In vivo multiplexed interrogation of amplified genes identifies GAB2 as an ovarian cancer oncogene


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High-grade serous ovarian cancers are characterized by widespread loss and gain of copy number alterations. Although some regions of copy number change harbor known oncogenes and tumor suppressor genes, the genes targeted by the majority of amplified or deleted regions in ovarian cancer remain undefined. Here we systematically tested amplified genes for their ability to promote tumor formation using an in vivo multiplexed transformation assay. We identified the GAB2-associated binding protein 2 (GAB2) as a recurrently amplified gene that potently transforms immortalized ovarian and fallopian tube secretory epithelial cells. Cancer cell lines overexpressing GAB2 require GAB2 for survival and show evidence of phosphatidylinositol 3-kinase (PI3K) pathway activation, which was required for GAB2-induced transformation. Cell lines overexpressing GAB2 were as sensitive to PI3K inhibition as cell lines harboring mutant PIK3CA. Together, these observations nominate GAB2 as an ovarian cancer oncogene, identify an alternative mechanism to activate PI3K signaling, and underscore the importance of PI3K signaling in this cancer.

Results

Amplicon-Based Pooled in Vivo Transformation Screen. To identify recurrently amplified genes that contribute to tumorigenicity in HGSOE cancers, we initiated a systematic study in which we used genome characterization data to identify recurrent amplified genes, created a lentivirally delivered collection of ORFs, and then screened for genes that induced tumorigenicity using a multiplexed in vivo transformation assay. We queried the copy number data generated by TCGA (3) to identify 1,017 recurrently amplified genes resident in the 63 recurrently amplified regions in HGSOE cancers. Using the Center for Cancer Systems Biology (CCSB)/Broad Institute lentiviral ORF expression collection (12), we created an arrayed collection of 587 ORFs representing 455 amplified ovarian genes (Dataset S1) including AKT1 that served as a positive control.

Significance

High-grade serous ovarian cancers are characterized by widespread loss and gain of copy number involving large numbers of genes; however, the functional consequences of many of these changes remain unclear. To determine which of the many amplified genes exhibited tumor-promoting behavior, we developed a novel in vivo method to systematically screen potential oncogenes for tumor formation. We identified GAB2, a signaling adaptor protein, as a potent oncogene that is also significantly amplified in ovarian and breast cancer. GAB2 overexpression activates the phosphatidylinositol 3-kinase (PI3K) pathway and confers sensitivity to PI3K pathway inhibition. These results credit GAB2 as a potent oncogene in ovarian cancer and emphasize the importance of PI3K signaling in this cancer.
We previously showed that human embryonic kidney cells immortalized by expression of the human catalytic subunit of telomerase (hTERT) and the SV40 Early Region (SV40 ER) (HA1E) (13, 14) (HA1E) are rendered tumorigenic by the expression of HRAS (15). To assess the expression of constitutively active alleles of MEK1-DD (HA1E-M cells) and AKT1 (myristoylated-AKT1) (8). We used this experimental model to identify IKKE and PAK1 as breast cancer oncogenes (8, 9). Here we introduced each of the 587 ORFs into HA1E-M cells by transducing each ORF separately in arrayed format. We created 26 pools composed of ORF-expressing cell lines representing 16–24 ORFs and implanted each group s.c. in six separate replicates in immunodeficient mice (Fig. 1A). We also tested a minipool containing individually infected HA1E-M-BRAFV600E, HA1E-M-HRASV12, and HA1E-M-KRASV12 and a mixture of HA1E-M-AKT1 cells with uninfected cells at 1:24 and 15:24 ratios as well as a pool of HA1E-M-EGFP cells mixed with uninfected HA1E-M cells at a 1:24 ratio. After 120 d, 11 of 26 (42%) injected pools formed tumors, including 1 pool that formed tumors at 3 of the 6 injected sites and 4 pools that formed tumors at 2 of the 6 injected sites (Fig. 1B). In total, we recovered 21 tumors from 149 injected sites for an overall tumor incidence of 14.1%. To determine the identities of the ORF sequences within each tumor, we used a massively parallel sequencing approach (SI Materials and Methods). We considered ORFs present if their sequences represented greater than 0.1% of the nonvector-aligned reads in the recovered tumor to identify weaker scoring genes in pools. Of the 455 ovarian cancer genes screened, 25 unique ovarian cancer ORF sequences were recovered. GAB2 was the only ORF identified in all three tumors that formed from its parental pool but 94.5% and 96.8% of the ORF sequences within the parental pool but 94.5% and 96.8% of the sequences within the two resulting tumors, respectively. These experiments illustrate the utility of a multiplexed in vivo approach to oncogene identification and identify several candidate oncogenes capable of transformation.

