GEO database (accession numbers GSE23400, GSE47404, GSE13898, GSE37203, GSE28000, GSE28722, GSE10141, GSE45436, GSE54236, GSE3141, GSE7849, GSE50948). R scripting was used to extract the expression values of genes of interests and clinical data from the data matrices as described by Yuen *et al* (23, 24). Gene expressions were further divided into high and low levels using median expression level as the cut-off point for Kaplan-Meier survival analyses.

Statistical analysis

The data were expressed as the mean ± SD and compared using ANOVA. The expression level of Id1, E2F1, and IGF2 in tumor samples and matched normal samples was compared using paired or unpaired t-test. Correlation between E2F1 and Id1 or IGF2 expression in the frozen tissues and TMA was assessed using Pearson's rank correlation coefficient and Fisher's Exact tests, respectively. The association between the expression level and patient survival was plotted using the Kaplan-Meier method, and statistical differences were compared using the log-rank test. P values < 0.05 were deemed significant. All *in vitro* experiments and assays were repeated at least three times.

180

Results

Up-regulation of Id1, IGF2 and E2F1 in 5-FU-chemoresistant esophageal cancer cell subpopulation and significance of E2F1 in 5-FU chemoresistance

The PI3K/AKT pathway is one of the most important pathways involved in the development of chemoresistance. Since our previous study showed that PI3K/AKT can be activated by Id1-induced IGF2 in cancer cells (1) , we hypothesized that Id1 and IGF2 may have a role in 5-FU resistance. Furthermore, since it was reported that E2F1 expression can increase the resistance of fibrosarcoma cells to 5-FU (5) , we speculated that E2F1 protein may also be differentially expressed upon acquisition of 5-FU chemoresistance. We therefore made use of 5-FU resistant sublines (designated KYSE150FR and KYSE410FR) which were established from ESCC cell lines KYSE150 and KYSE410 through continuous treatment with increasing doses of 5-FU (from 1.25 μM to 80 μM) for over 18 months (Fig. 1A) as cell models to test our hypothesis. The proliferation rate and migration ability of FR cells were similar or slightly higher compared with parental cells (Supplementary Figure S1). Tumor xenografts that were derived from FR cells were confirmed to exhibit robust resistance to 5-FU *in vivo* (Fig. 1B). Comparison of the FR cell lines and their parental cell lines showed up-regulation of Id1, IGF2, and E2F1 protein expression (Fig. 1C), as well as increased secretion of IGF2 in the FR cells (Fig. 1D). Increased mRNA expression levels of Id1 and IGF2, but not E2F1, were observed in the FR cells (Fig. 1E). ESCC cells with E2F1 overexpression or knockdown were treated with 5-FU, and then cell proliferation was measured. As expected, ectopic expression of E2F1 increased TS expression and 5-FU chemoresistance, whereas repressed expression of E2F1 had the opposite effects (Supplementary Fig. S2). These findings strongly support the rationale of using these FR sublines as cell models for

identifying chemoresistance-associated genes, and for studying the roles of Id1 and IGF2 in regulating 5-FU chemoresistance in ESCC.

Id1 confers 5-FU chemoresistance through E2F1-dependent induction of thymidylate synthase expression

Having established that Id1, IGF2 and E2F1 proteins were upregulated in FR cells, our next questions were whether Id1 plays an important role in 5-FU chemoresistance and whether E2F1 is involved in mediating this function. Gain- and loss-of function experiments were carried out to study the effect of Id1 on 5-FU chemoresistance, and on E2F1 and TS expression in ESCC cells. Rescue experiments were performed to determine whether E2F1 mediates the effect of Id1 in increasing 5-FU resistance. We also determined the clinical relevance of Id1 and E2F1 by analyzing their protein levels in 50 pairs of primary ESCC tumors and tumor-adjacent normal tissues by Western blot. The *in vitro* experiments showed that ectopic Id1 expression significantly enhanced the resistance of esophageal cancer cells to 5-FU (Supplementary Fig. S3A). Conversely, knockdown of Id1 expression significantly restored the sensitivity of FR cells to 5-FU (Supplementary Fig. S3B and C). Interestingly, we found that Id1 overexpression induced (Fig. 2A), whereas Id1 knockdown reduced (Fig. 2B), the expression levels of E2F1 and TS dose-dependently. The rescue experiments showed that the induction of TS by Id1 was abrogated by two different shRNAs against E2F1 (Fig. 2C, left), and that E2F1 overexpression restored the TS expression in Id1-repressed ESCC cells (Fig. 2C, right). In addition, higher Id1 and E2F1 expressions were observed in the majority of tumors compared with the corresponding normal tissues (Supplementary Fig. S4). There was also a positive correlation between expressions of Id1 and E2F1 in the 50 pairs of ESCC and normal esophageal tissues (Fig. 2D). Furthermore, our *in vitro* functional assays

showed that E2F1 knockdown and overexpression abolished the effects of Id1 overexpression and knockdown, respectively, on sensitivity of esophageal cancer cells to 5-FU *in vitro* (Fig. 2E). More importantly, the animal experiments showed that 5-FU treatment which exerted a markedly repressive effect on the size of vector control tumors had little effect on that of the Id1-overexpressing tumors, but knockdown of E2F1 significantly reduced the 5-FU resistance of Id1-overexpressing tumors (Fig. 2F, left; Supplementary Figure S5A). Conversely, although 5-FU treatment had no effect on growth of tumors derived from FR cells, there was an obvious response in the KYSE410FR-shId1 tumors, which was abolished when E2F1 was overexpressed (Fig. 2F, right; Supplementary Figure S5B). Taken together, these findings consistently showed that Id1 significantly increased TS expression and 5-FU chemoresistance in esophageal cancer cells through upregulation of E2F1.

Id1 protects E2F1 protein from degradation and increases its expression by competitive binding to Cdc20

Given that Id1 interacts with Cdc20 (7) , and that Cdc20 can target E2F1 for proteasomal degradation (8) , we hypothesized that Id1 might compete with E2F1 for interaction with Cdc20, therefore stabilizing E2F1 protein. Id1-overexpressing ESCC cells and the corresponding vector control cells were treated with protein synthesis inhibitor cycloheximide (CHX) for up to 8 h. Western blot data showed that E2F1 protein degradation was retarded in the Id1-expressing cells compared with the control cells (Fig. 3A), which suggests that Id1 overexpression leads to stabilization of E2F1 protein. We then performed immunoprecipitation on esophageal cancer cells co-transfected with the plasmids expressing Flag-Cdc20 and HA-Id1, and found that Cdc20 and Id1 were indeed interacting partners in esophageal cancer cells (Fig. 3B). Meanwhile, the physical interaction between Cdc20 and

E2F1 in esophageal cancer cells was also determined by immunoprecipitation and Western blot. HA-tagged E2F1 protein was detected in the Flag-Cdc20 immunoprecipitate in the cells co-transfected with Flag-Cdc20 and HA-E2F1 (Fig. 3C). In the reverse co-immunoprecipitation experiments, Cdc20 was detectable in E2F1- and Id1-immunoprecipitates, thus confirming that Cdc20 could directly bind to E2F1 and Id1 (Supplementary Figure S6A and B). More importantly, we co-transfected the plasmids expressing Flag-Cdc20 and HA-E2F1 together with HA-Id1-expressing plasmid or vector control, and found significantly lower E2F1 level in the Flag-Cdc20 immunoprecipitate of the Id1 transfectants (Fig. 3D, lane 4 vs lane 3), indicating that Id1-Cdc20 interaction inhibited the association between Cdc20 and E2F1. Similar results were observed when the cells were treated with 5-FU (Supplementary Figure S6C). On the other hand, immunoprecipitation assay failed to reveal any interaction between Id1 and E2F1 in either ESCC parental cells or FR cells (supplementary Fig. S7). Our results collectively demonstrated that Id1 could protect E2F1 protein degradation and increase its expression by competitive binding to Cdc20, as illustrated in Figure 3E.

