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Massachusetts Institute of Technology

Activation of GATA binding protein 6 (*GATA6*) sustains oncogenic lineage-survival in esophageal adenocarcinoma

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Gene amplification is a tumor-specific event during malignant transformation. Recent studies have proposed a lineage-dependency (addiction) model of human cancer whereby amplification of certain lineage transcription factors predisposes a survival mechanism in tumor cells. These tumor cells are derived from tissues where the lineage factors play essential developmental and maintenance roles. Here, we show that recurrent amplification at 18q11.2 occurs in 21% of esophageal adenocarcinomas (EAC). Utilization of an integrative genomic strategy reveals a single gene, the embryonic endoderm transcription factor *GATA6*, as the selected target of the amplification. Overexpression of *GATA6* is found in EACs that contain gene amplification. We find that EAC patients whose tumors carry *GATA6* amplification have a poorer survival. We show that ectopic expression of *GATA6*, together with *FGFR2* isoform IIIb, increases anchorage-independent growth in immortalized Barrett's esophageal cells. Conversely, siRNA-mediated silencing of *GATA6* significantly reduces both cell proliferation and anchorage-independent growth in EAC cells. We further demonstrate that induction of apoptotic/anoikis pathways is triggered upon silencing of *GATA6* in EAC cells but not in esophageal squamous cells. We show that activation of p38α signaling and up-regulation of TNF-related apoptosis-inducing ligand are detected in apoptotic EAC cells upon *GATA6* deprivation. We conclude that selective gene amplification of *GATA6* during EAC development sustains oncogenic lineage-survival of esophageal adenocarcinoma.

lineage-survival oncogene | transcriptional reprogramming | extrinsic apoptosis pathway | p38α and TNF-related apoptosis-inducing ligand activation

Esophageal adenocarcinoma (EAC) is a highly lethal malignancy of the distal esophagus with a 5-y survival rate of only 10–15%. The incidence of EAC has increased 300–500% in the past three decades in Western countries (1). Chronic gastroesophageal reflux disease is a major risk factor for development of Barrett's esophagus, a condition whereby normal squamous epithelia of the distal esophagus are replaced by epithelia of intestinal metaplasia. Barrett's esophagus may predispose patients to the development of EAC; rates of transformation to cancer are estimated at 0.5% per year for patients with Barrett's metaplasia and 10% per year for those with dysplasia (2).

Chromosomal aneuploidy and mutations/deletions of the tumor-suppressor genes, *p16/CDKN2A* and *TP53*, are prevalent and occur early in the progression from Barrett's metaplasia to EAC (3, 4). These somatic changes, however, are hallmarks for many, if not all, cancer types and lack specificity for EAC origin (5). DNA copy number increase is another common event in EAC, and individual amplified loci identified in EAC demonstrate tumor-type specificities that may be essential for the malignant transformation in this disease (6–8). To date, the key molecular pathways and mechanisms that underlie malignant transformation from Barrett's metaplasia to EAC remain undetermined.

Cancer development is a multistage process involving both activation of oncogenes and inactivation of tumor suppressor genes (9). Gene amplification is one mechanism for the activation of oncogenes, and this activation can be causative for tumorigenesis (10, 11). Despite the complexity of genetic, epigenetic, and chromosomal abnormalities in a given cancer, inactivation of a single or a few initiating oncogenes may impair tumor growth and survival, a phenotype termed "oncogene addiction" (12, 13). Recent studies of genomic amplification in cancer demonstrated that certain master regulatory factors, involved in both embryogenesis and subsequent tissue maintenance, are often selectively amplified in tumors arising from the lineages where the factors play an important developmental role (14–17). In the present study, we identify a highly amplified transcription factor, *GATA6*, in EAC using integrative genomic approaches and show that *GATA6* has properties of a lineage-survival oncogene in EAC.

MEDICAL SCIENCES

Results

Integrative Genomic Analysis in EAC Identifies Recurrent Amplification at 18q11.2 and a Single Selected Target Gene, *GATA6*. We performed array-based comparative genomic hybridization (array-CGH) in 20 EAC samples and identified genomic amplification at 18q11.2 as a recurrently amplified locus (Fig. 1*A*). Three of 20 EAC samples assayed by array-CGH showed amplification with one tumor (T8) containing an amplified unit about 706 kb that included only two genes, *GATA6* and *CTAGE1* (Fig. 1*A*, and *SI Appendix*, Fig. S1*A* and Table S1*A*). We next analyzed DNA dosage of five genes spanning a 1.5-Mb segment of the 18q11.2 region, including both *GATA6* and *CTAGE1*, in a cohort of 85 EACs using real-time PCR (qPCR) (Fig. 1*B* and *SI Appendix*, Fig. S2). The $2^{-\Delta\Delta Ct}$ calculation of cycle threshold was performed (18). The cutoff value was arbitrarily set at ≥1.9, which represented greater than 4N of the haploid genome, given ≥70% tumor cell content of the EAC samples studied. *GATA6* amplification was found in 18 of 85 EACs (21.2%), which is significantly higher than the other four genes coamplified in the amplicon ($P < 0.001$) (Fig. 1*C*, and *SI Appendix*, Fig. S1*C* and Table S1*C*). The highest DNA copy number (>36N) was also found in the GATA6 gene