**GAB2 Transforms Immortalized Ovarian and Fallopian Tube-Derived Cells.** Prior work has implicated GAB2 in NeuNT-driven murine mammary tumorigenesis and migration (16, 17). However, GAB2 overexpression failed to induce the transformation of immortalized MCF10A cells (16, 17). To validate our screening results, we reintroduced GAB2 into HA1E-M cells and found that tumors formed in mice at the same rate (7 of 12, 58%) as that induced by the ovarian cancer oncogene, ID4 (5) (8 of 14, 57%). To determine whether GAB2 expression also transforms immortalized cells relevant to ovarian cancer, we introduced GAB2 into human immortalized ovarian surface epithelial (IOSE) cells expressing the SV40 ER, hTERT, and an activated MEK1-DD allele and immortalized fallopian tube secretory epithelial cells (FTSEC) expressing hTERT and the SV40 large and small T antigens (18). Expression of GAB2 induced significantly larger tumors than control or LACZ-expressing control IOSE cells when injected into immunodeficient NOD/IL2Rγ−/−SCID mice (Fig. 2A), and immortalized FTSEC expressing GAB2 formed significantly more anchorage-independent colonies than did control cells. FTSEC expressing both GAB2 and activated MEK1-DD formed nearly as many colonies as FTSEC expressing constitutively active KRASV12 (Fig. 2B). These observations confirmed the transforming function of GAB2 in lineage-matched, physiologically-relevant contexts in which GAB2 is expressed at high levels similar to those found in GAB2-overexpressing cell lines.

**GAB2 Is Amplified and Overexpressed in Ovarian Cancer.** Prior work identified 11q14.1 as recurrently amplified in ovarian cancer (19), but several genes including CCND1, PAK1, and GAB2 have been nominated as potential targets of this amplification. We used the Genomic Identification of Significant Targets in Cancer Version 2.0 (GISTIC 2.0) algorithm (20) to identify recurrent copy number alterations in 562 samples within TCGA ovarian cancer dataset (3, 21). We found that GAB2 is amplified in 44% of samples. Among the samples that harbored this amplification, 24.2% demonstrated focal amplification, which reflects gain of a GAB2-containing region spanning less than 50% of the 11q arm. Furthermore, 13.7% of samples exhibited high-level gain, defined as amplification of more than 1 full copy of GAB2 (Fig. 3A). Collectively, these observations show that GAB2 is located at the center of a peak that also harbors KCTD21 and USP35 that is telomeric to CCND1 and PAK1 (Fig. 3B). This region on 11q14.1 is the fourth most frequently amplified region in ovarian cancer (Fig. 3B). This observation revealed that GAB2 is a significant recurrently amplified gene in ovarian cancer. In addition, because several prior studies (16, 22) found that a subset of breast cancers harbored GAB2 amplification, we applied GISTIC analysis to the TCGA breast cancer dataset to assess the copy number status of GAB2. Across 846 samples within the TCGA breast cancer dataset, GAB2 is amplified in 24.7% of samples in which 15% harbor a focal amplification and 8.4% harbor a high-level amplification (21). This region of amplification is slightly larger than in ovarian cancer and also includes PAK1 (9). There are also a small number of lung cancers and glioblastoma that harbor GAB2 amplification. Collectively, these observations show that 11q14.1 is a major region of amplification in both ovarian and breast cancers and suggest that GAB2 is one of several targets of this region of copy number gain.

To determine the relationship between GAB2 amplification, gene expression, and protein expression, we evaluated ovarian cancer samples in the TCGA for which there was available data on GAB2 amplification, expression, and protein expression. We found that 88% of samples with high-level GAB2 amplification showed high levels of GAB2 mRNA expression compared to tumors with low levels of amplification. Furthermore, by immunohistochemistry, we found that 95% of tumors with high-level GAB2 amplification also showed substantial GAB2 protein expression in the cytoplasm and nuclei of ovarian cancer cells. This observation suggests that the GAB2 protein is a target of the amplification and is likely driving the transformation of these cells.