E2F1 mediates Id1-induced upregulation of IGF2 by binding directly to IGF2 promoter

Although we have reported that Id1 induces the expression of IGF2 in cancer cells (1), the mechanism is still unknown. The above findings raised the question of whether there is a link between the regulation of E2F1 by Id1 and that of IGF2 by Id1. The effect of E2F1 on IGF2 was studied using Western blot. Ectopic E2F1 expression was found to induce IGF2 protein expression dose-dependently in KYSE150 and KYSE410 (Fig. 4A, left). Transient transfection of two different shRNAs against E2F1 successfully repressed E2F1 expression and inhibited IGF2 protein expression in KYSE270 and T.Tn ESCC cells (Fig. 4A, right),

indicating the positive regulation of IGF2 by E2F1. These effects were confirmed in other human cancer lines including colon and liver cancer cells (Supplementary Fig. S8). Moreover, the data from RT-PCR analysis showed that E2F1 overexpression increased (Fig. 4B, left), whereas E2F1 knockdown decreased (Fig. 4B, middle and right), the mRNA expression of IGF2 in ESCC cell lines, indicating that E2F1 regulates IGF2 expression at both protein and mRNA levels. Next, two software programs that predict transcription factor binding sites, namely Contra V2 and TRRD (25, 26), were used to search for potential E2F1 binding sites (BS) in the IGF2 promoter region, and three potential binding sites (designated BS1, BS2 and BS3) were identified by both software, which suggested that E2F1 may bind directly to the IGF2 promoter and activate IGF2 transcription (Fig. 4C). Then chromatin immunoprecipitation (ChIP) assay of endogenous E2F1 in esophageal cancer cells, followed by quantitative PCR, were performed to verify the physical binding of E2F1 to the individual binding sites on IGF2 promoter. The results showed that the DNA fragments containing BS1 and BS2, but not BS3, were detected in the E2F1-immunoprecipitated DNA fragments (Fig. 4C). To examine whether E2F1 directly activates IGF2 transcription, dual luciferase reporter assay was conducted by co-transfecting the luciferase reporter plasmid (pGL2-Luc-basic) containing the IGF2 promoter together with E2F1-expressing plasmid or vector control. The data showed that the luciferase activity of IGF2 promoter was significantly enhanced when co-transfected with wild type (WT) E2F1-expressing plasmid, compared with vector control (Fig. 4D). Mutations in BS1 or BS2, but not BS3, resulted in loss of promoter activity upon activation by E2F1 (Fig. 4D), indicating that E2F1 activates IGF2 transcription by binding to the BS1 and BS2, but not BS3 of IGF2. Furthermore, we investigated whether E2F1 mediates the effect of Id1 on IGF2 expression. Western blot data from KYSE150 and KYSE410 cells showed that knockdown of E2F1 by two different shRNAs against E2F1 attenuated the increase in expression levels of E2F1 and IGF2 induced by Id1 overexpression (Fig. 4E).

Conversely, E2F1 overexpression counteracted the inhibitory effect of Id1-knockdown on IGF2 expression in KYSE270 and T.Tn cells (Fig. 4F). Together, these results showed that E2F1, induced by Id1, could directly activate IGF2 transcription.

E2F1 and IGF2 are overexpressed and positively correlated with each other in human cancers

IGF2 is overexpressed in 81% of ESCC (27). The direct regulation of IGF2 by E2F1 demonstrated in the *in vitro* experiments above led us to postulate that E2F1 expression may be upregulated and positively correlated with IGF2 expression in ESCC. To study the significance of E2F1 and IGF2 expressions in human esophageal cancer, IGF2 expression was examined in 50 pairs of primary ESCC tumors and tumor-adjacent normal tissues by Western blot. Similar to E2F1 described above (Supplementary Fig. S4), higher IGF2 expression was found in the majority of the primary esophageal tumors relative to the corresponding normal tissues (Fig. 5A, left). The mean expression level of IGF2 in ESCC was about 4-fold higher than that in the normal esophageal tissue (0.99 ± 0.64 versus 0.28 ± 0.30; $P < 0.001$) (Fig. 5A, right). More importantly, the 50 pairs of ESCC and normal esophageal tissues showed a positive correlation between expressions of E2F1 and IGF2 (Fig. 5B). The correlation was further validated by analyzing the immunohistochemical expressions of E2F1 and IGF2 in a TMA containing 35 cases of primary ESCC tumor tissues (Fig. 5C). Furthermore, analysis of gene expression profiles of several cohorts of patients from Gene Expression Omnibus (GEO) database showed strong positive correlation between E2F1 and IGF2 expression in ESCC, colon, and breast cancers; and modest but statistically significant correlation in esophageal adenocarcinoma (EAC), hepatocellular carcinoma (HCC) and lung cancer (Fig. 5D). E2F1 mRNA expression was also positively correlated with TS

mRNA expression in the same GEO datasets (Supplementary Fig. S9). These results further support our findings that E2F1 may be important in regulating IGF2 expression and 5-FU chemoresistance.

IGF2 plays an important role in regulating esophageal cancer chemoresistance

Although our previous study showed that blockade of the IGF2 receptor IGF1R can sensitize ESCC cells to 5-FU treatment (1) , the function and mechanism of IGF2 in 5-FU chemoresistance remained unexplored. *In vitro* and *in vivo* experiments were carried out to determine if IGF2 is crucial for 5-FU chemoresistance in esophageal cancer. We found that addition of exogenous IGF2 to ESCC cells not only increased the expression levels of phosphorylated-AKT (p-AKT) and its downstream target TS (Supplementary Fig. S10A), but also protected the cells from 5-FU-induced apoptosis and enhanced their resistance to 5-FU, as indicated by the decrease in 5-FU-induced cleaved caspase-3 expression (Supplementary Fig. S10B) and increased cell proliferation (Supplementary Fig. S10C). These effects were abolished by the specific PI3K inhibitor LY294002. In addition, we stably transduced shRNA against IGF2 into the FR cell lines, KYSE150FR and KYSE410FR, to generate stable cell lines with repressed IGF2 expression and secretion (Fig. 6A, left and Supplementary Fig. S11), and obtained consistent data showing that knockdown of IGF2 significantly reduced p-AKT and TS expressions, increased 5-FU-induced cell death and cleaved caspase-3 expression compared with non-target control (shCON) (Fig. 6A), indicating restored sensitivity of FR cells to 5-FU by IGF2 silencing. These effects were revoked by addition of exogenous IGF2 to the culture media of IGF2-knockdown FR cells. Moreover, stable knockdown of IGF2 in two ESCC cell lines with relatively high endogenous IGF2 expression and 5-FU chemoresistance rendered the cells more apoptotic and sensitive to 5-FU treatment

(Supplementary Fig. S12A-D). The significance of IGF2 in chemoresistance was also tested *in vivo*. The results showed that knockdown of IGF2 significantly reduced the resistance of KYSE410FR and KYSE270FR tumors to 5-FU treatment in mice, as evidenced by the decreased tumor volume compared with the respective 5-FU-refractory control groups (Fig. 6B and Supplementary Fig. S12E), thus confirming that IGF2 plays an important role in acquired 5-FU chemoresistance. Furthermore, we found that blockade of IGF2 with shRNA or neutralizing antibody attenuated the effects of Id1 and E2F1 in increasing 5-FU chemoresistance (Fig. 6C). Taken together, these data suggest that IGF2 upregulates TS expression and thus enhances 5-FU chemoresistance in Id1-overexpressing tumors by signaling through the PI3K/AKT pathway (Fig. 6D).

