

# The RNA binding protein FXR1 is a new driver in the 3q26-29 amplicon and predicts poor prognosis in human cancers

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**Aberrant expression of RNA-binding proteins has profound implications for cellular physiology and the pathogenesis of human diseases such as cancer. We previously identified the Fragile X-Related 1 gene (*FXR1*) as one amplified candidate driver gene at 3q26-29 in lung squamous cell carcinoma (SCC). *FXR1* is an autosomal paralog of Fragile X mental retardation 1 and has not been directly linked to human cancers. Here we demonstrate that *FXR1* is a key regulator of tumor progression and its overexpression is critical for non-small cell lung cancer (NSCLC) cell growth in vitro and in vivo. We identified the mechanisms by which *FXR1* executes its regulatory function by forming a novel complex with two other oncogenes, protein kinase C,  $\epsilon$  and epithelial cell transforming 2, located in the same amplicon via distinct binding mechanisms. *FXR1* expression is a candidate biomarker predictive of poor survival in multiple solid tumors including NSCLCs. Because *FXR1* is overexpressed and associated with poor clinical outcomes in multiple cancers, these results have implications for other solid malignancies.**

3q amplification | *FXR1* | RNA binding protein | non-small cell lung cancer

**A**mplification of the chromosomal region 3q26-29 is the most frequent genomic alteration in primary squamous cell lung cancers (1) and occurs in many other cancers (2). The best studied oncogenes of this amplicon include phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) (3, 4), *TP63* (5), sex-determining region Y box 2 (*SOX2*) (6), epithelial cell transforming 2 (*ECT2*) (7), and protein kinase C,  $\epsilon$  (*PRKCI*) (8). In an effort to identify oncogenic drivers in lung cancer associated the 3q26-29 amplicon, we previously integrated genomic and gene expression analysis of multiple lung SCC datasets and identified Fragile X-related 1 (*FXR1*) as a potential new candidate driver gene (9). *FXR1* belongs to a small family of RNA-binding proteins that includes Fragile X mental retardation 1 (*FMR1*) and Fragile X-related 2 (*FXR2*) (10). Inactivation of *FMR1* expression is the cause of the Fragile X syndrome in humans, whereas alterations of *FXR1* are yet to be associated with the pathogenesis of human disease. RNA-binding proteins (RBPs) are essential in RNA metabolism, from synthesis to degradation. RBPs coordinate elaborate networks of RNA-protein and protein-protein interactions that link RNA metabolism to signal transduction pathways (11). Aberrant function of RBPs contributes to the progression of many human diseases including cancer. Nevertheless, few RBPs have been identified as oncogenes or tumor suppressors and clinical implications of these cancer related RBPs is largely unknown. *FXR1* is highly expressed in vertebrate muscle cells and *FXR1* knockout mice die early during embryogenesis, suggesting an

essential role for *FXR1* in development (12). In this study, we examined whether RNA binding protein *FXR1* is a regulator of tumor progression in non-small cell lung cancer (NSCLC) and a driver of the 3q amplicon. We tested this hypothesis across a large number of clinical specimens, in gain- and loss-of-function and mechanistic studies in vitro and in vivo. We investigated the translational relevance of our findings in NSCLC tissue microarrays and in datasets of multiple human cancers available in the public domain.

## Results

***FXR1* Is Coamplified and Coexpressed with *ECT2* and *PRKCI* in SCC of the Lung.** To assess *FXR1* copy number (CN) and expression, we performed genome-wide array comparative genomic hybridization (aCGH) and gene expression profiling on twenty-four untreated lung SCCs. High level amplification (log2 ratio > 0.8, 7 of 24) of *FXR1* gene was found in 30% of the samples (*SI Appendix, Fig. S1A*). *FXR1* mRNA levels were higher in tumors with CN gain ( $P < 0.0001$ ; *SI Appendix, Fig. S1B*). Using The Cancer Genome Atlas (TCGA) lung SCC data, *FXR1* amplification was

## Significance

**Altered expression of RNA binding proteins might contribute to cancer development. This study reveals the functional implications and clinical relevance of *FXR1*, an RNA binding protein, in non-small cell lung cancer (NSCLC). Our results demonstrate that *FXR1* promotes tumor progression by regulating two other oncogenes within the same chromosome 3q amplicon. To drive tumor progression, *FXR1* forms a new complex with protein kinase C,  $\epsilon$ , and posttranscriptionally stabilizes the expression of epithelial cell transforming 2. We show that increased *FXR1* expression in NSCLC is a candidate biomarker predictive of poor survival and might represent a novel therapeutic target. In addition, *FXR1* expression correlates with poor clinical outcome in multiple human cancers, suggesting broader implications of this RNA binding protein in cancer progression.**