**Fig. 1.** In vivo ORF transformation screen. (A) Schematic for the ORF screen. Individual ORFs were expressed in HA1E-M cells, pools created from these cells and implanted s.c. in immunodeficient mice. Genomic DNA from tumors was sequenced for ORF identification. (B) Tumor formation per ORF pool. Positive controls include a minipool containing individually infected HA1E-M-BRAFV600E, HA1E-M-HRASV12, and HA1E-M-KRASV12 and a mixture of HA1E-M-AKT1 cells with uninfected cells at 1:24 and 15:24 ratios. The negative control included HA1E-M-EGFP cells mixed with uninfected HA1E-M cells at a 1:24 ratio. (C) List of ORFs recovered in one, two, or three tumors. (D) Enrichment of ORFs from pools 7C, 7B, and 6C ORF sequence reads are shown as a percentage of the duplicated nonvector reads in either tumor or parental cell lines.
GAB2 protein levels by reverse phase protein array (RPPA). Of 326 analyzed samples, 58 of 326 (18%) displayed elevated protein expression and harbored elevated gene expression, gene amplification, or both characteristics (Fig. 3C). Of these high-expressing samples, 14 demonstrated the highest level of expression and were defined by both high GAB2 gene amplification and elevated levels of GAB2 mRNA. Having documented GAB2 overexpression by RPPA, we performed an independent analysis of protein expression via immunohistochemical analysis of GAB2 expression in tissue microarrays (TMAs) generated from patients with high-grade serous ovarian adenocarcinoma. Of 132 tumors assessed, we found 89 of 132 (67%) to be GAB2-negative with staining scores of 0 or 1+ (Fig. 3D). However, 43 of 132 (33%) samples were GAB2-positive (scores 2+ and 3+), with expression predominantly noted in the cytoplasm. These findings paralleled what we observed using RPPA. Finally, using the copy number and expression data in the Cancer Cell Line Encyclopedia (23), we identified ovarian cancer cell lines in which GAB2 was amplified and overexpressed (ONCODG1 and NIH:OVCAR3) and not amplified but overexpressed (IGROV1 and FUOV1). In contrast, the OV56 ovarian and MDA-MB-453 breast cancer cell lines expressed low levels of GAB2 (Fig. 3E). The lower molecular weight GAB2 seen in ONCODG1 and NIH:OVCAR3 likely represents a smaller isoform of GAB2 that lacks the first exon of the GAB2 gene and is likely translated from an internal methionine of the second exon (Fig. S1). Together, these results show that GAB2 is a significant target of amplification in human ovarian cancer and is both amplified and also overexpressed in human cancer cell lines and primary tumor samples.

Ovarian Cancer Cell Dependency on GAB2. The finding that GAB2 induced tumorigenicity and is amplified and overexpressed in a subset of ovarian cancers suggested that GAB2 was one target of the 11q14 amplicon. We investigated whether cell lines that harbor GAB2 amplification and/or overexpression required GAB2 expression for proliferation to establish that GAB2 is one target of the amplicon on chromosome 11q. We used a panel of ovarian cancer cell lines that overexpress GAB2 (NIH:OVCAR3, FUOV1, and IGROV1) and representative ovarian (OV56) and breast (MDA-MB-453) cancer cell lines with low GAB2 expression and expressed two independent shRNAs targeting GAB2 or a control shRNA construct targeting LacZ (shLACZ, Fig. 4A). Cell lines that overexpress GAB2 exhibited significantly decreased proliferation compared with cells expressing the shRNA targeting LacZ (Fig. 4B). In contrast, suppression of representative cell lines with low GAB2 expression failed to inhibit cell proliferation. Although these observations are representative of a range of cell lines tested, we also identified a subset of cell lines with lower GAB2 expression that were sensitive to GAB2 suppression (Fig. S2A and B), likely due to the involvement of GAB2 in other cancer-relevant signaling pathways such as those driven by receptor tyrosine kinases. These observations support the notion that GAB2 represents a dependency in GAB2-altered ovarian cancer cell lines.

Signaling Pathways Activated by GAB2. Prior work has implicated GAB2 as a signaling intermediate in both SHP2-dependent activation of MAPK signaling (16, 17) and activation of phosphatidylinositol 3-kinase (PI3K) (24). To assess the effects of suppressing GAB2 on these signaling pathways in cell lines expressing elevated or normal levels of GAB2, we interrogated the phosphorylation levels of PI3K/AKT/mTOR pathway components AKT1 and S6 as well as the MAPK pathway component ERK1/2. When we suppressed GAB2 with 2 independent GAB2-specific shRNAs in NIH:OVCAR3 and FUOV1 cell lines, we found decreased levels of phospho-AKT1, phospho-S6, and phospho-ERK1/2 compared with cells expressing a control shRNA, shLacZ (Fig. 4C). In contrast, suppression of GAB2 in the MDA-MB-453 and BT20 cell lines, both of which express lower levels of GAB2 and also harbor PIK3CA mutations, failed to induce changes in the observed levels of phospho-AKT1 or phospho-S6, and induced only a minimal decrease in phospho-ERK1/2 levels. Also, suppression of GAB2 in two additional low-expressing lines, OV7 and OVK18, failed to induce changes in pathway activation (Fig. S2A). These observations suggest that the PI3K/AKT1/mTOR and MAPK pathways are activated by GAB2 selectively in cell lines that overexpress this protein.