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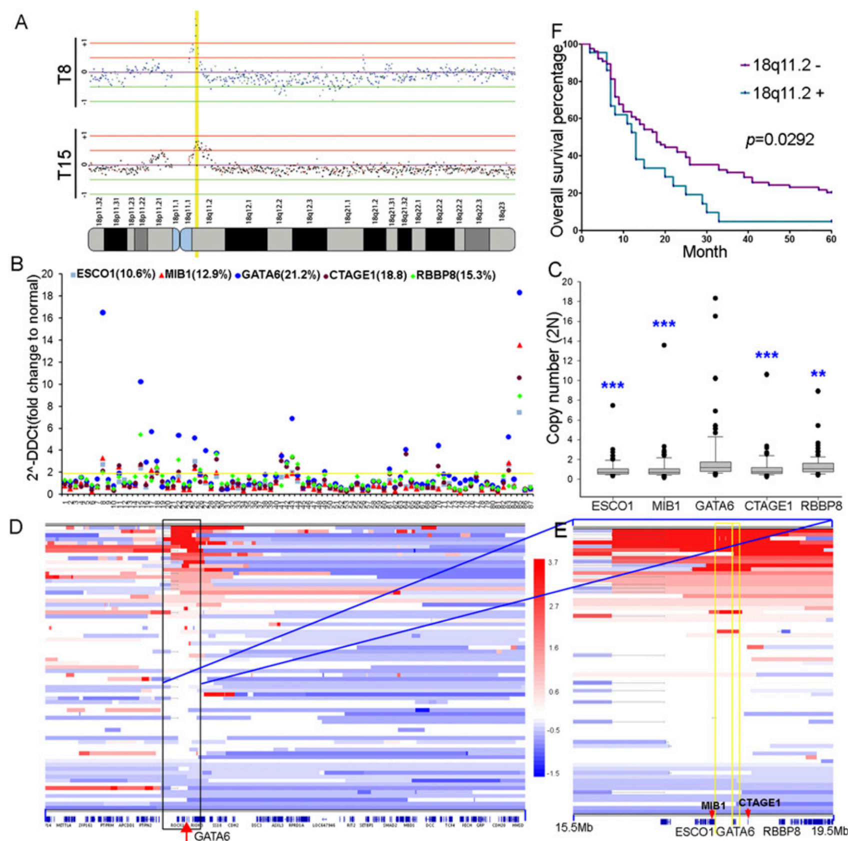

Fig. 1. Integrative genomic analysis of the recurrent amplification at chromosomal 18q11.2. (*A*) Array-CGH analyses of two representative EACs. A confined region with DNA copy number increase at 18q11.2 is identified. Yellow line highlights the core amplified-domain. (*B*) qPCR analyses of five genes spanning 1.5 Mb of the 18q11.2 amplicon in 85 EACs. The *y* axis shows an algorithm of $2^{-\Delta\Delta Ct}$ indicating the fold-change of a 2N genome and the *x* axis lists the tumor ID, of which sample 1 is a mean normal value. Numbers in parentheses represent amplification percentiles of the genes examined in 85 EACs. Yellow line highlights the cutoff value. All qPCR reactions were repeated in triplicate. (*C*) Boxplot of the qPCR data. *GATA6* demonstrates higher interquartile range, a larger upper whisker and more extreme upper-outliers than other genes within the amplicon and shows significant difference from the other four genes (***P* < 0.01, ****P* < 0.001; two-tailed, paired *t* test). (*D*) Recurrent amplifications of chromosome 18q11.2 in 73 EACs from SNP array data visualized in hg18 genome build using the IGV software. The *y* axis shows a descending $\log_2$ copy number ratio in 73 EACs. Horizontal bars represent individual tumor samples. The boxed area shows a 4-Mb region in the vicinity of the 18q11.2 amplicon with the arrow indicating the location of *GATA6*. (*E*) Magnified view of the 4-Mb region of the 18q11.2 amplicon from *D*. Boxed region with yellow lines shows the smallest amplified unit defined in 73 EACs. (*F*) Kaplan–Meier survival plots estimate a poorer clinical outcome (*P* = 0.0292) in EAC patients bearing the 18q11.2 amplicon in their tumors.

(Fig. 1 *B* and *C*). We further validated the 18q11.2 amplification in 73 of the 85 EAC samples using genome-wide 250 K Sty I SNP arrays. Consistent with array-CGH and qPCR results, the 18q11.2 amplicon was found to be a confined chromosomal segment with the core amplified-domain about 93 kb and including only *GATA6* (Fig. 1 *D* and *E*, and *SI Appendix*, Fig. S1*B* and Table S1*B*). *GATA6* was found to be amplified in 15 of 73 EACs examined (20.5%), with the cutoff value of $\log_2$ ratio ≥ 0.848 (16). The results of *GATA6* amplification in these samples assayed both by SNP array and qPCR were highly correlated (r = 0.92, P < 0.0001). Kaplan–Meier survival analysis in 97 EAC samples indicated that patients with the *GATA6* amplicon had a poorer survival (P = 0.0292) (Fig. 1*F*) and the amplification was not related to tumor stage (x^2 = 2.962, P = 0.0853) (*SI Appendix*, Table S3). In addition, we did not find any consistent deletion at the *GATA6* locus in 73 EAC SNP arrays (Fig. 1 *D* and *E*). We further examined eight additional SNP markers dispersed in the GATA6 gene region and sequenced the full-length *GATA6* coding region in 22 EACs (*SI Appendix*, Table S4). We did not find any mutations in the *GATA6* coding sequence or deletions at the *GATA6* locus.