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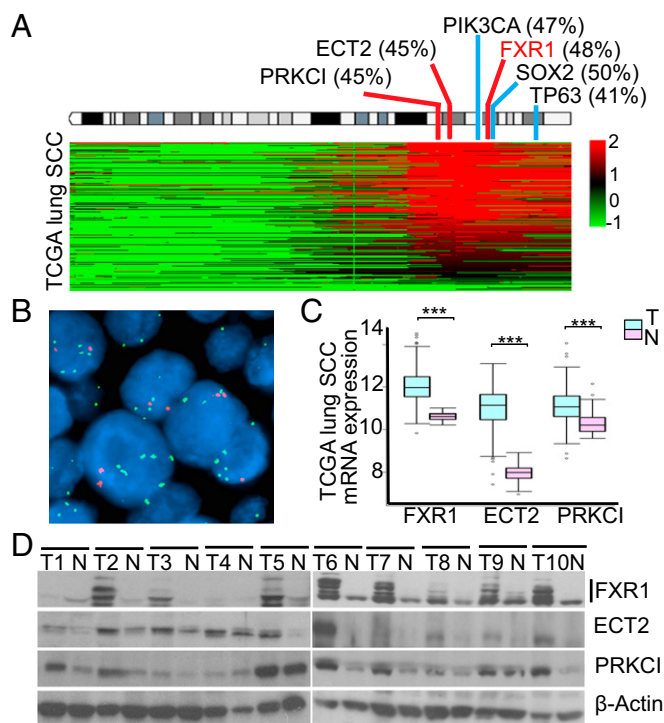
The authors declare no conflict of interest.

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Data Deposition: Microarrays have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession nos. [GSE40048](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40048) and [GSE40074](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40074)).

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**Fig. 1.** (A) Copy number alteration of *FXR1*, *ECT2*, and *PRKCI* in 343 TCGA lung SCCs (only 182 samples harboring 3q amplicon were shown). “2” is a high-level amplification ( $\log_2$  ratio  $> 0.8$ ), “1” indicates a low-level gain ( $\log_2$  ratio  $> 0.3$ ), “0” is diploid, “-1” is a single-copy loss (heterozygous deletion). Frequencies of high-level amplification are shown as a percentage of all cases. (B) Representative dual color FISH of the *FXR1* gene (3q26.3, green spots) and a chromosome 3 centromeric probe (red spots) in lung SCCs. (C) Lung SCCs (T) express higher *FXR1*, *ECT2*, and *PRKCI* mRNA levels than matched normal tissues (N) in TCGA dataset ( $n = 50$ ). \*\*\* $P < 0.0001$ . (D) Immunoblot analysis of ten primary SCCs and matched normal lung tissue for *FXR1*, *ECT2*, *PRKCI*, and  $\beta$ -actin.

confirmed in 48% of SCCs (Fig. 1A). Significant correlation of CN gain and mRNA expression was observed ( $R^2 = 0.72$ ,  $P < 0.0001$ , *SI Appendix*, Fig. S1C). *FXR1* amplification was further verified in 30% (14 of 47) of lung tumors by fluorescence in situ hybridization (FISH) analysis in an independent tissue microarray (TMA) of SCCs (Fig. 1B). To identify potential targets of *FXR1*, we searched our previously published lung SCC gene expression dataset (13) and found that *FXR1* expression was highly correlated with *ECT2* and *PRKCI* (*SI Appendix*, Table S1). *PRKCI* is also one of top 20 candidate driver genes we identified using a novel integrative computational analysis (9). *ECT2* and *PRKCI* have been reported to promote lung tumor cell growth through the extracellular signal-regulated kinases (ERK) signaling pathway by forming a complex with PARD6A (14). The overexpression and coexpression of these three genes were validated in two independent gene expression datasets that have both SCCs and matched normal samples including TCGA (Fig. 1C) and GSE31552 (*SI Appendix*, Fig. S1D and Tables S2 and S3). The coamplification of three genes was observed in TCGA and GSE20393 datasets (*SI Appendix*, Table S4). In addition, results from ten representative matched normal and lung SCCs samples analyzed by Western blotting confirmed the findings at the protein level (Fig. 1D). In contrast to *FXR1*, *FMR1* and *FXR2* genes were neither amplified (*SI Appendix*, Fig. S1E) nor overexpressed in lung SCCs (*SI Appendix*, Fig. S1F and G). Lastly, our findings were confirmed in 147 lung cancer cell lines from the Cancer Cell Line Encyclopedia (15) (*SI Appendix*, Fig. S1H and I). Thus, *FXR1* is the only *FMR1*-family member altered in lung SCCs; its