PI3K Activation in GAB2-Mediated Transformation/Dependency. "PI3Kness" appears to be a critical feature of both type I and type II ovarian cancers (25). However, analysis of the TCGA dataset revealed that alterations in known PI3K pathway components including PTEN, PIK3CA, AKT1, and AKT2 were present in only about one-third of ovarian cancer samples (3). Although prior studies have shown that GAB2-mediated migration of melanoma (24) and ovarian cancer cells (26) can be reduced by PI3K inhibitors, we hypothesized that GAB2 activation of signaling pathways such as those driven by receptor tyrosine kinases. These observations support the notion that GAB2 represents a dependency in GAB2-altered ovarian cancer cell lines.
amplification represents an additional mechanism of PI3K activation in ovarian cancer. We tested whether increased GAB2 expression activated AKT1. GAB2 overexpression in IOSE cells led to increased serine 473 phosphorylation of AKT1 compared with IOSE cells expressing control LacZ or the GAB2-3YF mutant protein, which impairs the ability of GAB2 to recruit p85 (Fig. S4). These observations show that increased GAB2 levels induced AKT1 phosphorylation, likely via the recruitment of the PI3K complex. This phosphorylation event required the activity of the mTOR complex; treatment of IOSE cells overexpressing GAB2 with Torin2, an inhibitor of both the mTOR complex and the p85-binding mutant GAB2-3YF, abolished phospho-AKT1 and phospho-S6, and AZD-6244 treatment expressing cell lines, GDC-0941 treatment abolished levels of phospho-ERK 1/2 (Fig. 6A). We extended these observations by exploring whether in-dependent activation of the down-stream PI3K/AKT pathway could rescue cells dependent upon GAB2 function in which GAB2 levels had been suppressed. We introduced a myristoylated, constitutively active form of AKT1 (myrAKT1) or a control vector into NIH:OVCAR3 cells and subsequently expressed a control lacZ shRNA or four independent GAB2-specific shRNAs and examined for differences in cell proliferation. Compared with GFP-expressing NIH:OVCAR3 cells in which we had suppressed GAB2, NIH:OVCAR3 cells overexpressing constitutively active myrAKT1 showed an ~20% increase in proliferation that was statistically significant with each GAB2-specific shRNA tested (Fig. 5C). These observations suggest that GAB2 likely acts, at least in part, through AKT1.

Finally, because GAB2 activates the PI3K pathway, we investigated the pattern of genomic alterations of PI3K signaling in primary ovarian cancers. Hanrahan et al. (28) recently reported a substantial number of PI3K pathway alterations in ovarian cancer. We extended this analysis by analyzing a substantially larger number of samples and also incorporating GAB2 in our analysis. Specifically, we analyzed the ovarian cancer TCGA dataset to determine the incidence and type of alterations in PIK3CA, PIK3CB, GAB2, AKT1, AKT2, AKT3, PTEN, and PDK1. Of the 562 samples analyzed, we found that nearly 54% harbored significant copy number alterations or mutations in these genes. Similar to our prior analysis, 13% of samples harbored GAB2 amplifications, some of which co-occurred with amplification of other pathway components.

These observations suggest that amplification of GAB2 also leads to activation of PI3K signaling.

### Sensitivity of GAB2-Altered Cell Lines to PI3K Inhibition

Having shown that the PI3K pathway was required for GAB2-mediated transformation and that activated AKT1 could partially rescue GAB2-dependent cell lines depleted of GAB2, we next determined whether GAB2 alterations represented a feature that correlated with enhanced sensitivity to PI3K pathway inhibition. Several studies suggest that PI3K inhibition attenuated GAB2-dependent migration phenotypes (24, 26) and pointed to the types of pathway alterations that activate AKT1 (28). However, although recent work has been directed at correlating PI3K pathway alterations with sensitivity to pathway inhibition in breast cancer (29), it is unclear whether increased GAB2 expression increases cell sensitivity to PI3K pathway inhibition in a manner similar to the sensitivity conferred by canonical mutations in PIK3CA and PTEN. We performed dose–response experiments to determine the IC50 of inhibitors against PI3K (GDC-0941) or MEK1 (AZD-6244) in a panel of 12 ovarian and 11 breast cancer cell lines 3 d posttreatment. In GAB2-overexpressing cell lines, GDC-0941 treatment abolished levels of both phospho-AKT1 and phospho-S6, and AZD-6244 treatment abolished phospho-ERK 1/2 (Fig. 6A). We observed similar effects in cell lines with low GAB2 expression (Fig. S2A). We observed that 10 cell lines that harbored amplification over-expression of GAB2 formed a distinct subgroup from 9 cell lines that harbored mutation of PIK3CA or loss of PTEN, with the
enrichment is likely due to the stochastic selection of clones that
phenotypic readouts using ORF collections that do not require
and quantitate their enrichment. This approach could be applied
to any cancer phenotype. We used a next-generation sequencing
discovery in ovarian cancer, this methodology can be applied to
context emphasizing other signaling cascades. Although we
and fallopian tube epithelial cells indicating that the HA1E-M
allele, thereby facilitating the identification of PI3K-activating
formation rate. The HA1E-M model expresses an active MEK
ability to transform an immortalized human cancer cell line using
a pooled high-throughput in vivo approach and identified 26
genes that induced tumorigenicity. We demonstrated that GAB2
induces the transformation of ovarian and fallopian tube cells by
activating PI3K signaling. Ovarian cell lines that harbor GAB2
amplifications or overexpress GAB2 are dependent on GAB2 for
proliferation and exhibit sensitivity to PI3K inhibition. These
observations identify GAB2 as an ovarian cancer oncogene and
underscore the importance of PI3K signaling in ovarian cancer.