High expression of Id1 and IGF2 is correlated with poor survival in cancer patients

Given that Id1 and IGF2 play important roles in regulating 5-FU chemoresistance, we postulated that Id1 and IGF2 may be potential prognostic markers for cancer patients. We therefore investigated whether a high level of Id1 and IGF2 expression in cancer is associated with survival of cancer patients. Firstly, expression levels of Id1 and IGF2 in ESCC were determined using qRT-PCR in a cohort of esophageal cancer patients with survival data, and the results showed that the patients with high Id1 and IGF2 expression had shorter survival (median survival = 15.61 months) than patients with low Id1 and IGF2 expression (median survival = 29.77 months). Log-rank analysis showed that high Id1 and IGF2 mRNA level was significantly correlated with shorter survival (Log rank = 4.880, P = 0.027; Fig. 6E), although it was not correlated with tumor stage or tumor differentiation (Supplementary Table S1). Likewise, analysis of colon cancer patient cohort from GEO datasets revealed that patients with high Id1 and IGF2 expression had shorter survival (median survival = 49.2

373 months) than patients with low Id1 and IGF2 expression (median survival = 85.3 months),
374 with a significant correlation between concurrent high Id1/IGF2 mRNA level and shorter
375 survival (Log rank = 6.534, $P = 0.011$). Similar results were obtained in cohorts of HCC, lung
376 cancer, and breast cancer patients (Fig. 6F). Collectively, our results indicated that concurrent
377 high expression of Id1 and IGF2 may predict poor prognosis of cancer patients.

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Discussion

Acquired chemoresistance contributes to poor treatment response and cancer recurrence. Chemoresistant cancer cell lines have been successfully used as models to efficiently identify key genes and signaling pathways associated with chemoresistance in human cancer (28-30). Establishment of chemoresistant cell lines from chemosensitive parental human ESCC cells *in vitro* mimics the *in vivo* process in which esophageal tumors acquire resistance to cytotoxic drugs after initial chemotherapy. A combination of 5-FU and cisplatin is one of the most commonly used regimens as first-line treatment of advanced esophageal cancer. The FR cells established in our laboratory showed increase in expression levels of Id1, IGF2, and E2F1. E2F1 has been documented to directly activate TS transcription and expression (6). The positive correlation between E2F1 and TS expression, and the association between E2F1 overexpression and poor prognosis in a variety of cancers including ESCC have been reported (31-33). By confirming the role of E2F1 in conferring 5-FU chemoresistance in esophageal cancer cells, we have justified the use of FR cell models as tools for identification of chemoresistance-associated genes and novel drug targets. Here, we report for the first time that Id1 can increase TS expression and promote 5-FU chemoresistance in human cancer, and that E2F1 mediates this effect. To our knowledge, this is the first report on the function of Id1 in ESCC chemoresistance.

E2F1 has primarily been recognized for its pivotal role in transcriptional regulation of genes related to cell cycle and apoptosis. Dysregulation of E2F1 is common in human cancer including esophageal cancer (34), but amplification of *E2F1* in cancer is rare. As in the case for many transcription factors, E2F1 is mainly regulated by post-translational modification. The pRb protein, which functionally inactivates E2F1 on one hand but protects it from degradation on the other, was thought to be the most crucial regulator of E2F1 (35). However,

after dissociation from pRb, interaction with other proteins may be vital for the stability of E2F1 protein. In this study, the gain- and loss-of-function experiments showed that ectopic Id1 expression induced, whereas Id1 knockdown reduced, the expression of E2F1 in multiple cancer cell lines, thus strongly suggesting that Id1 can regulate E2F1. Our results from CHX chase and immunoprecipitation experiments give novel insight into the regulation of E2F1 by providing the first evidence that Id1 competes with E2F1 for Cdc20 binding, thereby protecting E2F1 from Cdc20-mediated degradation. As discussed below, our data also revealed that this mechanism plays an important role in upregulating IGF2 in esophageal cancer.

Overexpression of IGF2 and its clinical significance in human cancer is well documented (36-38). Increased IGF2 expression in Taxol-resistant ovarian cancer cell line and the feasibility of IGF2 as a potential therapeutic target in Taxol-resistant ovarian cancer have been validated recently (39-41), but the functional role of IGF2 in 5-FU chemoresistance has not been elucidated. We found for the first time that IGF2 can significantly increase, whereas knockdown of IGF2 can decrease, TS expression. E2F1 is an important target of chemotherapeutic drugs, and aberrant expression of TS is significantly associated with the resistance of tumors to chemotherapy (42, 43). Our data showed that both intrinsic and acquired 5-FU chemoresistance of ESCC cells could be achieved by knocking down IGF2 to reduce TS expression. In addition, our *in vitro* and *in vivo* data from gain- and loss-of-function experiments provide novel evidence to support that IGF2 plays an important role in mediating the effects of Id1 in regulating the sensitivity of cancer cells to 5-FU. We recently reported that Id1 induces IGF2 expression and secretion (1), but the molecular mechanisms by which Id1 regulates IGF2 is still unknown. In this study, using ChIP, dual luciferase reporter, and rescue assays, we show for the first time that E2F1 mediates the positive

regulation of Id1 on IGF2 by directly binding to the IGF2 promoter, thereby activating IGF2 transcription and expression.

Overall, our results suggest that besides directly inducing the transcription and expression of TS, there exists a parallel mechanism in which Id1 and E2F1 can indirectly upregulate TS by transcriptional activation of IGF2, thus engaging the PI3K/AKT pathway in mediating 5-FU chemoresistance. The strong positive correlation between Id1 and E2F1, and between E2F1 and IGF2 protein expressions observed in esophageal tumor tissues, as well as between Id1 and IGF2 mRNA expressions in esophageal cancer and a variety of other cancer types further suggest that this regulatory mechanism has clinical significance in human cancer. More importantly, analysis of gene expression profiles of multiple cancer types indicated that simultaneous high Id1 and IGF2 expression in the tumors is significantly correlated with shorter survival of cancer patients. Taken together, this study suggests that dysregulation of E2F1 and IGF2 due to Id1 overexpression is important in cancer progression, and that the Id1-E2F1-IGF2 regulatory axis may be a valid gene expression signature for prognostic prediction and a target for new treatment strategies.

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Figure Legends

Figure 1. 5-FU-resistant (FR) esophageal cancer sublines have increased expression of Id1, IGF2 and E2F1, and form 5-FU-resistant tumors *in vivo*. **A**, diagram depicting the establishment of FR sublines from esophageal cancer cells. **B**, nude mice bearing KYSE410FR- or KYSE410-derived tumor xenografts were treated with 5-FU (20 mg/kg) twice weekly for three weeks (n = 6). **C** and **D**, FR cells and parental cells were compared for expression levels of Id1, IGF2, and E2F1 in cell lysate by Western blot (**C**) and for IGF2 concentration in the conditioned medium by ELISA (**D**). **E**, the mRNA expression levels of Id1, E2F1, and IGF2 were determined in FR cells and parental cells by real-time RT-PCR. Bars, SD; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. Id1 increases thymidylate synthase (TS) expression and 5-FU chemoresistance through E2F1. **A** and **B**, KYSE150 and KYSE410 cells were transfected with different doses of pcDNA3-Id1 supplemented with pcDNA3 (**A**), whereas KYSE150FR and KYSE410FR cells were transfected with siRNA against Id1 or the vector expressing shRNA against Id1 (**B**), then Western blot was performed. **C**, E2F1 knockdown markedly abrogated the effects of Id1 overexpression on TS expression, whereas E2F1 re-overexpression significantly alleviated the inhibitory effects of Id1 knockdown on TS expression. **D**, the expression levels of Id1 and E2F1, determined using Western blot, were significantly correlated in the 50 pairs of human esophageal tumor and normal specimens. Right panel, Western blot of Id1, E2F1 and actin in six representative pairs of esophageal tumor tissues (T) and their matched normal tissues (N). **E**, parental and FR esophageal cancer cells with stable expression of indicated plasmids were treated with 5-FU (10 μM) or DMSO for 48 h and then subjected to BrdU incorporation assay. **F**, left panel, comparison of KYSE410-CON, KYSE410-Id1, and