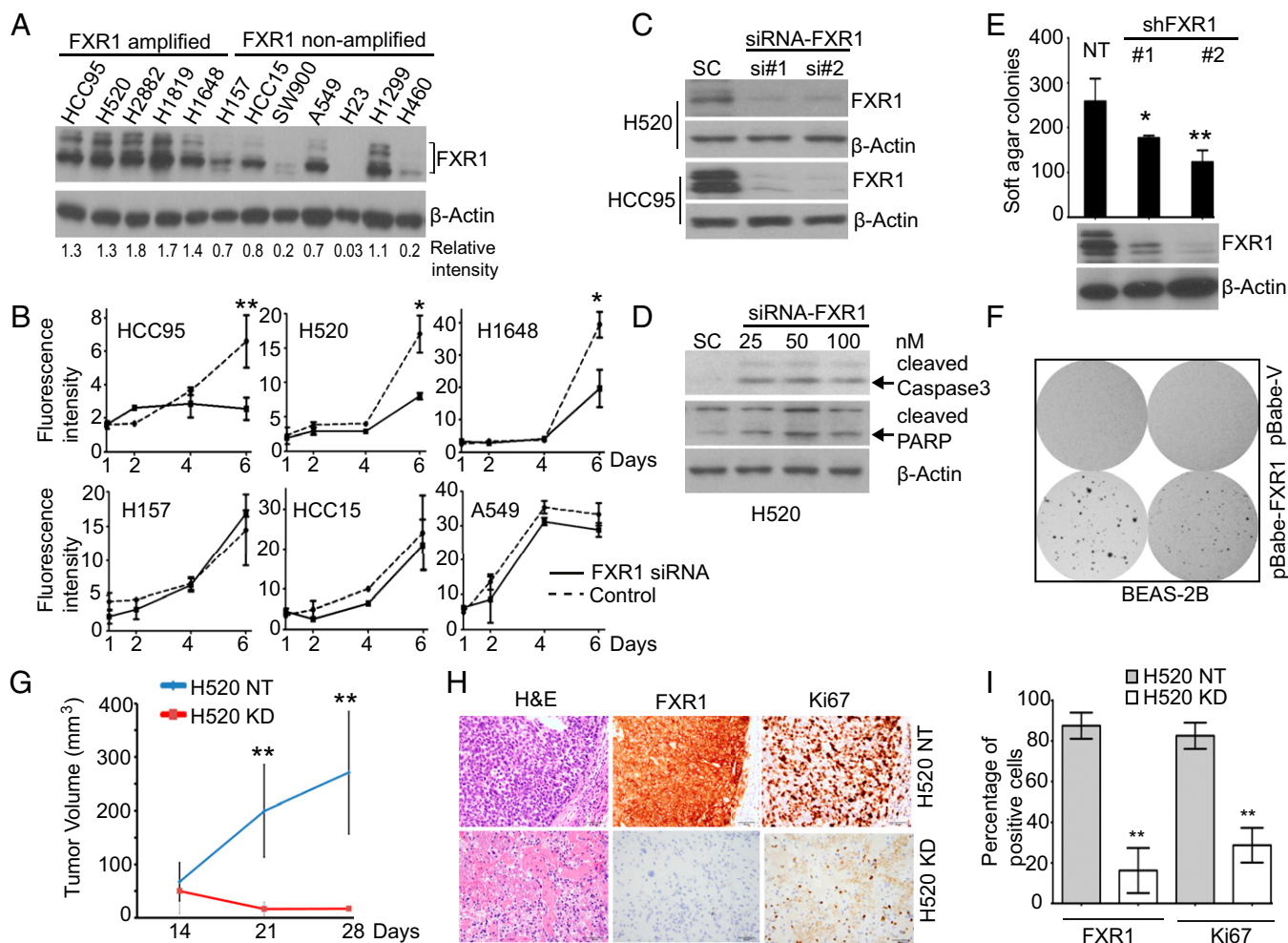
amplification and overexpression are associated with those of oncogenes *ECT2* and *PRKCI*.

**FXR1 Regulates Lung Cancer Cell Growth in Vitro and in Vivo.** To investigate the role of *FXR1* in lung cancer progression, we first tested the effect of *FXR1* on lung cancer cell growth and survival in cell lines with and without the 3q amplicon (Fig. 2A) by loss of function studies. Using a siRNA approach, we found that *FXR1* silencing inhibited cell growth in three *FXR1*-amplified cell lines but had no significant effect in three nonamplified NSCLC lines (Fig. 2B and C and *SI Appendix*, Fig. S2A). *FXR1* silencing also induces apoptosis, demonstrated by increased PARP cleavage in H520 cells (Fig. 2D). We further stably transfected H520 cells with three lentiviral *FXR1* shRNA constructs knockdown (KD) or a nontarget (NT) shRNA control and demonstrated that *FXR1* depletion led to significant inhibition of anchorage-independent colony formation on H520 cells (Fig. 2E and *SI Appendix*, Fig. S2B). Flow cytometry analysis revealed an increase in the percentage of H520 *FXR1*-KD cells in S phase, a decrease in G1/G0 and an increase in the sub-G1 population (*SI Appendix*, Fig. S2C). In addition, *FXR1*-KD cells exhibited impaired invasion commensurate with *FXR1*-KD level (*SI Appendix*, Fig. S2D). To test its tumorigenic properties, we ectopically overexpressed *FXR1* into two immortalized human bronchial epithelial cell lines BEAS-2B and HBEC3KT without the 3q amplicon (16) (*SI Appendix*, Fig. S2E) and observed enhanced anchorage-independent cell growth in soft agar of BEAS-2B (Fig. 2F) but not HBEC3KT overexpressing *FXR1*.