Multiplexed in Vivo Transformation Screens. We used a human
embryonic kidney cell line expressing the activated MEK162D
gene to facilitate a high-throughput, stringent in vivo transformation
screen. This model is useful due to its rapid in vivo growth kinetics
for oncogene discovery and its low background transformation rate. The HA1E-M model expresses an active MEK allele, thereby facilitating the identification of PI3K-activating genes such as GAB2. Further systematic follow-up will be necessary to validate additional candidate oncogenes identified using this system that may vary in strength of transforming capacity. We note that GAB2 also induced transformation in both ovarian and fallopian tube epithelial cells indicating that the HA1E-M model enabled the identification of ovarian cancer drivers. Additional oncogenes may be uncovered if screened in a cellular context emphasizing other signaling cascades. Although we describe a gain-of-function approach focused on oncogene discovery in ovarian cancer, this methodology can be applied to any cancer phenotype. We used a next-generation sequencing approach that allowed us both to identify ORFs within tumors and quantitate their enrichment. This approach could be applied easily to a range of scalable ORF-expressing pool sizes and phenotypic readouts using ORF collections that do not require additional barcoded cassettes. The observation that one of the three GAB2-scoring tumors displayed a lower level of GAB2 enrichment is likely due to the stochastic selection of clones that eventually form tumors at each site, owing to a slight variation in starting pooled inoculum or interactions with the local microenvironment. In addition to GAB2, we also identified additional candidates, such as N4RF and ASB10, as genes that induced tumors when overexpressed in HA1E-M cells that merit further investigation. Taken together, these studies provide further evidence that large-scale functional genomics approaches complement ongoing structural approaches to decipher genes and pathways involved in cancer pathogenesis.

Features of the 11q14 Amplion. GAB2 resides on the fourth most
significant amplicon in high-grade serous ovarian cancer (3), the
focal peak located on 11q14.1. This amplified region has been
identified as a recurrent alteration in breast (9, 22) and ovarian
cancer (19) as well as in metastatic melanoma (24). Across over
6,300 different TCGA samples, this region is among the 26 most
amplified regions in all cancers (21). This amplicon is slightly
broadier in breast cancer, encompassing both GAB2 and PAK1
(9, 21) as well as the nearby region harboring CCND1. Whereas
GAB2 amplification does not appear to be correlated with survival, there is a significant association with GAB2 amplification and the Her2-enriched (P = 0.042) and luminal B (P = 0.028) breast cancer subsets described by TCGA in normal breast.
GAB2 amplification and the 11q14.1 amplicon at is smaller and centered over GAB2. Although prior studies in ovarian cancer have highlighted the 11q14.1 amplification (3, 19), the identities of the candidate gene targets of amplification were not well clarified. These studies do not preclude the possibility that several genes on the same amplicon may cooperate to drive oncogenic programs. GAB2 may also contribute to the pathogenesis of individual ovarian cancers in which the center of the region of copy number gain does not fall on GAB2 itself but on neighboring candidate genes, such as RSFI (30), PAK1 (9, 19), or CCND1. Also, there are a significant number of samples that harbor elevated GAB2 gene expression in the absence of amplification, supporting the idea that amplification is one of several mechanisms underlying GAB2 protein overexpression and tumorigenesis. We note that GAB2 is somatically mutated in four TCGA samples, although these amino acid changes do not affect known functional domains.

GAB2 Alterations in Cancer. GAB2 plays critical roles in several
different cancers. GAB2 is required for BCR/ABL-mediated transformation in chronic myeloid leukemia (CML) (31) and also HER2-mediated mammary carcinogenesis through ERK activation (16). Moreover, GAB2 is overexpressed in breast cancer (9, 22) and some metastatic melanomas (24) and promotes survival in breast cancer (22) and migration in both malignancies (17, 24). Several ovarian cancer cell lines also overexpress GAB2, which was linked to increased migration and the epithelial-mesenchymal transition (26). Davis et al. also demonstrated that a panel of ovarian cancer cell lines with GAB2 amplification were susceptible to siRNA-mediated GAB2 knockdown (32), consistent with our findings. Although these prior studies suggest that GAB2 expression is altered in cancer, the observations presented herein show that GAB2 is a bona fide oncogene important for both tumor initiation and maintenance.

GAB2-Mediated Signaling and the PI3K Pathway. Recent work suggests that over 70% of ovarian cancers exhibit activation of the PI3K pathway (33), but there are likely more alterations that influence this pathway than have been identified. We identified PI3K pathway alterations in 54% of the high-grade serous ovarian cancer samples we analyzed from the TCGA dataset. PIK3CA amplification was a common event and has been seen in other cancers (34, 35). Co-occurrence of GAB2 amplification and PI3K pathway component alteration was observed in a subset of ovarian cancer samples. Co-occurrence and ASB10 altered pathways components has been observed in other PI3K-altered cancer types and could potentiate PI3K-mediated signaling. Based on our findings, GAB2 overexpression represents a subset of pathway alterations that result in dysregulated PI3K signaling. Several studies have addressed PI3K/akt pathway inhibition in