Gene Amplification Drives the Overexpression of *GATA6* in EACs. Transcriptional expression of *GATA6* among 30 EACs, including all amplified tumor specimens available, was assessed using quantitative RT-PCR (qRT-PCR) (Fig. 2*A* and *SI Appendix*, Fig. S2*C*). The value of $2^{-\Delta\Delta Ct} \geq 2$ (twofold) relative to normal intestinal RNA was set as the threshold for gene overexpression. Fourteen of these 30 EAC samples contained *GATA6* amplification; among them, 13 (93%) were found to overexpress *GATA6* (r = 0.850, P < 0.0001). Only 9 of 30 EACs were found to have *MIB1* amplification (*SI Appendix*, Fig. S1*C*), and five of these nine samples overexpressed *MIB1* (r = 0.073, P = 0.8406) (Fig. 2 *A* and *B*). The change in *GATA6* expression in tumors containing *GATA6* amplification was significantly greater than that in tumors without *GATA6* amplification (P < 0.001) or in tumors with or without *MIB1* amplification (P < 0.001) (Fig. 2*B*). Overexpression of GATA6 protein in these cases was confirmed using both Western blot and immunohistochemistry with an esophageal tissue microarray (TMA) (Fig. 2 *C* and *D*). Ten of 13 *GATA6*-amplified EAC TMA cores demonstrated strong staining, whereas only seven were found to contain *MIB1*amplification and four of the seven *MIB1*-amplified EACs had positive MIB1 staining (Fig. 2*D*). Furthermore, when we analyzed a multicancer study of gene-expression profiling using the Oncomine database (www.oncomine.com), we found that *GATA6* was one of the signature genes with high expression that distinguishes gastrointestinal carcinomas from other tumor types (*SI Appendix*, Fig. S3).

Ectopic Expression of *GATA6* Increases Anchorage-Independent Growth in Immortalized Barrett's Cells in Collaboration with *FGFR2*IIIb. Given that *GATA6* is an embryonic gut lineage transcription factor and that gene amplification of *GATA6* is selected during development of EAC, we hypothesized that *GATA6* exerts an oncogenic lineage-survival role in EAC. We found that *GATA6* alone was not transforming, as determined by anchorage-independent assays in 3T3, RK3E, and immortalized Barrett's CP-A (Fig. 3*A*) cells. We then examined whether *GATA6* was transforming in collaboration with other genetic events. Analysis of our EAC U133A array data showed that expression of *FGFR2*, a receptor tyrosine kinase and an oncogene amplified in gastric cancer (19), was one of the top 50 genes significantly correlated with *GATA6* expression (r = 0.58, P < 0.0001) (*SI Appendix*, Fig. S4*A*). We also found that *FGFR2* and *GATA6* were coamplified in one EAC (*SI Appendix*, Fig. S4*B*). Additionally, we recently reported that when *FGFR2*IIIb was ectopically coexpressed with *SOX2*, a foregut lineage-survival oncogene in squamous epithelial malignancies, transformation of immortalized tracheobronchial epithelial cells was observed (16). The cooperative transforming effect between *GATA6* and *FGFR2*IIIb was assessed using soft-agar assays in both transiently transduced CP-A ($p16^{-/-}/TP53^{WT}$) Barrett's cells (Fig. 3*A*) and in CP-A/*FGFR2*IIIb stable cells that were infected with the *GATA6*

Fig. 2. Overexpression of *GATA6* driven by gene amplification in EACs. (*A*) qRT-PCR analysis of 30 EACs. The *y* axis shows fold-changes ($2^{-\Delta\Delta Ct}$) of gene expression relative to the normal intestinal tissue (IntN) as *GATA6* expression was found to be extremely low or absent in esophageal squamous epithelia (e.g., 43N, and N27 in *C*). Overexpression of *GATA6* was detected in 13 of 14 EACs containing the *GATA6* amplicon. *GATA6* up-regulation was also observed in a subset of dysplastic Barrett's samples (e.g., 19B). All qRT-PCR reactions were repeated three times. (*B*) Boxplot analysis of the qRT-PCR data. The *y* axis represents fold-changes in gene expression relative to the expression of normal intestinal RNA (***$P < 0.001$, Student's *t* test). (*C*) Western blot analysis. Only a small set of primary tissues were examined because of sample availability. Overexpression of GATA6 is shown in a *GATA6*-amplified EAC (T27) but not in EACs without the *GATA6* amplicon (T34 and T78). Samples T27 (EAC), G27 (normal gastric), and N27 (normal esophageal squamous mucosa) were derived from the same patient. (*D*) Immunohistochemistry of GATA6 and MIB1 in EAC TMAs. Overexpression of GATA6 protein was detected in EACs with amplified *GATA6* (representative T27, T70, and T83) compared with EAC without *GATA6* amplification (T9) (Magnification ×10). MIB1 expression was only observed in tumor T83 that contains MIB1 amplification (Magnification ×20).

Fig. 3. Colony formation assays following ectopic expression of *GATA6*. (*A*) Enhanced cell transformation in immortalized Barrett's CP-A cells following transient cotransduction of *GATA6* and *FGFR2IIIb* compared with $KRAS^{12v}$ positive control. (*B*) CP-A/*FGFR2IIIb* stable cells transduced with *GATA6* pBMN6 compared with LacZ control. Significant increase of anchorage-independent growth was observed in CP-A/*FGFR2IIIb* stable cells transduced with *GATA6*. Colony count was performed using ImageJ software. qRT-PCR of *FGFR2* and *GATA6* was used to monitor transduction efficiency (Magnification ×1.5; **$P < 0.01$, ***$P < 0.001$, Student's *t* test). All transduction and colony formation assays were conducted in triplicate.

construct pBMN6 (Fig. 3*B*). We found that *GATA6* conferred significantly enhanced anchorage-independent growth in CP-A cells in the presence of *FGFR2*IIIb (Fig. 3), although the mechanism of interdependency for cellular transformation between *GATA6* and *FGFR2IIIb* is yet to be determined.