KYSE410-Id1-shE2F1 tumor xenografts for 5-FU sensitivity in nude mice (n = 6). Right Panel, E2F1 overexpression counteracted the inhibitory effect of Id1-knockdown on 5-FU chemoresistance of KYSE410FR tumors in nude mice (n = 6). Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3. Id1 protects E2F1 protein from degradation through competitive binding to Cdc20. **A**, KYSE150-Id1, KYSE410-Id1 and their respective vector control cells were treated with cycloheximide (CHX, 50 μg/ml). The cell lysates were collected at the indicated time points and compared for E2F1 expressing using Western blot. E2F1 signals were quantified by densitometry and the degradation rate was shown as the ratio of E2F1 level at each time point to the respectively original level (0 h). The half-life (t1/2) of E2F1 was 6.08 h and 3.01 h in Id1-overexpressing KYSE150 cells and corresponding vector control cells respectively; t1/2 values were 13.23 h and 3.97 h in Id1-overexpressing KYSE410 cells and vector control cells respectively. **B** and **C**, the indicated Flag/HA-tagged plasmids or pcDNA3 empty vector were transfected into KYSE150 cells. Immunoprecipitation was performed using an anti-Flag antibody or IgG as control, and Western blot carried out on the total cell lysate or immunoprecipitate using the indicated antibodies showed that Cdc20 co-immunoprecipitated with Id1 and E2F1. **D**, the constructs expressing Flag-tagged Cdc20 and HA-tagged E2F1 were co-transfected with HA-tagged Id1 construct or vector control into KYSE150 cells. Immunoprecipitation assay was performed on the cell lysates using an anti-Flag antibody or IgG as a control, followed by Western blot to detect protein expressions. **E**, a proposed model illustrating the mechanism by which Id1 induces E2F1 stabilization through competitive binding with Cdc20 to activate IGF2 transcription and expression.

Figure 4. E2F1 directly binds to IGF2 promoter and increases IGF2 transcription and expression, thereby mediating the regulation of IGF2 by Id1. **A** and **B**, Western blot (**A**) and RT-PCR (**B**) analyses of IGF2 in the esophageal cancer cells transfected with different doses of pcDNA3-E2F1, or plasmids expressing shE2F1#1 or shE2F1#2. The pcDNA3 empty vector was transfected as control. **C**, upper panel, schematic illustration of putative E2F1-binding sites in the IGF2 promoter region. TSS represents transcription start site. BS1, BS2, and BS3 indicate the predicted E2F1-binding sites. Lower panel, ChIP assay was conducted to pull down potential E2F1-binding DNA fragments in KYSE270 cells using E2F1 antibody or IgG antibody. qPCR was performed to determine the abundance of DNA fragments in the putative IGF2 promoter region. **D**, upper panel, a diagram representing the IGF2 promoter region inserted upstream of firefly luciferase gene in pGL2-basic vector, and the mutations at the predicted E2F1-binding sequences. Lower panel, E2F1-expressing plasmid or vector control was co-transfected with the wild type (WT) or mutant reporter construct into KYSE150 cells, and luciferase activity was measured 48 h after transfection. **E**, Western blots of KYSE150 and KYSE410 cells that were co-transfected with Id1-expression or pBabe control vector, and indicated plasmids expressing shE2F1#1, shE2F1#2 or shCON performed. **F**, Western blot indicated that knockdown of Id1 inhibited E2F1 and IGF2 expressions in KYSE270 and T.Tn cells, and that transfection with E2F1-expressing plasmid abolished this effect. Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control cells unless otherwise indicated.

Figure 5. Positive correlation between E2F1 and IGF2 in human cancers. **A**, IGF2 and actin expressions were determined in 50 pairs of esophageal tumor and matched normal tissues by Western blot and densitometry. The boxes in the right panels contain the values between 25th

669 and 75th percentiles of the 50 cases, and the whiskers extend to the highest and lowest values.
670 The lines across the boxes indicate the median values, and the white diamonds inside the
671 boxes represent the mean values. **B**, the expression levels of E2F1 and IGF2 were
672 significantly correlated in the 50 pairs of human esophageal tumor and normal specimens.
673 Right panel, Western blot of E2F1, IGF2 and actin in six representative pairs of esophageal
674 tumor tissues (T) and their matched normal tissues (N). **C**, two consecutive sections of a
675 human ESCC tissue microarray were immunostained for E2F1 and IGF2 expression. The
676 correlation between the immunostaining intensity of the proteins was determined by Fisher's
677 Exact test (left panel), and two representative cases showing strong (Case 1) and weak (Case
678 2) staining are shown in the right panel. **D**, Gene Expression Omnibus (GEO) cancer datasets
679 were acquired for analyzing the correlation between relative levels of E2F1 and IGF2 mRNA
680 using Pearson's rank correlation coefficient analysis. E2F1 and IGF2 expressions were
681 significantly correlated in all the datasets examined in this study including ESCC
682 (GSE23400/47404), EAC (GSE13898/37203), colon cancer (GSE28000/28722), HCC
683 (GSE10141/45436/54236), lung cancer (GSE3141), and breast cancer (GSE7849/50948).

684

685 **Figure 6.** Significance of IGF2 in 5-FU chemoresistance and impact of high Id1 and IGF2
686 expression on survival of cancer patients. **A**, left panel, Western blot showed that IGF2
687 knockdown significantly reduced p-AKT and thymidylate synthase (TS) expressions. Middle
688 and right panels, the FR cells stably transfected with shIGF2 or non-effective shRNA
689 expression plasmids were treated with 5-FU (20 μM) or DMSO in the presence or absence of
690 exogenous IGF2 (50 ng/ml) for four days; cell proliferation was determined by BrdU
691 incorporation assay, and the expression levels of caspase-3 and cleaved caspase-3 were
692 compared by Western blot. **B**, 5-FU treatment for three weeks significantly reduced the size

693 of the KYSE410FR-shIGF2 tumors, but not the KYSE410FR-shCON tumors (n = 6). **C**,
694 esophageal cancer cells with ectopic Id1 (left panel) or E2F1 (right panel) expression and the
695 vector control cells were treated with 5-FU (10 μM) or DMSO for 48 h, and cell proliferation
696 compared using BrdU incorporation assay. Note that shRNA or neutralizing antibody against
697 IGF2 (0.5 μg/ml) ameliorated the Id1- and E2F1-induced chemoresistance to 5-FU. **D**,
698 proposed model illustrating the regulatory roles of Id1 and IGF2 in 5-FU chemoresistance. **E**,
699 Kaplan-Meier curves comparing survival rates of ESCC patients (n = 35) dichotomized into
700 high Id1/high IGF2- and low Id1/low IGF2-expressing groups. **F**, Kaplan-Meier plots based
701 on GEO datasets of colon cancer (GSE28722; n = 125), HCC (GSE54236; n = 81), lung
702 cancer (GSE3141; n = 111), and breast cancer (GSE7849; n = 78) patients. The results
703 consistently showed that high Id1 and IGF2 expression is significantly associated with shorter
704 survival. Bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control cells
705 unless otherwise indicated.