Fig. 6. Effects of a PI3K inhibitor on cell proliferation. (A) Effect of increasing doses of the PI3K inhibitor GDC-0941 or the MEK inhibitor AZD-6244 on AKT1, ERK1/2, and S6 phosphorylation in GAB2-overexpressing cell lines NIH- OVCAR3, FUOV-1, and IGROV1. (B) Half-maximal inhibitory concentrations (IC50) for GDC-0941 (Left) and AZD-6244 (Right) in 23 ovarian and breast cancer cell lines. Genomic alterations are depicted by color as shown in Insert.
ovarian cancer. In one study, a subset of ovarian cancer cell lines was sensitive to inhibition of AKT1 and AKT2, whereas those expressing AKT3 required inhibition of all three isoforms (28). In a separate study, modest clinical responses were seen in a phase-I trial incorporating mTOR inhibitors in the patients with PIK3CA mutant compared with PIK3CA wild-type cancers (36). These findings point to the importance of careful genetic annotation in tailoring pathway-specific therapies. Our findings suggest that cell lines characterized as GAB2 overexpressing are as sensitive to PI3K pathway inhibition as cell lines harboring classical PIK3CA mutations, and subsequent work has shown that additional cell lines with low GAB2 expression, OV7 and OVK1, exhibit nearly fourfold higher IC50 values in response to PI3K inhibitor treatment. These observations provide a rationale to consider inhibition of this pathway in human ovarian cancers as well as breast cancers and metastatic melanomas exhibiting appropriate pathway-specific genomic features.

**Adaptors as Therapeutic Targets.** In addition to its ability to activate the PI3K pathway, GAB2 can also activate the ERK pathway through Shp2. In a mammary epithelial cell model, NeuNT-driven multicellular structure formation through GAB2 requires Shp2/ERK signaling (16). However, we failed to observe a decrease in transformation using a GAB2 mutant unable to bind Shp2 and did not find that ovarian cancer cell lines were as sensitive to MEK inhibition as they were to PI3K inhibition. Together, these observations suggest that signaling downstream of GAB2 may be context or lineage specific. Further work will be necessary to address these possibilities.

Adaptor proteins amplify receptor-initiated signaling events by recruiting downstream modular signaling proteins. Thus, genes such as GAB2 and CRKL (37) are powerful transforming oncogenes because of the number of pathways influenced by their overexpression. As was demonstrated in CRKL-overexpressing mutant EGFR lung cancer cells resistant to EGFR inhibition (37), cells overexpressing GAB2 may be resistant to inhibitors of upstream receptor tyrosine kinases, such as HER2, with which GAB2 associates. Adaptor proteins may represent a class of cancer-relevant targets that warrant further study. The observation that GAB2 copy number alterations correlate with sensitivity to PI3K pathway inhibition supports the prospective annotation of GAB2 amplification as a relevant GAB2 protein expression in clinical trials of PI3K inhibitors.

**Materials and Methods**

*Pooled in Vivo ORF Screen.* Five hundred eighty-eight ORFs representing genes recurrently amplified in the ovarian cancer TCGA dataset as well as controls were obtained from the CCSB/Broad Institute ORF collection (12). HA1E-M cells were infected in arrayed fashion, pooled, and injected into three sites each in two Ncr nude mice (Tacomic) per pool. Growing tumors were harvested, and ORFs were amplified from genomic DNA and recombined into common vectors, and pooled recombined plasmids were recovered from transformed bacteria and subjected to next-generation sequencing. Extended details are described in SI Materials and Methods.

**Additional Materials and Methods.** Plasmids, cell lines and reagents, chemicals, immunoblotting, cell proliferation and anchorage-independence assays, TMA and immunohistochemistry, genomics/proteomics analysis, and animal injections are described in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

**Plasmids.** All ORFs used in the screen were cloned into the pLX304 vector backbone and obtained from the Center for Cancer Systems Biology (CCSB)/Broad Institute ORF collection (1). The pLX304-GAB2-3YF mutant was generated by serial overlapping site-directed mutagenesis via a protocol similar to that described previously (2). Y452F, Y476F, and Y854F mutations were introduced using the following primers: Y452F-For (5′-CAG CAC CAA TTC TGA AGA CAA CTT TGT GCC CAT GAA-3′) and Y452F-Rev (5′-ATG ATG GCC ACA AAG TGG TCT TCA GAA TTG GTG CTG-3′), Y476F-For (5′-ATT CCC AGA GCG TCT TCA TCC CAA TGG AGC CCC-3′) and Y476F-Rev (5′-GGG CTC ATT GGG ATG AAG ACG CTC TGG GAA T-3′), Y854F-For (5′-CAG GAG ACA CGG AAG AGA ACT TTG CTC GTA GAA TTG GTG CTG-3′) and Y854F-Rev (5′-GCA TAG GGA CAA AGT TCT CTT CCG TGT CTC CTG-3′), and pLX-for (5′-CACA CAA AAT CAG CGG GAC TT-3′) and pLX-rev (5′-CAACA CAC GAG ATT GTC AG-3′). The pLX-LacZ, pLX-KRAS(5′), and pLX-HRAS (5′) plasmids were obtained from the CCSB/Broad Institute ORF collection. The pLentiv.3-blasticidin-GFP has been described previously (3). The retroviral plasmid pBabe-Puro-MEK was obtained from the Broad Institute (4). A 1.5-kb myristoylated Flag epitope tagged AKT plasmid was generated by subcloning the myristoylated Flag epitope tagged AKT1 (myr-Flag-AKT1) cassette (4) into the pBabe-GFP vector (Addgene plasmid 10668). All short hairpin RNA (shRNA) constructs were obtained from The RNAi Consortium (Broad Institute, Cambridge, MA) and have the following clone reference numbers: shLacZ (TRCN0000231710), shGAB2 #6 (TRCN0000155921), shGAB2 #7 (TRCN0000154991), shGAB2 #9 (TRCN0000155271), and shGAB2 #10 (TRCN0000154706).