There are only a few EAC cell lines available worldwide. The cell lines used in this study, including Flo-1 and OE33 (*SI Appendix*, Table S5) EAC lines, CP-A and CP-B immortalized Barrett's cells (20), and Het-A1 and TE13 esophageal squamous cell lines, expressed different levels of *GATA6* and none have *GATA6* amplification, except for TE13, which has 4–5N copy numbers of *GATA6* relative to Flo-1 cells (*SI Appendix*, Fig. S5 and Table S6). We investigated whether a high level of ectopic expression of *GATA6* would alter cell proliferation in cultured EAC cells. When Flo-1 cells that expressed very limited endogenous *GATA6* were transduced with *GATA6* pBMN6 (*SI Appendix*, Fig. S5*B*), a consistent increase in cell proliferation was not observed (*SI Appendix*, *SI Note*, and Fig. S6 *A–C*). However, ectopic expression of *GATA6* in cells significantly increased DNA synthesis and S-phase cell cycle distribution in BrdU incorporation assays (*SI Appendix*, Fig. S6 *D* and *E*). A subsequent reduction of S-phase fraction at 48 h following *GATA6* transduction was also observed and we speculated that a temporal onset of inducible *p21*/*INKN1A* (21) might account for this observation (*SI Appendix*, Fig. S6 *F* and *G*), indicating the complexity of *GATA6* function in these cells.

We further investigated *GATA6* modulated tumorigenicity and tumor growth in immune compromised NOD SCID-γ (NSG) mice. Immortalized Barrett's CP-A cells or EAC FloA cells (FloA cells are derived from Flo-1 cells through soft agar selection) were transiently transduced with expression constructs either pBMN-GATA6(pBMN6) or pBMN-LacZ (pBMN-Z) and subjected to subcutaneous implantation (*SI Appendix*, Fig.S7*A*). Xenograft tumor growth was not observed when 2×10^6 CP-A or CP-A/*FGFR2*IIIb cells with pBMN6 or pBMN-Z were injected in NSG mice for 7 wk. Although most of the tumors that formed at 7 wk in NSG mice after injection with either 5×10^6 of FloA/pBMN-Z or FloA/pBMN6 cells had no significant difference in tumor volume and weight, one of six FloA/pBMN6 xenografts grew aggressively with significantly large tumors that invaded through adjacent muscle tissue to the abdomen and the bone of the hind leg (*SI Appendix*, Fig. S7 *A–E*).

GATA6 Sustains EAC Cell Growth and Survival in a Lineage-Specific Manner. We next analyzed the growth- and survival-dependency of *GATA6* in EAC, Barrett's and esophageal squamous cell lines that endogenously or exogenously express *GATA6* (*SI Appendix*, Fig. S5*B* and Table S6) using siRNA-mediated knockdown assays. Cell proliferation in both Barrett's cells (CP-A and CP-B) and EAC cells (OE33 and Flo-1 with stably transduced *GATA6*)

was decreased following knockdown of *GATA6*, with more significant reduction observed in EAC cells than in Barrett's cells (Fig. 4 *A* and *B*). In contrast, this reduction of cell proliferation upon knockdown of *GATA6* was not found in esophageal squamous cells Het-1A and TE13 (*SI Appendix*, Fig. S8). We further observed that siRNA-mediated silencing of *GATA6* significantly reduced anchorage-independent growth in OE33 cells (Fig. 4*C*). Morphological changes characteristic of cell death were observed in EAC cells but not in squamous cells (Fig. 4*D*). *GATA6* silencing induced significant DNA fragmentation indicative of cellular apoptosis in EAC cells (OE33) but not in non-EAC lines (TE13 and Het-1A), as determined by BrdU/TUNEL assays (Fig. 4 *E*–*G*). To further determine the apoptotic phenotypes induced upon silencing of *GATA6*, we assessed anoikis, a specific type of apoptosis (22), in Flo-1/*GATA6* stable cells. We found that silencing of *GATA6* enhanced anoikis as determined by increased poly(ADP-ribose) polymerase (PARP) cleavage, an indicator of caspase 3 activation (Fig. 5*A*). To validate the effects of siRNA-mediated silencing of *GATA6*, we performed assays using two additional siRNAs that targeted different coding sequences of *GATA6* (*SI Appendix*, Table S7). PARP cleavage was observed in all three siRNA-treated EAC OE33 cells, but not in squamous TE13 cells (Fig. 5*B*). In addition, all three siRNA fragments targeting *GATA6* significantly increased caspase 3 activity in OE33 cells compared with TE13 cells (Fig. 5*C*) and produced similar apoptotic phenotypes following *GATA6* knockdown (Fig. 5*D* and *SI Appendix*, Fig. S9*A*). Changes in cellular senescence were not found in non*GATA6*-expressing EAC Flo-1 cells upon ectopic expression of *GATA6* or in *siGATA6*-transfected OE33 cells using senescence-associated β-galactosidase staining (*SI Appendix*, Fig. S9*B*).