To examine the in vivo effect of targeting *FXR1* in H520 cells, we injected H520 NT and H520 *FXR1*-KD cells s.c. into the flanks of athymic nude mice and found H520 *FXR1*-KD cells exhibited impaired tumor growth compared with control (Fig. 2G and *SI Appendix*, Fig. S2F). Histological analysis of the xenograft tissues confirmed strong *FXR1* protein expression in control tumor tissues but nearly undetectable levels in *FXR1*-KD xenografts. Ki-67 staining was remarkably reduced in *FXR1*-KD tumors (Fig. 2H and I, and *SI Appendix*, Fig. S2G). The growth inhibition due to loss function of *FXR1* in vivo was also observed in another lung cancer cell line H1299 without 3q amplicon but overexpressing *FXR1* (*SI Appendix*, Fig. S2H). Together, these results indicate that *FXR1* regulates proliferation and survival of NSCLC cells in vitro and in vivo, a process dependent on *FXR1* gene overexpression but not only on genomic amplification.

**FXR1 Regulates *ECT2* and *PRKCI* Expression via a *FXR1*/*PRKCI*/mRNA Complex in NSCLC Cells.** To investigate the mechanisms of *FXR1*-dependent regulation of lung tumorigenesis, we examined the levels of *ECT2* and *PRKCI* protein expression after knockdown of *FXR1* expression in three *FXR1*-overexpressing NSCLC cell lines. Silencing *FXR1* led to reduced expression of *ECT2*, *PRKCI*, and phospho-ERK1/2 (Fig. 3A and *SI Appendix*, Fig. S3A), whereas the converse effect was observed in HBEC3KT cells exogenously overexpressing *FXR1* (*SI Appendix*, Fig. S2E). These findings demonstrate that *FXR1* regulates the expression *ECT2* and *PRKCI*.

To gain insights into the mechanisms by which *FXR1* regulates *ECT2* and *PRKCI*, we used a series of reciprocal immunoprecipitation (IP) experiments and found that *FXR1* physically binds to *PRKCI* protein but not to *ECT2* or *PRAD6A* protein in NSCLC cells (Fig. 3B and *SI Appendix*, Fig. S3B–F). The binding of *FXR1* to *PRKCI* was not observed in immortalized normal lung epithelial cells (*SI Appendix*, Fig. S3G). We further found that *FXR1* is phosphorylated and binds to phosphorylated *PRKCI* in NSCLC cells (*SI Appendix*, Fig. S3H–L). When a Flag-tagged full length of *FXR1* and a hemagglutinin-tagged (HA) constitutively active wild type (WT) of *PRKCI* (17) or a HA-tagged dominant negative (DN) mutant of *PRKCI* were cotransfected into HEK293T cells, we found that *FXR1* binds only to active WT-*PRKCI* but not *PRKCI* DN mutant (Fig. 3C



**Fig. 2.** *FXR1* regulates lung cancer cell growth in vitro and in vivo. (A) Immunoblot analysis of a panel of NSCLC cell lines for *FXR1* and  $\beta$ -actin. Relative *FXR1* protein level was normalized to  $\beta$ -actin. (B) Effect of *FXR1*-specific siRNA on viable cells over time. Cells were measured 1, 2, 4, and 6 d after plating using the Cyquant assay. \* $P < 0.05$ ; \*\* $P < 0.01$ . (C) Down-regulation of *FXR1* protein in H520 and HCC95 cells treated with siRNA against *FXR1* after 48 h. SC: scrambled siRNA control. si#1, si#2: two individual *FXR1* siRNAs. (D) Activated Caspase 3 and PARP in H520 cells treated with pooled *FXR1*-siRNA after 72 h. SC: scrambled siRNA control. (E) Effect of *FXR1*-shRNA on H520 anchorage-independent soft agar colony formation. H520 cells were infected with three lentiviruses containing shRNA targeting *FXR1* or a nontarget (NT) sequence. Two stable *FXR1* knockdown (KD) cell lines are shown. H520/*FXR1* KD cells grow significantly slower than H520/NT cells (\* $P < 0.05$ , \*\* $P < 0.01$ ). (F) Effect of *FXR1* overexpression on BEAS-2B anchorage independent cell growth compared with vector control. pBabe-V, BEAS-2B-vector group; pBabe-*FXR1*, BEAS-2B cells stably transfected with pBabe-Flag-*FXR1*. (G) Effects of *FXR1* knockdown on tumorigenicity in nude mice. H520 NT and H520 *FXR1* KD cells were injected s.c. into the flanks of nude mice ( $n = 10$ ). Tumor volume was measured twice a week in all experiments by caliper and calculated by the formula:  $V = 3.14 \text{ (smaller diameter)}^2 \text{ (larger diameter)} / 6$ . The quantification of tumor volume changes over a 4-wk period is shown; \*\* $P < 0.01$ . (H) Representative H&E staining, immunohistochemical staining of *FXR1* and Ki67 in tumors formed by H520 NT and H520 KD in one mouse. Scale bars, 50  $\mu\text{m}$ . (I) Quantification of *FXR1* and Ki67 immunohistochemical staining shown by the percentage of positively stained cells compared with total number of cells per field. The  $P$  value (Student's  $t$  test) relative to NT is shown. \*\* $P < 0.01$ . Data are mean  $\pm$  SEM.