**Cell Lines and Reagents.** Immortalized human embryonic kidney epithelial cells overexpressing MEK (HA1E-M) cells (4), immortalized fallopian tube epithelial cells (FTSECs) (5), immortalized ovarian surface epithelial (IOSE) cells (6), immortalized fallopian tube secretory epithelial cells (FTSECs) (7), and immortalized ovarian surface epithelial (IOSE) cells (6) were described previously. NCI-H1435 and MDA-MB-468 cell lines were purchased from the American Type Culture Collection. NIH-OVCAR3, COV362, FUOVI1, IGROV1, MDA-MB-453, BT20, A2780, HCC1187, OVCA8, Kuramochi, EFO21, and EFO27 were generated at the Broad Institute (7). All cell lines were maintained in DMEM (Mediatech) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Cellgro).

**Pooled in Vivo ORF Screen.** The 588 ORFs representing genes amplified in the ovarian cancer The Cancer Genome Atlas (TCGA) dataset as well as positive and negative controls were obtained from the CCSB/Broad Institute ORF collection. HA1E-M cells were plated at a density of 6,800 cells per well in 96-well flat-bottom plates (VWR) and infected in arrayed fashion with lentiviruses generated from the lentiviral ORF plasmids. After 2 d, singly infected confluent wells were pooled into collections of 22–24 ORF-expressing HA1E-M cells, expanded into T-150 tissue culture flasks (VWR), and injected. Positive controls 1:24 and 15:24 mixtures of pLX304-AKT1–infected HA1E-M cells with uninfected cells as well as a mixture of HA1E-M cells infected with pLX304-BRAF(V600E) or pLX304-HRAS(5′) and pLX304-EGFP–infected HA1E-M cells with uninfected HA1E-M cells. A replicate of infected HA1E-M cells was treated with 8 μg/mL blasticidin and monitored for cell death. Each pool of HA1E-M cells was injected into three sites each in two NCr nude mice (Taconic) per pool. Cells were trypsinized, resuspended in PBS, and injected at a concentration of 10 × 10^6 cells per mL in 200 μL (2 × 10^6 cells) per site.

**Identification of ORF Sequences in Tumors.** Tumors were resected when they reached a minimum largest diameter of 1 cm. Tumors were minced to homogeneity in culture medium, and genomic DNA was isolated from two independent tissue sections using the DNeasy Blood & Tissue Kit (Qiagen). ORF sequences were amplified by PCR from 150 ng genomic DNA by amplifying across the ORF sequence using pLX304-specific flanking primers pLX-for and pLX-rev for 35 cycles using KOD polymerase (EMD Millipore). PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen), and 200 ng of purified amplicon DNA from each single reaction were cloned into 150 μg of the pDONR223 vector (Invitrogen) using the BP reaction (Invitrogen) for 4 h at room temperature. DH5α Escherichia coli cells were transformed with the entire reaction and plated on LB agar plates containing 50 μg/mL spectinomycin. After 12 h, bacterial cells were harvested by scraping, and pDONR223 plasmids harboring recombinant ORF sequences were purified using the QIAexpress Spin Miniprep Kit (Qiagen). The pDONR223 ORF plasmids from each tumor were sheared, barcoded, pooled, and sequenced on a HiSeq system (Illumina). ORF incidence per tumor was calculated by dividing the number of duplicated ORF reads by the total number of nonvector duplicated ORF reads in a given pool. ORFs were scored as present if they comprised at least 0.1% of the nonvector sequences.

**Chemicals.** GDC-0941 and AZD-6424 were purchased from Selleck Chemicals. Torin 2 was obtained from Tocris Bioscience.

**Immunoblotting.** Immunoblotting was performed as described (8). Antibodies to the following proteins used for immunoblotting were purchased from Cell Signaling: GAB2 (no. 3239), AKT (no. 9272), phospho-S473 AKT (no. 4060L), mTOR (no. 2972), S6 kinase (no. 9202), phospho-S6 kinase (no. 9205), ERK1/2 (no. 9102), and phospho-ERK1/2 (no. 9101). Additional antibodies for immunoprecipitation and immunoblotting GAB2 included an anti-C-terminal antibody (no. sc-9313, Santa Cruz) and an anti-N-terminal antibody (no. AP6908a, Abgent). Antibody to β-actin (sc-7778) was purchased from Santa Cruz Biotechnology.