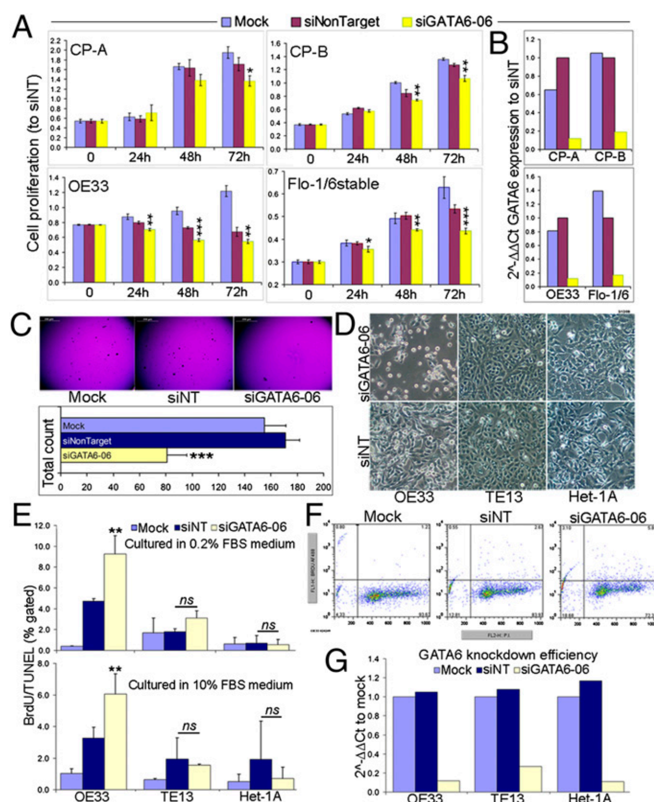

Fig. 4. Cell proliferation, anchorage-independent growth, and DNA fragmentation assays following siRNA-mediated silencing of *GATA6*. (*A*) Significant reduction of cell proliferation upon silencing of *GATA6* was observed in both immortalized Barrett's cells (CP-A and CP-B) and EAC OE33 and Flo-1/*GATA6* stable cells. WST-1 assays were conducted in quadruplicate (see *SI Appendix*, Fig. S8 for the nonlineage TE13 and Het-1A cells). (*B*) qRT-PCR of the matched experiments was performed to monitor the knockdown efficiency (up to 85–90%). (*C*) Significantly decreased colony formation was observed in *siGATA6*-06-treated OE33 cells compared with siNonTarget controls in soft-agar assays performed in triplicate (Magnification ×1.25). The *x* axis reflects number of colonies. (*D*) Brightfield microscopic images of the siRNA-mediated knockdown of *GATA6* in esophageal cells at 72 h (Magnification ×10). (*E*) A significant increase in DNA fragmentation upon *GATA6* knockdown, assayed by BrdU/TUNEL flow cytometry, was observed in OE33 cells in both 0.2% and 10% FBS media compared with esophageal squamous TE13 and Het-1A cells. (*F*) Representative images of BrdU/TUNEL flow cytometry assays in OE33 cells. An increased upper right quadrant cell population is shown in *GATA6* knockdown cells. (*G*) Quantitative verification of *GATA6* knockdown using qRT-PCR (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

Activation of p38α Signaling and Up-Regulation of TNF-Related Apopstosis-Inducing Ligand upon siRNA-Mediated *GATA6* Withdrawal in EAC Cells. Because *GATA6* is a spatial and temporal master regulator in embryonic development (23) and its deprivation causes massive apoptosis in embryonic ectoderm (24), we speculate that gene amplification-induced differential expression of *GATA6* in EAC may cause transcriptional reprogramming in tumor genomes. We used two model EAC cell lines, endogenously-limited *GATA6*-expressing Flo-1 cells and *GATA6*-expressing OE33 cells (*SI Appendix*, Fig. S5*B*), for transcriptional profiling using Affymetrix U133A arrays. Cell RNA was harvested at 24 h to assess an acute response and at 72 h for analysis of the sustained effect of *GATA6* regulation following transient transduction of *GATA6* (>900-fold) in Flo-1 cells or siRNA-mediated knockdown of *GATA6* (>90%) in OE33 cells (*SI Appendix*, Fig. S10*A*). Analysis of array data revealed that many genes in diverse cellular pathways were transcriptionally reprogrammed upon differential expression of *GATA6* (*SI Appendix*, Fig. S10*B*). We were particularly interested in the genes relative to apoptotic pathways following *GATA6* silencing in OE33 cells (*SI Appendix*, Table S8). The proapoptotic genes, *TNFSF10/TNF-related apopstosis-inducing ligand* (*TRAIL*), *XAF1*, and *DAPK2*, were among the top 100 genes up-regulated upon *GATA6*-silencing (Fig. 6*A* and *SI Appendix*, Table S8), and the results were validated in an

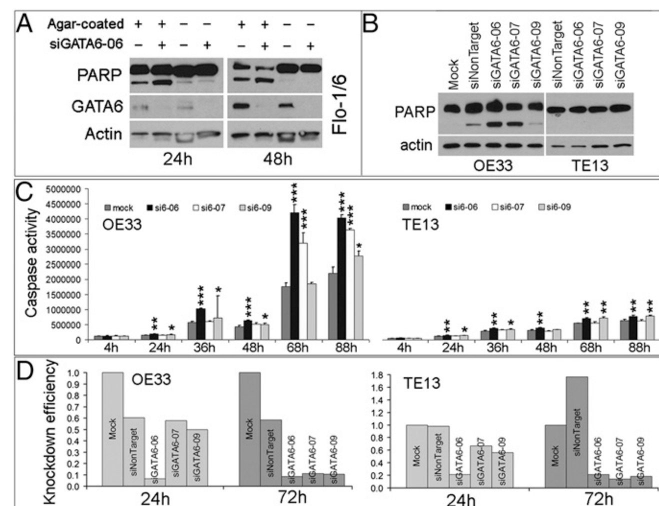

Fig. 5. Induction of apoptosis in *GATA6*-silenced esophageal cells. (*A*) Western blot analysis of PARP cleavage following *GATA6*-silencing was indicative of anoikis. Flo-1/*GATA6* stable cells were cultured on agar-coated plates followed by *GATA6* knockdown. (*B*) PARP cleavage by Western blot analysis was observed in OE33 but not in TE13 cells transfected with various *GATA6* siRNA fragments against three different *GATA6* coding sequences (*SI Appendix*, Table S7). (*C*) Caspase-Glo3/7 assays demonstrated that transfection of all three siRNA fragments targeting *GATA6* caused significant increases in caspase activity in OE33 cells compared with squamous TE13 cells (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001). (*D*) qRT-PCR assays to monitor knockdown efficiency of all three siRNA fragments targeting *GATA6* in OE33 and TE13 cells.