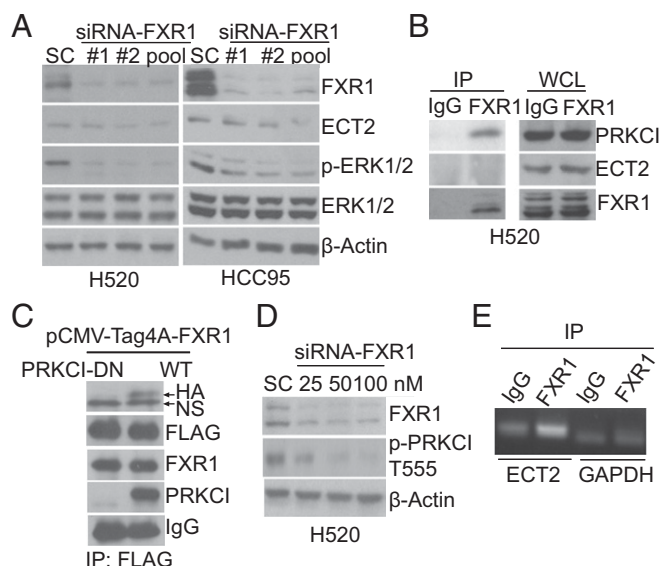
and *SI Appendix*, Fig. S3M). These results indicate that *FXR1* directly binds to active PRKCI and this binding might be a common event in NSCLC cells. In addition, we discovered that the knockdown of *FXR1* leads to the down-regulation of phosphorylated PRKCI in H520 cells (Fig. 3D). In contrast, the inhibition of PKC activity with chelerythrine resulted in a time- and dose-dependent decrease in phosphorylated PRKCI/T555, *FXR1* and *ECT2* expression (*SI Appendix*, Fig. S3N and O). Altogether, these results suggest that *FXR1* is phosphorylated by PRKCI and that there is a reciprocal regulation between PRKCI and *FXR1* in lung cancer cells.

Because *FXR1* regulates *ECT2* expression without binding to the *ECT2* protein, we tested whether *FXR1* binds to *ECT2* mRNA. We recovered endogenous *ECT2* mRNA transcripts by RT-PCR in H520 cell lysates immunoprecipitated with anti-*FXR1* (Fig. 3E and *SI Appendix*, Fig. S3P). To further determine the role of *FXR1* in *ECT2* gene expression, we performed

actinomycin D chase experiments (18) to measure *ECT2* mRNA half-life in H520-*FXR1*-KD cells and found that the half-life of *ECT2* mRNA in H520-*FXR1*-KD cells was much shorter than those observed in control cells ( $P < 0.01$ , *SI Appendix*, Fig. S3Q). These results demonstrate that *FXR1* regulates *ECT2* expression by binding to and stabilizing *ECT2* mRNA in H520 cells.

**FXR1 Overexpression Predicts Overall Survival in NSCLC.** To test the translational potential of *FXR1* as a biomarker, we studied the association of *FXR1* expression with clinical outcomes by immunohistochemistry in 292 NSCLCs (*SI Appendix*, Table S5). *FXR1* protein was localized to the cytoplasm in lung cancer cells (Fig. 4A). *FXR1* expression was significantly and positively correlated with squamous histology, disease stage and smoking history (*SI Appendix*, Fig. S4). Elevated *FXR1* expression was associated with shorter overall survival (OS) independent of age, sex, stage, and smoking history ( $P = 0.0024$ , HR, 1.28, 95% CI,





**Fig. 3.** FXR1 regulates ECT2 and PRKCI expression via distinct mechanisms. (A) Down-regulation of ECT2 and phospho-ERK1/2 in FXR1 knockdown NSCLC cells. (B) Immunoprecipitation (IP) of FXR1 reveals that FXR1 binds to PRKCI protein but not to ECT2 in H520 cells. (C) HEK293T cells were cotransfected with Flag-tagged pCMV-Tag4A-FXR1 and either HA-tagged pHACE-PRKCI-WT or pHACE-PRKCI-DN for 48h. Cell lysates were then subjected to IP with anti-HA, anti-Flag, anti-FXR1 or anti-PRKCI. The IP experiments reveal that FXR1 binds to active PRKCI but not the PRKCI DN mutant. NS, nonspecific band. (D) Reduced phosphorylated PRKCI T555 is observed in FXR1 knockdown H520 cells. (E) IP using either anti-FXR1 or normal IgG in H520 under conditions that preserve the association of RNA-binding proteins with target mRNAs. IP was followed by RT-PCR analysis to detect endogenous *ECT2* mRNA; PCR products were resolved by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