706

707

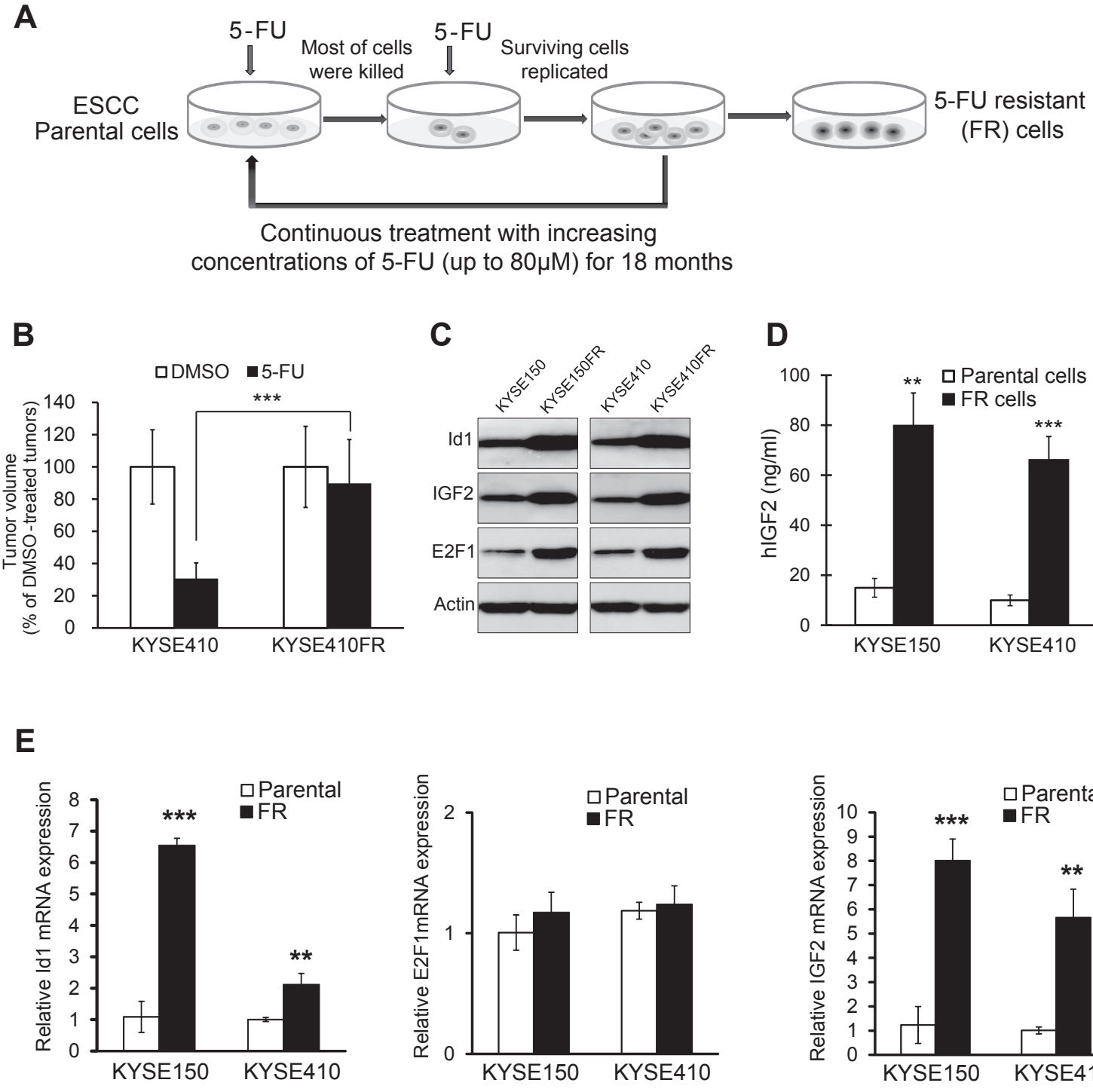

A
5-FU
Most of cells were killed
5-FU
Surviving cells replicated
ESCC Parental cells
5-FU resistant (FR) cells
Continuous treatment with increasing concentrations of 5-FU (up to 80μM) for 18 months
B
DMSO
5-FU
***
Tumor volume (% of DMSO-treated tumors)
KYSE410
KYSE410FR
C
KYSE150
KYSE150FR
KYSE410
KYSE410FR
Id1
IGF2
E2F1
Actin
D
Parental cells
FR cells
**
***
hIGF2 (ng/ml)
KYSE150
KYSE410
E
Parental
FR
Relative Id1 mRNA expression
Relative E2F1mRNA expression
Relative IGF2 mRNA expression
KYSE150
KYSE410