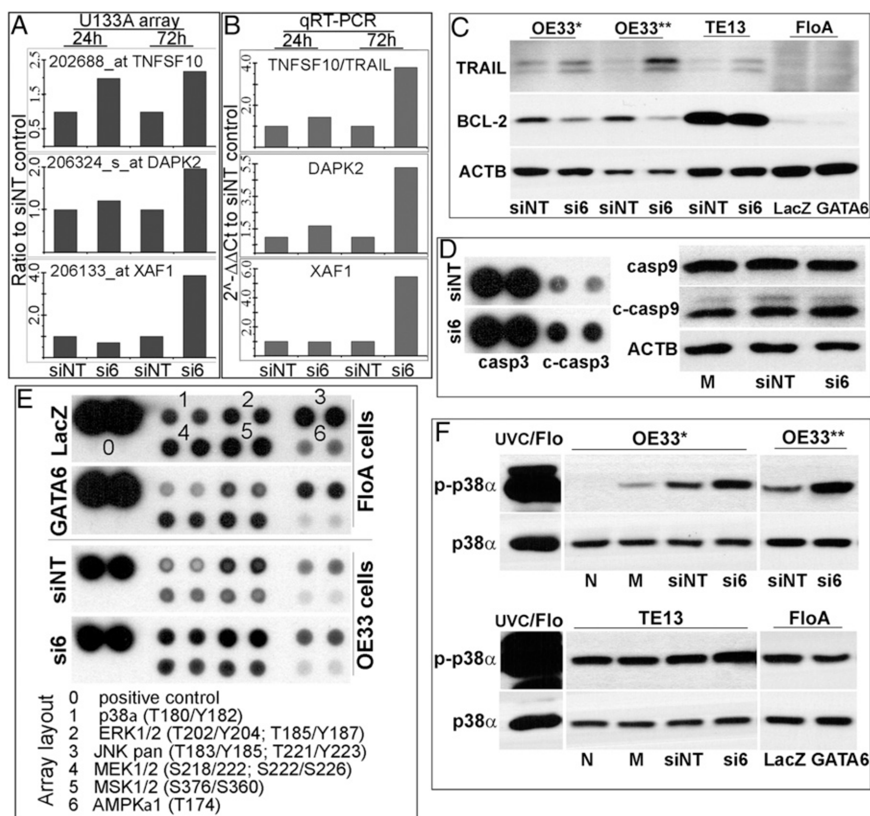

Fig. 6. Differentially regulated pro- and anti-apoptotic signals following *GATA6* modulation in EAC cells. (*A*) Three proapoptotic genes that were up-regulated upon silencing of *GATA6* in OE33 cells using U133A array assays. (*B*) Validation of expression profiling using real-time RT-PCR in an independent set of experiments. Ratios represent comparisons of *siGATA6*-treated cells to matched cells treated with siNonTarget control at the same time point. (*C*) Up-regulation of TRAIL and down-regulation of BCL-2 in cells treated with either *siGATA6* or pBMN6. OE33* and ** represent two independent experiments. (*D*) Analyses of procaspase 3 (casp3) and cleaved caspase 3 (c-casp3) expression in an apoptosis antibody array (*Left*) and Western blot of procaspase 9 (casp9) and cleaved caspase 9 (c-casp9) expression (*Right*) in OE33 cells treated with either siNonTarget control or *siGATA6* for 60h (M, mock). (*E*) Representative kinase activation from the analysis of 46 phosphorylated kinases in EAC cells with either ectopic expression (FloA) or silencing (OE33) of *GATA6* for 60 h using human phospho-kinase antibody array. Each phospho-kinase antibody is dotted in doublet. Sample layout is numbered in the upper panel and listed underneath. (*F*) Western blot analysis of p38α activation. Both OE33 and TE13 cells were treated for 60 h with *siGATA6* or controls and FloA cells were transduced with either pBMN-Z or pBMN6 for 60 h. Total p38α and phospho-p38α (p-p38α) were examined and protein extracted from UVC-irradiated Flo-1 cells was used as a positive control. (N, not treated; M, mock).

independent experiment with *siGATA6*-treated OE33 cells using real-time RT-PCR (Fig. 6*B*). TNFSF10/TRAIL is a death ligand of the TNF family and has been shown to preferentially induce apoptosis in transformed tumor cells (25). TRAIL protein was up-regulated whereas the prosurvival protein BCL-2 was down-regulated following 60-h treatment with *siGATA6* in apoptotic OE33 cells (Fig. 6*C*). The fact that cleaved caspase 3 but not caspase 9 was increased (Fig. 6*D*) in apoptotic OE33 cells treated with *siGATA6* indicates that *siGATA6*-induced apoptosis in EAC cells may be through the extrinsic apoptosis pathway. Using a phosphokinase array, we confirmed that the differential expression of *GATA6* in FloA or OE33 EAC cells led to modulations of many diverse kinase signaling pathways, including the three MAPK pathways, MEK1/2-ERK1/2, JNK, and p38α (Fig. 6*E*). In particular, we observed that stress-activated protein kinase p38α/MAPK14, which can induce apoptosis through several mechanisms, including activation of proapoptotic proteins and inactivation of prosurvival signals (26), was down-regulated in *GATA6*-transduced FloA cells and up-regulated in siGATA6-silenced OE33 cells (Fig. 6*E*), and the results were further validated by Western blot analyses (Fig. 6*F*).