**Cell Proliferation Assay.** The 1,700 IGROV1, FUOVI1, and OV56 cells; 1,500 NIH-OVCAR3; 2,000 OV7 and OVK18; and 3,000 MDA-MB-453 cells were seeded into each well of 96-well plates 24 h before infection. Six replicate infections were performed for control shRNAs targeting LacZ (shLacZ) and GAB2-specific shRNAs in the presence of 4 μg/mL polybrene for 24 h followed by selection with 2 μg/mL puromycin. The ATP content was measured at 6 d postinfection by using the CellTiter-Glo luminescent cell viability assay (Promega).

**Anchorage-Independent Growth Assay.** Growth in soft agar was determined by plating 1 × 10^6 cells in triplicate in 5 mL medium containing 0.4% Noble agar (BD Biosciences) which was placed on top of 4 mL solidified 0.6% agar. Colonies greater than 100 μm in diameter were counted 4 wk after plating.

**High-Density Tissue Microarray and Immunohistochemistry.** A tissue microarray (TMA) comprised 134 cases of high-grade, late-stage ovarian serous carcinoma as well as immunohistochemistry methods.
were described previously (9, 10). For immunohistochemistry, antibody to human GAB2 (sc-9313, Santa Cruz Biotechnology) was used at a dilution of 1:500. GAB2 staining was scored on a scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) intensity. All tissue review was performed in a blinded manner by pathologists.

Analysis of Primary Human TCGA Genomics and Reverse Phase Protein Array Data. We used the Broad Institute Firehose analysis run from August 25, 2012, which may be found in the TCGA Data Coordination Center (https://confluence.broadinstitute.org/display/GDAC/Home), for all primary cancer data files (copy number, expression, protein levels) and analysis results [Genomic Identification of Significant Targets in Cancer (GISTIC) result files]. Screenshots of the segmented primary breast and ovarian cancer copy number data (from Affymetrix SNP6 arrays) with scores from TCGA ovarian specific GISTIC analysis were taken using the Integrative Genome Viewer (www.broadinstitute.org/igv).

A total of 326 primary ovarian cancer samples from TCGA had reverse phase protein array (RPRA), mRNA expression and copy number data available. GAB2 mRNA levels were pulled from mRNA expression data, using Agilent G4502A arrays. High expression was defined as 1 SD above the mean of GAB2. Protein levels of GAB2 were determined from RPRA arrays. Amplification of GAB2 was pulled from the ovarian cancer GISTIC results; only those amplifications larger than the arm level amplifications observed for the sample were considered highly amplified.

To analyze GAB2 and additional components of the phosphatidylinositol 3-kinase (PI3K) pathway, copy number pipeline methodology developed at the Broad Institute was applied to the copy number data obtained on the Affymetrix platform across 481 OV samples from the TCGA study deposited at Broad Genome Data Analysis Center (GDAC) Firehose database. We called high-level somatic copy number alternations based on GISTIC 2.0 (11) for GAB2 and the PI3K pathway genes PIK3CA, PIK3CB, AKT1, AKT2, AKT3, PTEN, PIK3R1, and PDK1.

Animal Injections/Tumorigenicity. Cell line xenograft experiments were performed as described (4, 8). Animal protocols were approved by the Dana–Farber Cancer Institute Institutional Care and Use Committee. HA1E-M cells were grown as described above, trypsinized, resuspended in PBS, and injected s.c. at an inoculum of 2 × 10^6 cells per site in 6-wk-old Ncr-nude mice (Taconic). IOSE-derived cells were grown as described above, resuspended in 400 μL 1× PBS mixed with 400 μL of Matrigel Basement Membrane Matrix (BD Biosciences), and injected s.c. at an inoculum of 2 × 10^6 cells per site in 6-wk-old NOD/IL2Rγ/scid mice (Taconic) and monitored for tumor formation.

Fig. S2. Effects of GAB2 suppression in cells expressing low levels of GAB2. (A) OV7 and OVK18 cells were transduced with shLacZ, shGAB2 #6, and shGAB2 #7 and then monitored for levels of GAB2 protein as well as phosphorylated levels of AKT1, S6, and ERK1/2. (B) Effects of suppressing GAB2 expression on cell proliferation in a GAB2-overexpressing IGROV1 cell line and two cell lines with low GAB2 expression. **P < 0.0001. Error bars reflect standard deviation. (C) Effect of increasing doses of the PI3K inhibitor GDC-0941 or the MEK inhibitor AZD-6244 on AKT1, ERK1/2, and S6 phosphorylation in GAB2 low-expressing OV7 and OVK18 cell lines.

Other Supporting Information Files

Dataset S1 (XLS)