Figure 1

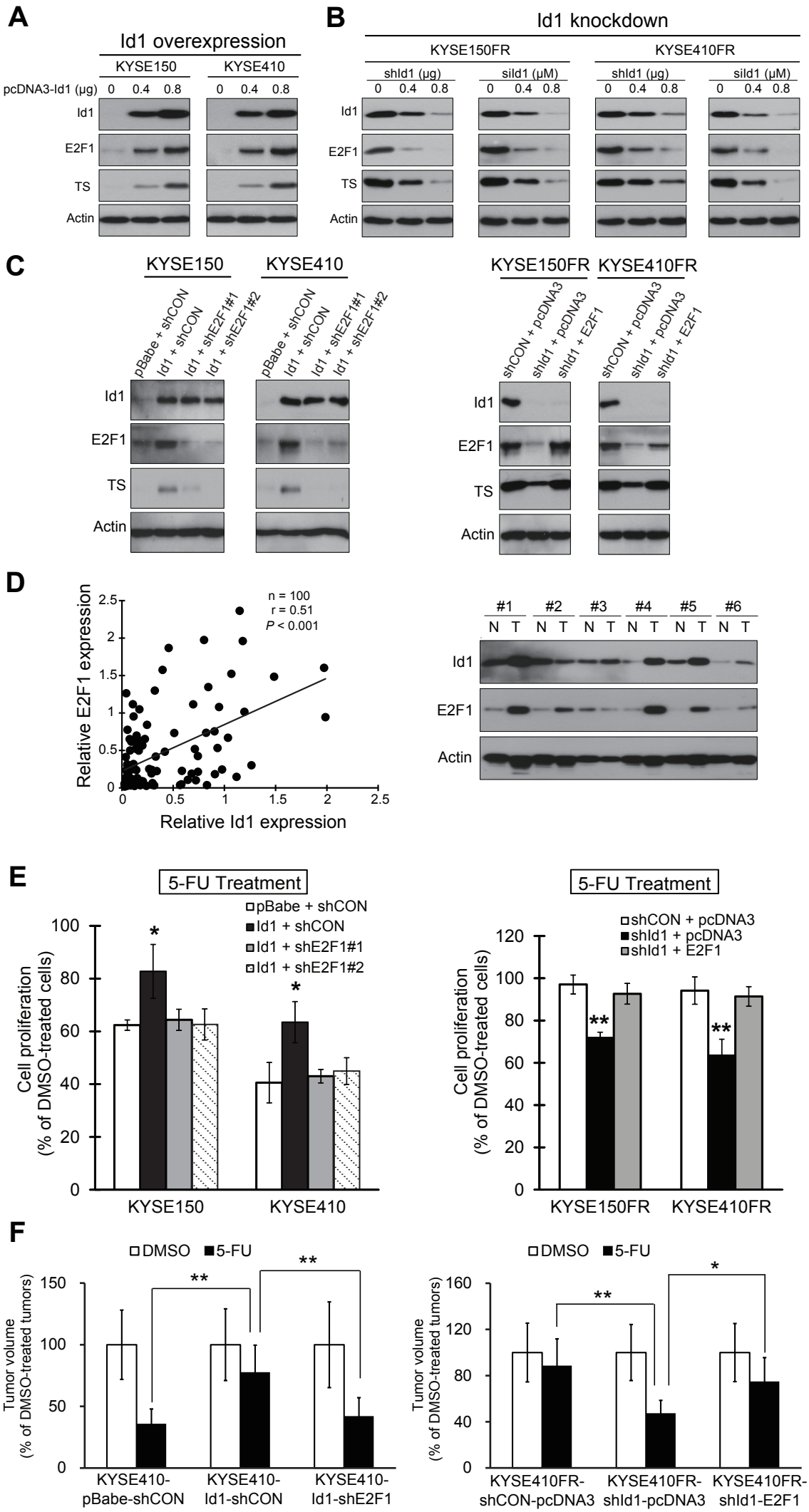
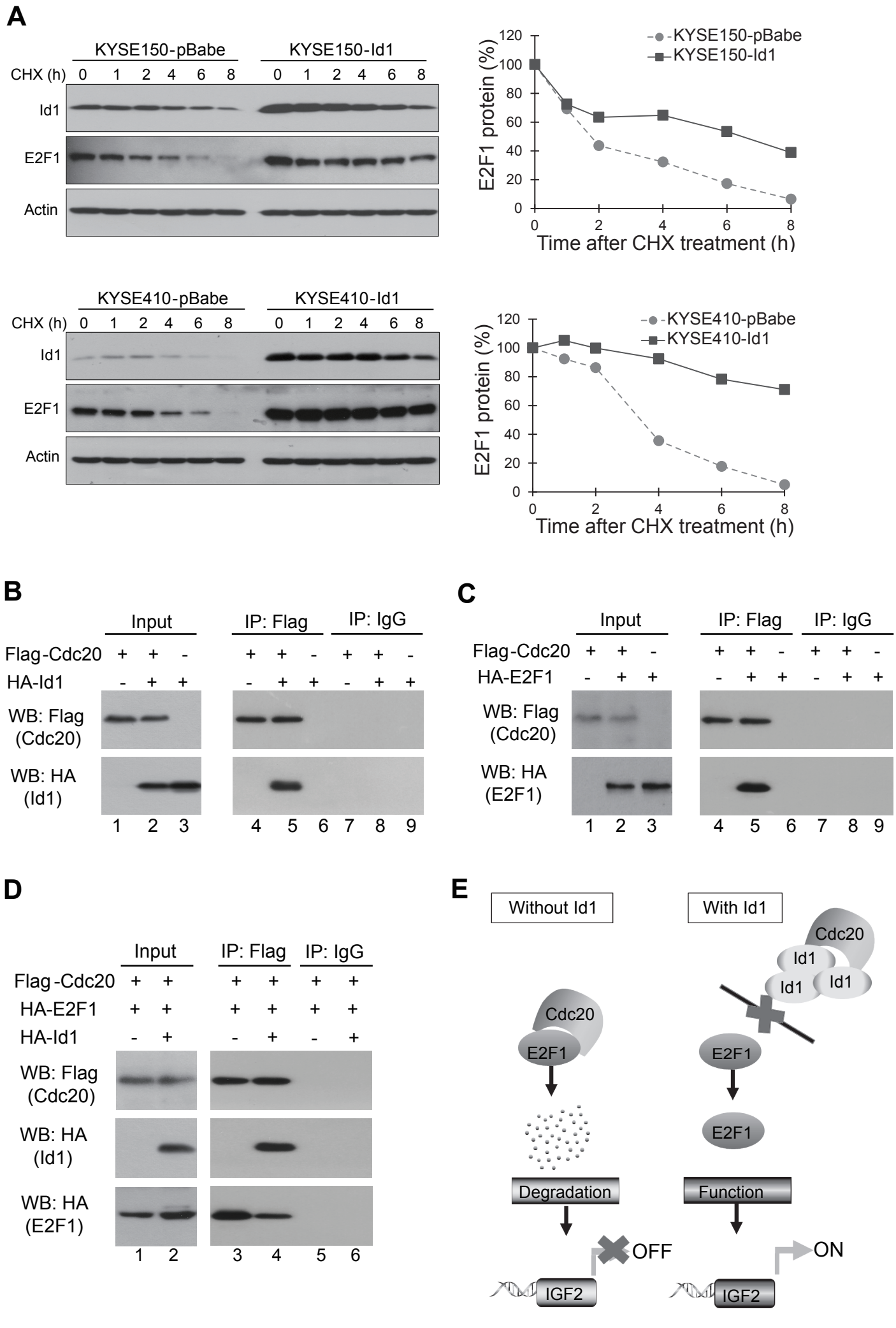

A
Id1 overexpression
KYSE150
KYSE410
pcDNA3-Id1 (μg) 0 0.4 0.8 0 0.4 0.8
Id1
E2F1
TS
Actin
B
Id1 knockdown
KYSE150FR
KYSE410FR
shId1 (μg)
siId1 (μM)
0 0.4 0.8
Id1
E2F1
TS
Actin
C
KYSE150
KYSE410
pBabe + shCON
Id1 + shCON
Id1 + shE2F1#1
Id1 + shE2F1#2
KYSE150FR
KYSE410FR
shCON + pcDNA3
shId1 + pcDNA3
shId1 + E2F1
Id1
E2F1
TS
Actin
D
n = 100
r = 0.51
P < 0.001
Relative E2F1 expression
Relative Id1 expression
#1 #2 #3 #4 #5 #6
N T N T N T N T N T N T
Id1
E2F1
Actin
E
5-FU Treatment
pBabe + shCON
Id1 + shCON
Id1 + shE2F1#1
Id1 + shE2F1#2
Cell proliferation (% of DMSO-treated cells)
KYSE150
KYSE410
5-FU Treatment
shCON + pcDNA3
shId1 + pcDNA3
shId1 + E2F1
Cell proliferation (% of DMSO-treated cells)
KYSE150FR
KYSE410FR
F
DMSO
5-FU
Tumor volume (% of DMSO-treated tumors)
KYSE410-pBabe-shCON
KYSE410-Id1-shCON
KYSE410-Id1-shE2F1
DMSO
5-FU
Tumor volume (% of DMSO-treated tumors)
KYSE410FR-shCON-pcDNA3
KYSE410FR-shId1-pcDNA3
KYSE410FR-shId1-E2F1

Figure 2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KYSE150-Id1
CHX (h) 0 1 2 4 6 8 0 1 2 4 6 8
Id1
E2F1
Actin
KYSE150-pBabe
KYSE150-Id1
E2F1 protein (%)
Time after CHX treatment (h)
KYSE410-pBabe
KYSE410-Id1
CHX (h) 0 1 2 4 6 8 0 1 2 4 6 8
Id1
E2F1
Actin
KYSE410-pBabe
KYSE410-Id1
E2F1 protein (%)
Time after CHX treatment (h)
B
Input
IP: Flag
IP: IgG
Flag-Cdc20 + + - + + - + + -
HA-Id1 - + + - + + - + +
WB: Flag (Cdc20)
WB: HA (Id1)
1 2 3 4 5 6 7 8 9
C
Input
IP: Flag
IP: IgG
Flag-Cdc20 + + - + + - + + -
HA-E2F1 - + + - + + - + +
WB: Flag (Cdc20)
WB: HA (E2F1)
1 2 3 4 5 6 7 8 9
D
Input
IP: Flag
IP: IgG
Flag-Cdc20 + + + + + +
HA-E2F1 + + + + + +
HA-Id1 - + - + - +
WB: Flag (Cdc20)
WB: HA (Id1)
WB: HA (E2F1)
1 2 3 4 5 6
E
Without Id1
With Id1
Cdc20
E2F1
Id1
Degradation
Function
OFF
ON
IGF2