Discussion

GATA6 is a member of the highly conserved GATA family, which is composed of six zinc-finger transcription factors that regulate lineage-restricted development, differentiation, and cellular aging (27–29). *GATA1-3* are essential for formation and differentiation of pluripotent and multipotent hematopoietic stem cells (30), whereas *GATA4-6* are indispensable for the lineage-specific development and differentiation of cells of endodermal and mesodermal origin (24, 31). Inactivation of *GATA6* in the mouse embryo causes embryonic lethality (24, 32). *GATA6* is thought to be a master regulator because inactivation of *GATA6* resulted in loss of expression of all hepatocyte nuclear factors in knockout mice (23, 33). In the adult gastrointestinal tract, *GATA6* is more localized and expressed within the proliferative and lineage stem-cell zone at the bottom of the gut crypts (34, 35).

Consistent with the idea that *GATA6* amplification is a lineage-specific activation, *GATA6* amplification was not observed in esophageal squamous carcinoma, as reported in our recent study (16). Interestingly, Kwei et al. (36) and Fu et al. (37) reported that 18q11.2 gain/amplification with overexpression of *GATA6* is detected in 9–19% of pancreatic carcinomas. Both the pancreas and distal esophagus are derived from the embryonic endodermal foregut, making it plausible that these two tumors may share a common lineage-survival oncogene. Although *GATA6* has been reported to be a tumor suppressor in glioma (38) and ovarian (39) cancers, which are tissues of nonendodermal origin, recent comprehensive studies have failed to uncover any evidence for genomic alterations of *GATA6* in these diseases (40, 41).

Amplification of lineage-survival oncogenes imposes survival mechanisms in tumor cells. These factors are otherwise involved in lineage precursor cell development and differentiation (14–17). We hypothesized that *GATA6* is a lineage-survival oncogene in EAC based on the fact that *GATA6* is a master regulator and stem cell-lineage transcription factor in embryogenesis and that *GATA6* amplification is a selective event during the development and progression of EAC. We demonstrated that siRNA-mediated silencing of *GATA6* decreased both cell proliferation and anchorage-independent growth in EAC cells and caused a variety of apoptotic phenotypes. The fact that direct tumorigenicity was not affirmed in immortalized Barrett's CP-A cells indicates that amplification-led overexpression of *GATA6* in EAC may impose survival and "stemness" to the esophageal cells under chronic attack from gastro-esophageal reflux and subsequent inflammatory environment, rather than play a role in EAC initiation or formation. We observed that modifying the expression of *GATA6* in EAC cells induced broad cellular responses. Specifically, we demonstrated that differential expression of *GATA6* caused changes in p38α activation, as well as modulation in the TRAIL-mediated apoptotic pathway. Clearly, further experiments are required to fully understand the oncogenic lineage-survival role of *GATA6* in cellular transformation and progression

of esophageal adenocarcinoma. In light of the lineage-addiction model of human cancer, our present study suggests that therapeutic deprivation of GATA6 in *GATA6*-amplified EAC patients may improve patient survival.

Materials and Methods

Patients and EAC Samples. All animal studies were conducted under the guidelines and approved protocols from the University Committee on Use and Care of Animal of the University of Michigan. Written consent was obtained from each patient according to the approval and guidelines of the University of Michigan institutional review board . Tissues were obtained from patients undergoing esophagectomy for adenocarcinoma at the University of Michigan Health System between 1991 and 2004. Patients in this study had no preoperative radiation or chemotherapy. Specimens were fresh-frozen in liquid nitrogen and stored at −80 °C until use. Cellularity of metaplastic, dysplastic, and tumor samples were assured to be greater than 70% before sample DNA, RNA, or protein was isolated. DNA, RNA, and protein isolation procedures are in *SI Appendix, SI Materials and Methods*.

Tiling Path Array-CGH and Data Analysis. DNA copy number profiles were generated for 20 EACs using a whole-genome tiling path array, as previously described (42). Data analysis details are in *SI Appendix, SI Materials and Methods*.

SNP Array Experiments and Analysis. SNP arrays were performed as previously described (16). Briefly, 73 EAC DNAs were genotyped using the Genome-Wide Human Sty I 250K SNP Array (Affymetrix). Copy number analyses with SNP arrays were performed as a $\log_2$ copy number ratio exceeding 0.848 for amplifications and −0.737 for deletions. Genomic positions were mapped in the hg18 genome build. SNP data were visualized using the software IGV 1.3.1 (Integrative Genomics Viewer, www.broadinstitute.org/igv).

Immunohistochemistry of TMAs. Briefly, TMA arrays contained 122 sections from 73 EAC patients, including 63 EAC sections, 18 mixed sections of EAC and dysplasia, 22 Barrett's metaplastic and dysplastic sections, 9 metastatic lymph nodes, and 10 normal sections of various tissue types. Procedure details are in *SI Appendix, SI Materials and Methods*.

Cell Lines and Culture Conditions. CP-A and CP-B cell lines were kind gifts from Peter Rabinovitch (University of Washington, Seattle, WA). CP-A and CP-B were derived from Barrett's metaplasia and high-grade dysplasia, respectively, and were immortalized through induction of *hTERT* (20). Procedure details are in *SI Appendix, SI Materials and Methods*.

Statistical Analysis. Kaplan-Meier survival was computed using the GraphPad Prism5 software and *P* values were determined by a log-rank test. Box plot analyses were determined using Sigma-Plot software. Analyses in *t*-test, one-way ANOVA, and correlation coefficient were applied for all necessary experiments.