1.09–1.49, *SI Appendix*, Table S6). Multivariate analysis further indicates that FXR1 expression is a better predictor for OS than other clinical variables tested in patients with stage I NSCLC treated surgically as primary treatment modality (HR, 1.31, 95% CI, 1.05–1.63,  $P = 0.018$ ), particularly in lung SCC subtype (HR, 1.44, 95% CI, 1.03–2.01,  $P = 0.032$ , Fig. 4B, *SI Appendix*, Tables S7 and S8). To evaluate the clinical relevance of FXR1 expression in lung adenocarcinoma (ADC), we analyzed FXR1 mRNA expression in six independent cohorts consisting of 697 stage I lung ADC patients (*SI Appendix*, Table S9). A meta-analysis indicated higher FXR1 mRNA is associated with shorter OS in lung ADC stage I patients (Fig. 4C). Together, these results suggest that FXR1 expression is a novel prognostic biomarker in NSCLC.

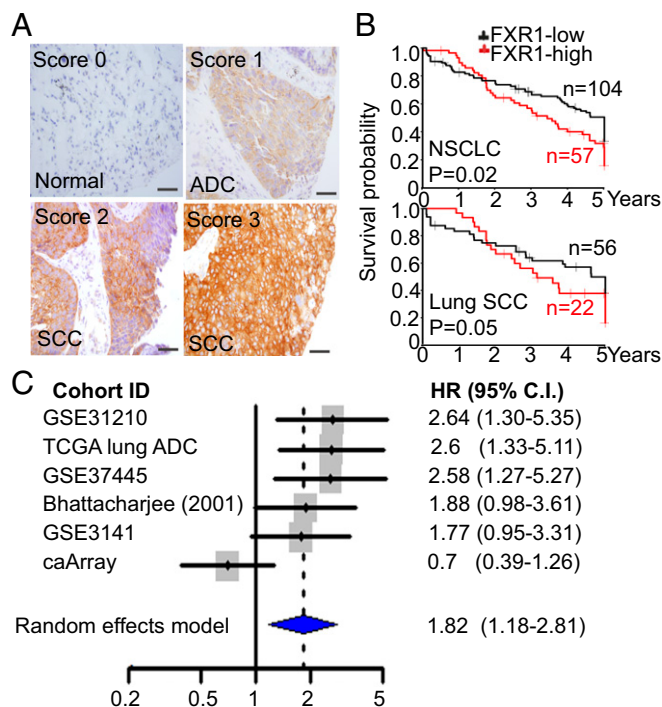
**FXR1 Overexpression Correlates with Poor Clinical Outcomes in Multiple Cancers.** To determine whether FXR1 may be involved in other solid tumors, we interrogated its gene copy number alteration and gene expression in 18 types of human cancers in the TCGA database. We found FXR1 amplification in 19–48% of SCCs of different origin including lung, cervix, and head and neck squamous carcinoma (HNSC) (*SI Appendix*, Fig. S5A and Table S10). Elevated FXR1 mRNA levels were observed in 10 types of cancers including breast, ovarian and HNSC cancers compared with control tissues (*SI Appendix*, Table S10 and Fig. S5B). In breast cancer, FXR1 mRNA expression was tested as a prognostic biomarker using multivariate Cox proportional hazards analysis in eight independent breast cancer cohorts including GSE3494 ( $n = 250$ ), GSE4922 ( $n = 249$ ), GSE2034 ( $n = 286$ ), GSE2603 ( $n = 121$ ), GSE11121 ( $n = 200$ ), TCGA ( $n = 483$ ), TRANSBIG consortium ( $n = 198$ ), and METABRIC consortium ( $n = 1,992$ , Fig. 5A) (*SI Appendix*, Tables S11–S18). The association of FXR1 mRNA with poor outcome was significantly in all these

cohorts (*SI Appendix*, Tables S19–S26). In ovarian cancer, FXR1 mRNA level was significantly associated with worse relapse free survival (RFS) in early stage of ovarian cancer patients in combined GSE9891 and TCGA cohorts (HR, 5.38, 95% CI 1.95–14.82,  $P = 0.001$ , Fig. 5A and *SI Appendix*, Tables S27 and S28). In HNSC, multivariate Cox analysis indicates that FXR1 mRNA level was independently associated with poor overall survival (TCGA, HR, 1.56,  $P = 0.037$ , Fig. 5A) and metastasis-free survival (E-TABM-302, HR, 2.25,  $P = 0.01$ ) (*SI Appendix*, Tables S29–S32).