Figure 3

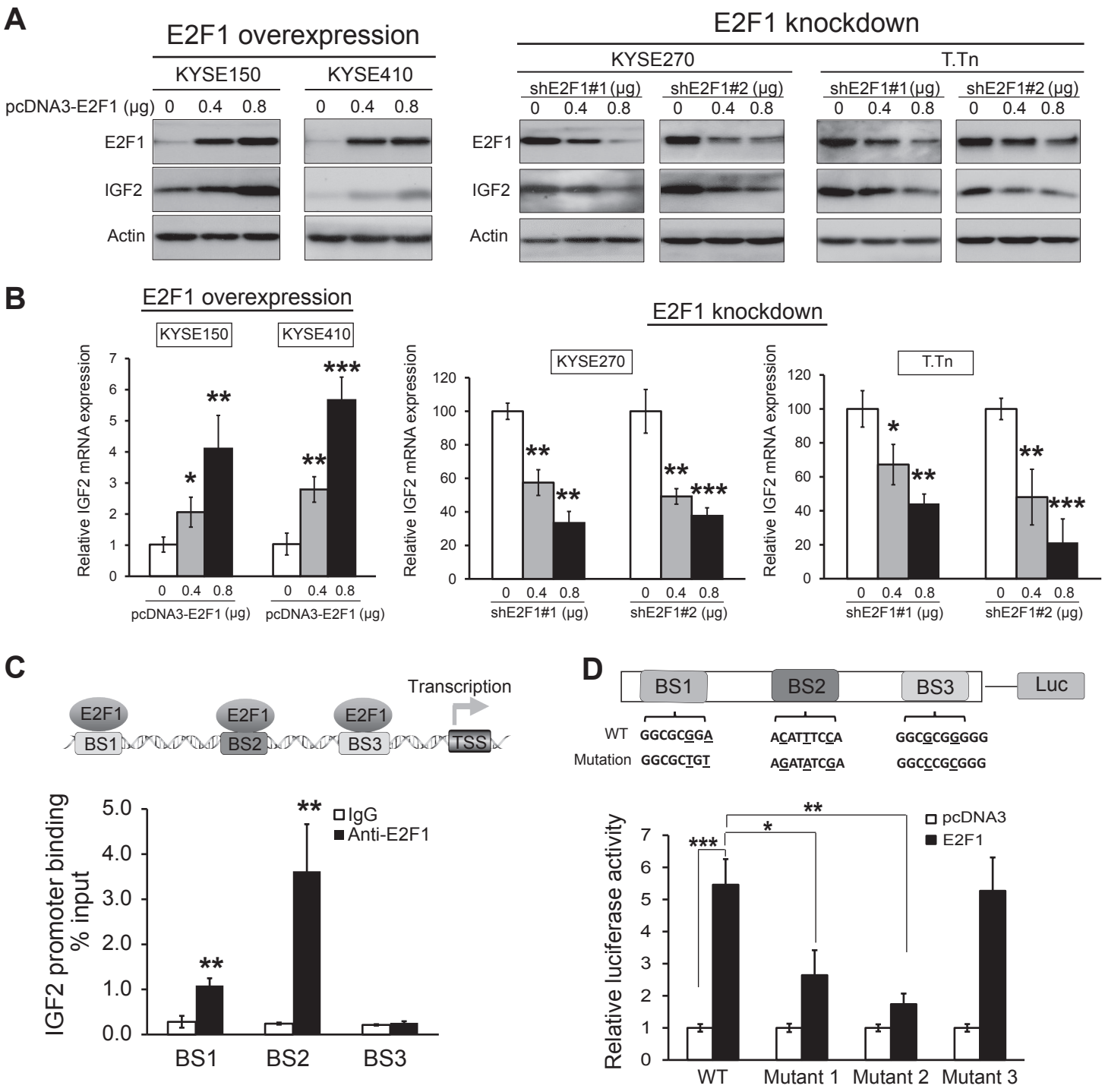
A
E2F1 overexpression
KYSE150
KYSE410
pcDNA3-E2F1 (μg) 0 0.4 0.8
E2F1
IGF2
Actin
E2F1 knockdown
KYSE270
T.Tn
shE2F1#1 (μg)
shE2F1#2 (μg)
B
E2F1 overexpression
Relative IGF2 mRNA expression
pcDNA3-E2F1 (μg)
E2F1 knockdown
shE2F1#1 (μg)
shE2F1#2 (μg)
C
Transcription
E2F1
BS1
BS2
BS3
TSS
IGF2 promoter binding % input
IgG
Anti-E2F1
D
Luc
WT GGCGCGGA ACATTCCA GGCGCGGGGG
Mutation GGCGCTGT AGATATCGA GGCCCGCGGG
Relative luciferase activity
pcDNA3
E2F1
WT
Mutant 1
Mutant 2
Mutant 3
E
KYSE150
KYSE410
pBabe + shCON
Id1 + shCON
Id1 + shE2F1#1
Id1 + shE2F1#2
Id1
E2F1
IGF2
Actin
F
KYSE270
T.Tn
shCON+ pcDNA3
shId1+ pcDNA3
shId1 + E2F1

Figure 4

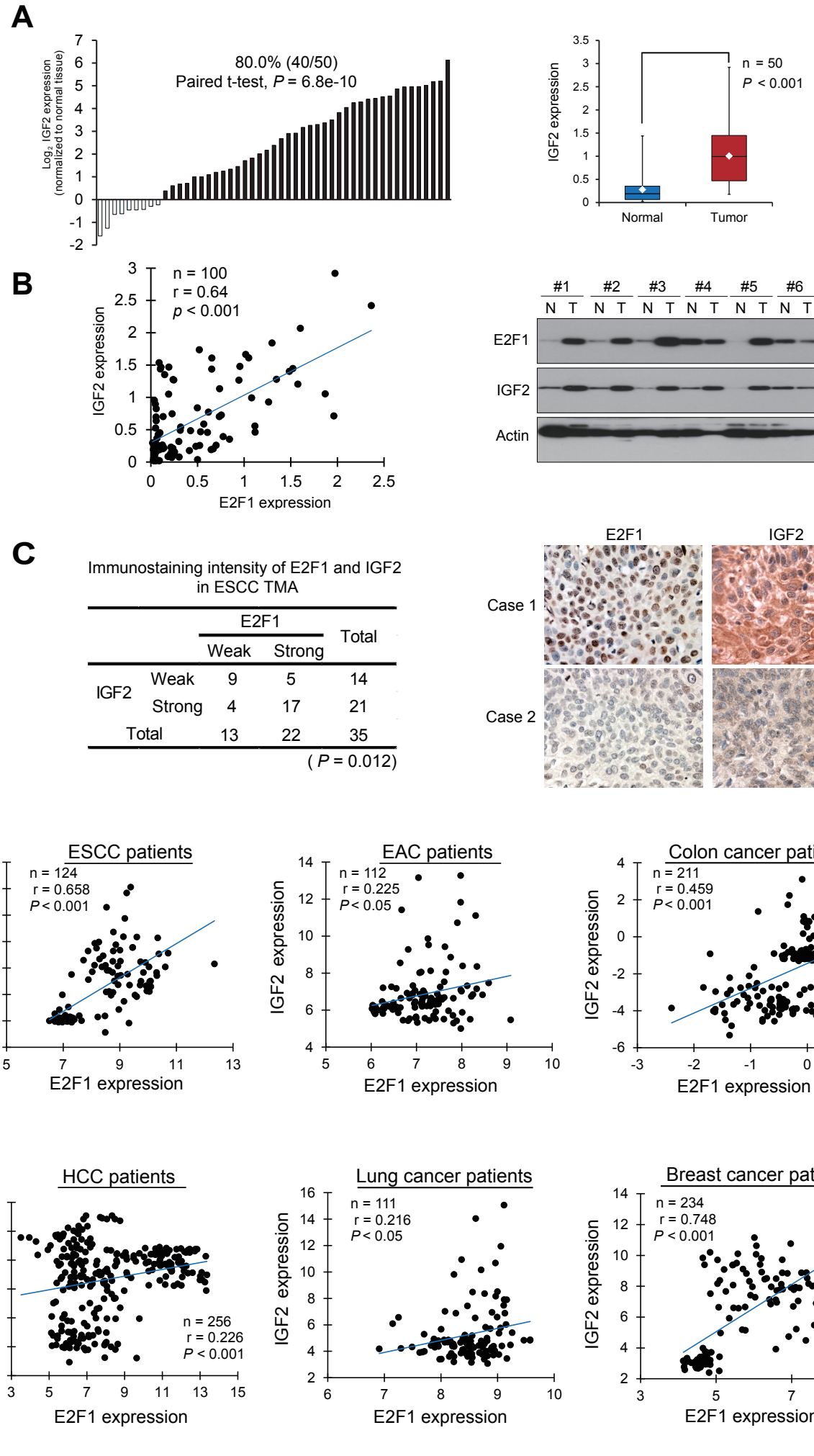
A
80.0% (40/50)
Paired t-test, P = 6.8e-10
Log2 IGF2 expression (normalized to normal tissue)
n = 50
P < 0.001
IGF2 expression
Normal
Tumor
B
n = 100
r = 0.64
p < 0.001
IGF2 expression
E2F1 expression
#1 #2 #3 #4 #5 #6
N T N T N T N T N T N T
E2F1
IGF2
Actin
C
Immunostaining intensity of E2F1 and IGF2 in ESCC TMA
E2F1
Weak Strong Total
IGF2 Weak 9 5 14
Strong 4 17 21
Total 13 22 35
(P = 0.012)
E2F1
IGF2
Case 1
Case 2
D
ESCC patients
n = 124
r = 0.658
P < 0.001
EAC patients
n = 112
r = 0.225
P < 0.05
Colon cancer patients
n = 211
r = 0.459
P < 0.001
HCC patients
n = 256
r = 0.226
P < 0.001
Lung cancer patients
n = 111
r = 0.216
P < 0.05
Breast cancer patients
n = 234
r = 0.748
P < 0.001
IGF2 expression
E2F1 expression