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1. Brown LM, Devesa SS, Chow WH (2008) Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. *J Natl Cancer Inst* 100:1184–1187.
2. Shaheen NJ, Richter JE (2009) Barrett's oesophagus. *Lancet* 373:850–861.
3. Barrett MT, et al. (1999) Evolution of neoplastic cell lineages in Barrett oesophagus. *Nat Genet* 22:106–109.
4. Wild CP, Hardie LJ (2003) Reflux, Barrett's oesophagus and adenocarcinoma: Burning questions. *Nat Rev Cancer* 3:676–684.
5. Negrini S, Gorgoulis VG, Halazonetis TD (2010) Genomic instability—An evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11:220–228.
6. Miller CT, et al. (2003) Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. *Clin Cancer Res* 9:4819–4825.
7. Lin L, et al. (2000) A minimal critical region of the 8p22-23 amplicon in esophageal adenocarcinomas defined using sequence tagged site-amplification mapping and quantitative polymerase chain reaction includes the *GATA-4* gene. *Cancer Res* 60:1341–1347.
8. Lin L, et al. (2002) The hepatocyte nuclear factor 3 alpha gene, HNF3alpha (FOXA1), on chromosome band 14q13 is amplified and overexpressed in esophageal and lung adenocarcinomas. *Cancer Res* 62:5273–5279.
9. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70.
10. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 224:1121–1124.
11. Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* 396:643–649.
12. Weinstein IB (2002) Cancer. Addiction to oncogenes—The Achilles heal of cancer. *Science* 297:63–64.
13. Weinstein IB, Joe A (2008) Oncogene addiction. *Cancer Res* 68:3077–3080, discussion 3080.
14. Garraway LA, Sellers WR (2006) Lineage dependency and lineage-survival oncogenes in human cancer. *Nat Rev Cancer* 6:593–602.
15. Garraway LA, et al. (2005) Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 436:117–122.
16. Bass AJ, et al. (2009) SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat Genet* 41:1238–1242.
17. Kendall J, et al. (2007) Oncogenic cooperation and coamplification of developmental transcription factor genes in lung cancer. *Proc Natl Acad Sci USA* 104:16663–16668.
18. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–408.
19. Yoshida T, Sakamoto H, Terada M (1993) Amplified genes in cancer in upper digestive tract. *Semin Cancer Biol* 4:33–40.
20. Palanca-Wessels MC, et al. (2003) Extended lifespan of Barrett's esophagus epithelium transduced with the human telomerase catalytic subunit: A useful in vitro model. *Carcinogenesis* 24:1183–1190.
21. Perlman H, Suzuki E, Simonson M, Smith RC, Walsh K (1998) GATA-6 induces p21(Cip1) expression and G1 cell cycle arrest. *J Biol Chem* 273:13713–13718.
22. Liotta LA, Kohn E (2004) Anoikis: Cancer and the homeless cell. *Nature* 430:973–974.
23. Morrisey EE, Ip HS, Lu MM, Parmacek MS (1996) GATA-6: A zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev Biol* 177:309–322.
24. Morrisey EE, et al. (1998) GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev* 12:3579–3590.
25. Almasan A, Ashkenazi A (2003) Apo2L/TRAIL: Apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev* 14:337–348.
26. Dolado I, Nebreda AR (2008) Regulation of tumorigenesis by p38 MAP kinase. *Stress-Activated Protein Kinases, Topics in Current Genetics*, eds Posas F, Nebreda, AR (Springer, New York), Vol 20, pp 99–128.
27. Huggon IC, et al. (1997) Molecular cloning of human GATA-6 DNA binding protein: High levels of expression in heart and gut. *Biochim Biophys Acta* 1353:98–102.
28. Budovskaya YV, et al. (2008) An elt-3/elt-5/elt-6 GATA transcription circuit guides aging in *C. elegans*. *Cell* 134:291–303.
29. Burch JB (2005) Regulation of GATA gene expression during vertebrate development. *Semin Cell Dev Biol* 16:71–81.
30. Orkin SH (1992) GATA-binding transcription factors in hematopoietic cells. *Blood* 80:575–581.
31. Capo-Chichi CD, et al. (2005) Perception of differentiation cues by GATA factors in primitive endoderm lineage determination of mouse embryonic stem cells. *Dev Biol* 286:574–586.
32. Koutsourakis M, Langeveld A, Patient R, Beddington R, Grosveld F (1999) The transcription factor GATA6 is essential for early extraembryonic development. *Development* 126:723–732.
33. Fujikura J, et al. (2002) Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev* 16:784–789.
34. Gao X, Sedgwick T, Shi YB, Evans T (1998) Distinct functions are implicated for the GATA-4, -5, and -6 transcription factors in the regulation of intestine epithelial cell differentiation. *Mol Cell Biol* 18:2901–2911.
35. Haveri H, et al. (2008) Transcription factors GATA-4 and GATA-6 in normal and neoplastic human gastrointestinal mucosa. *BMC Gastroenterol* 8:9.
36. Kwei KA, et al. (2008) Genomic profiling identifies GATA6 as a candidate oncogene amplified in pancreatobiliary cancer. *PLoS Genet* 4:e1000081.
37. Fu B, Luo M, Lakkur S, Lucito R, Iacobuzio-Donahue CA (2008) Frequent genomic copy number gain and overexpression of GATA-6 in pancreatic carcinoma. *Cancer Biol Ther* 7:1593–1601.
38. Kamnasaran D, Qian B, Hawkins C, Stanford WL, Guha A (2007) GATA6 is an astrocytoma tumor suppressor gene identified by gene trapping of mouse glioma model. *Proc Natl Acad Sci USA* 104:8053–8058.
39. Caslini C, et al. (2006) Histone modifications silence the GATA transcription factor genes in ovarian cancer. *Oncogene* 25:5446–5461.
40. Anonymous; Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455:1061–1068.
41. Parsons DW, et al. (2008) An integrated genomic analysis of human glioblastoma multiforme. *Science* 321:1807–1812.
42. Lockwood WW, et al. (2008) DNA amplification is a ubiquitous mechanism of oncogene activation in lung and other cancers. *Oncogene* 27:4615–4624.