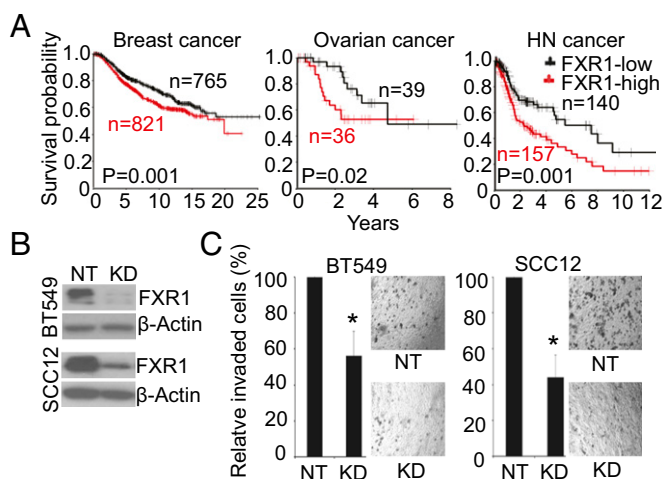
Finally, to demonstrate that FXR1 overexpression was a biological plausible biomarker of tumor progression in these other tumor types, we examined its expression in seven breast cancer cell lines, five HNSC lines and two ovarian cancer cell lines (*SI Appendix*, Fig. S5C). shRNA knockdown of FXR1 expression in three cell lines (Fig. 5B and *SI Appendix*, Fig. S5D), each of different histological subtype and overexpressing FXR1 protein (without genomic amplification) significantly impaired cell invasion of BT549 (breast cancer line) and SCC12 (HNSC line) in vitro (Fig. 5C). The growth inhibition of A2780 (ovarian cancer line) and SCC12 upon FXR1 depletion were also confirmed in mouse xenograft models (*SI Appendix*, Fig. S5E–G). Together, these results suggest that FXR1 is a newly identified candidate prognostic marker and may play critical role in other human cancers including breast, ovarian and head and neck cancers.

## Discussion

Here we described, to our knowledge for the first time, the functional and clinical significance of FXR1 overexpression in NSCLC and other solid tumors. We and others have previously identified FXR1 gene is located at the peak of 3q26-29 amplicon



**Fig. 4.** FXR1 overexpression is associated with poor overall survival in NSCLC. (A) Representative immunohistochemical staining of FXR1 protein expression in sections of formalin-fixed paraffin-embedded normal lung tissue, lung ADC, and SCCs. (B) Kaplan–Meier plots of overall survival of stage I NSCLC patients ( $n = 161$ ) or SCC ( $n = 78$ ) stratified by FXR1 protein expression. The log-rank  $P$  values are shown. (C) FXR1 mRNA level is associated with poor overall survival in stage I lung ADC patients indicated by a meta-analysis in six independent cohorts (*SI Appendix*, Table S9). Patients who received adjuvant chemotherapy were excluded.



**Fig. 5.** FXR1 is associated with poor outcome in multiple human cancers. (A) FXR1 overexpression is associated with worse disease specific free survival in METABRIC breast cancer cohort, worse relapse-free survival in early stage of ovarian cancer (combined GSE9891 and TCGA) and worse overall survival in TCGA HNSC cohort. The log-rank *P* values are shown. The datasets were described in detail in *SI Appendix, Tables S17, S27, and S29*. (B) Down-regulation of FXR1 protein in BT549 and SCC12 cells treated with shRNA against FXR1. NT: nontarget shRNA control. KD: shRNA FXR1 knockdown. (C) Effect of FXR1-shRNA on BT549 and SCC12 cell invasion. Significant differences between NT and FXR1 KD cells are indicated; \**P* < 0.05.

in squamous cell carcinoma of the lung (9, 19). *FXR1* promotes lung epithelial cell growth and its overexpression is critical for lung cancer cell proliferation, survival and invasion. We discovered that *FXR1* engages with two novel binding partners (*PRKCI* and *ECT2*), genes located in the same 3q amplicon, thereby providing a mechanism of action and opening new potential therapeutic avenues in 3q amplified lung tumors. Loss of *FXR1* substantially reduced the size of multiple types of tumors in mouse xenograft models. *FXR1* is highly expressed in many human cancers and its overexpression is associated with poor outcomes in NSCLC, breast, ovarian cancers and HNSC (Table 1), suggesting a role across multiple tumor types. In contrast, the expression of *FMRI* and *FXR2* is significantly lower than *FXR1* in normal or squamous carcinoma cells (*SI Appendix, Fig. S6*). Only *FXR1* expression correlates with smoking history (*SI Appendix, Fig. S7*) and predicts poor prognosis in lung adenocarcinoma and HNSC (*SI Appendix, Tables S33 and S34*), further suggesting a unique role of *FXR1* in the pathogenesis of airway epithelial cell cancers (20).