Figure 5

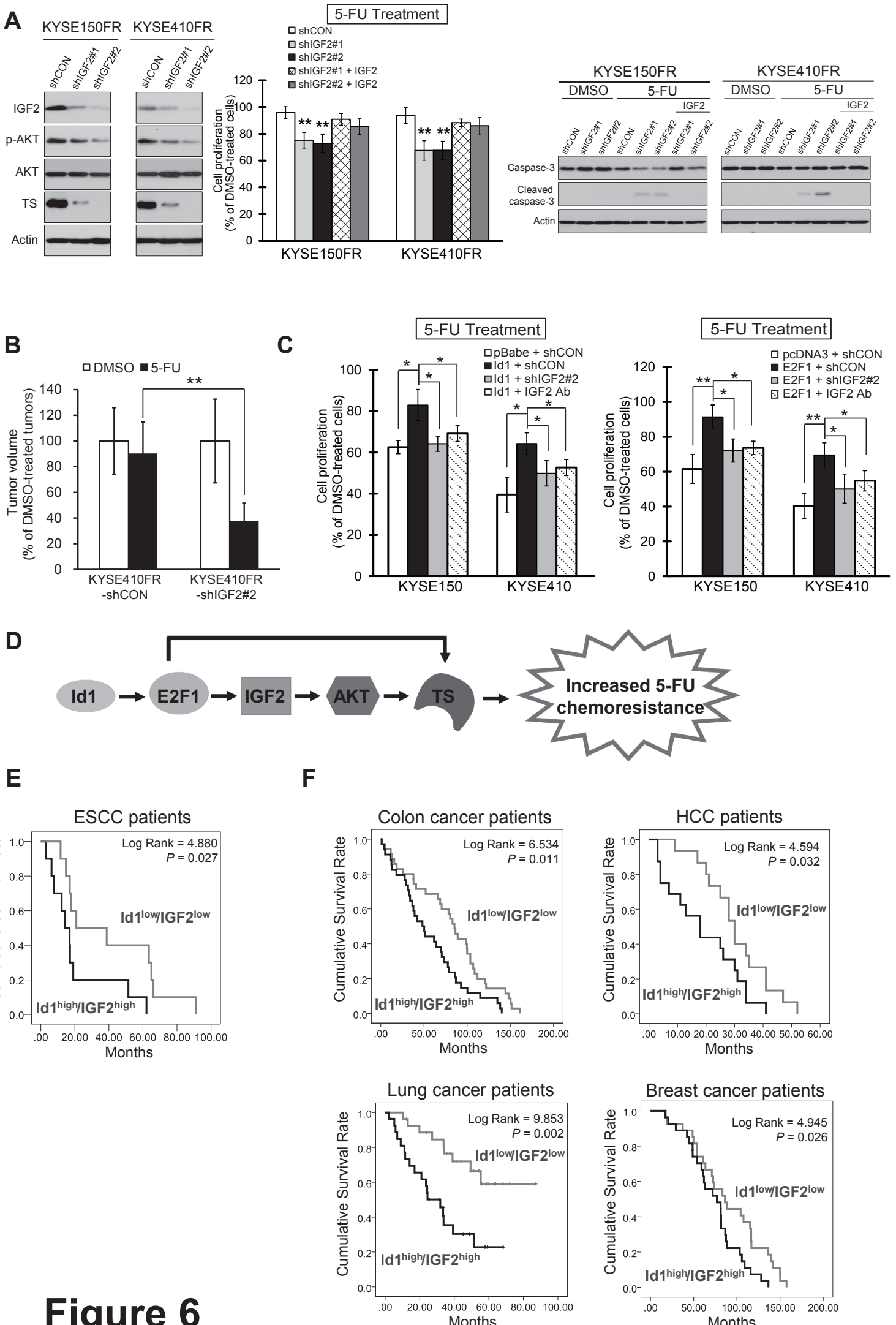
A
KYSE150FR
KYSE410FR
shCON
shIGF2#1
shIGF2#2
IGF2
p-AKT
AKT
TS
Actin
5-FU Treatment
shCON
shIGF2#1
shIGF2#2
shIGF2#1 + IGF2
shIGF2#2 + IGF2
Cell proliferation (% of DMSO-treated cells)
KYSE150FR
KYSE410FR
DMSO
5-FU
IGF2
Caspase-3
Cleaved caspase-3
Actin
B
DMSO
5-FU
Tumor volume (% of DMSO-treated tumors)
KYSE410FR -shCON
KYSE410FR -shIGF2#2
C
5-FU Treatment
pBabe + shCON
Id1 + shCON
Id1 + shIGF2#2
Id1 + IGF2 Ab
Cell proliferation (% of DMSO-treated cells)
KYSE150
KYSE410
5-FU Treatment
pcDNA3 + shCON
E2F1 + shCON
E2F1 + shIGF2#2
E2F1 + IGF2 Ab
Cell proliferation (% of DMSO-treated cells)
KYSE150
KYSE410
D
Id1
E2F1
IGF2
AKT
TS
Increased 5-FU chemoresistance
E
ESCC patients
Log Rank = 4.880
P = 0.027
Id1low/IGF2low
Id1high/IGF2high
Cumulative Survival Rate
Months
F
Colon cancer patients
Log Rank = 6.534
P = 0.011
HCC patients
Log Rank = 4.594
P = 0.032
Lung cancer patients
Log Rank = 9.853
P = 0.002
Breast cancer patients
Log Rank = 4.945
P = 0.026
Cumulative Survival Rate
Months

Figure 6