FMR members regulate mRNA translation through mRNA-protein or microRNA-protein complexes (21, 22). To date, the FXR1-associated complex has not been identified in human cancer cells. In this report we provide mechanistic studies suggesting a novel role of *FXR1* in the progression of NSCLC. *FXR1* regulates ERK signaling pathway through interaction with *PRKCI* and *ECT2*, two known oncogenes within same 3q26-29 amplicon (7, 23). *PRKCI* has been reported to be activated by PI3 Kinase, PDK1, RAS and SRC either alone or in association with the PAR complex, although the signaling mechanisms involved appear to differ in different tumor types (24). Our results demonstrate that *PRKCI* is a new binding partner of FXR1 in lung cancer cells. The detailed binding sites of FXR1 to *PRKCI* and the effect of FXR1-*PRKCI* interaction on FXR1-bound transcripts in lung cancer cells remain to be elucidated. *ECT2*, a proposed oncogene in NSCLC (7), is the other newly discovered binding partner of FXR1. *ECT2* is phosphorylated by *PRKCI* and associated with PAR complex to drive transformed lung cancer cell growth and invasion via activation of ERK signaling cascade (14, 25). Here, we provide compelling evidence for *ECT2* as a novel mRNA target of *FXR1* in lung cancer. The change in the expression of FXR1 involved in SCC exemplifies how a derangement in the mRNA stabilization process may be associated with tumor progression. Together, our findings unravel a new important mechanistic insight into how FXR1 participates in transformation by forming a FXR1/*PRKCI*/mRNA complex. Further profiling of FXR1 RNA protein complexes to identify potential binding proteins or mRNAs in cancer versus normal cells may provide new insights into the role of *FXR1* in the regulation of specific signaling pathways in human cancer.

The importance of *FXR1* in this study is suggested by its co-operation with other driver genes in the 3q amplicon. We report the results of functional studies in five cell lines overexpressing FXR1 with or without gene amplification and confirmed the critical driver properties provided by overexpression of FXR1. Our results demonstrate that *FXR1* is a critical member of this large amplicon (176 genes included between 3q26-29) and contributes to the pathogenesis of lung cancer. The single gene driver theory has been challenged by recent studies (26, 27), supporting that in fact multiple oncogenes work in concert to drive the cancer, sometime as a driver, sometime a passenger gene. Genomic amplifications happen in different size (Mb) and intensity (copy number between 4 and 25). The mechanisms underlying these differences are poorly understood. Chromothripsis is a possible mechanism to explain these genomic amplifications (28). Other multistep evolutionary process starting from single chromosome ancestral amplicons could explain the process (29). Therefore, in large amplicon like this one (~32 Mb), the pressure may be such that it is not one gene but a set of

**Table 1.** FXR1 expression is associated with poor outcomes in multiple human cancers

Cancer type	Outcome	Sample size	HR*	<i>P</i>	Biomarker	Platform
NSCLC stage I	Overall survival	161	1.31	0.018	Protein	IHC
Lung SCC stage I	Overall survival	78	1.44	0.032	Protein	IHC
Lung ADC stage I <sup>†</sup>	Overall survival	697	1.82	0.007	mRNA	Microarray and RNA-seq
Breast cancer (TCGA)	Overall survival	483	2.75	0.03	mRNA	RNA-seq
Breast cancer (METABRIC)	Disease-specific free survival	1,992	1.43	0.03	mRNA	Microarray
Ovarian cancer (stage I-II) <sup>‡</sup>	Relapse-free survival	81	5.38	0.001	mRNA	Microarray
Head and neck SCC (TCGA)	Overall survival	300	1.54	0.04	mRNA	RNA-seq
Head and neck SCC <sup>§</sup>	Metastasis-free survival	81	2.25	0.01	mRNA	Microarray
Total		3,795				

\*Hazard ratio (HR) was calculated using multivariate Cox proportional-hazards analysis.

<sup>†</sup>HR was derived from a meta-analysis of six independent lung ADC cohorts (*SI Appendix, Table S9*).

<sup>‡</sup>HR was derived from a combined two independent cohorts (GSE9881 and TCGA).

<sup>§</sup>HR was derived from E-TABM-302 cohort.



genes that can drive the genomic alteration and that many neighboring genes are indeed coamplified as bystanders of the process. However, some of the bystanders functionally are critical to tumor progression. These genes, drivers and passengers, might be functionally associated and their cooperation might be a key mechanism to promote cancer progression. More evidence that multiple oncogenic drivers in 3q amplicon cooperate to promote tumorigenesis is indeed emerging. Justilien et al. first reported a mechanism by which PRKCI forms a complex with ECT2-PAR6A to drive transformed growth through activation of a RAC1-PAK-MEK1-ERK1,2 signaling axis in NSCLC (14, 25). Recently, the same group reported a functional link of PRKCI and SOX2 to activate hedgehog signaling in lung SCC (30). Hagerstrand et al. reported SEC62 and SKIL as new cancer drivers in 3q amplicon and co-overexpression of SEC62 and SKIL induces transformation independently of PIK3CA or SOX2 (31). Interestingly, PIK3CA, ECT2, and SKIL were among 34 3q genes identified as potential mRNA targets of FXR1 in human embryonic kidney 293 cells (32), further implying the regulatory mechanisms driven by RNA binding protein FXR1 other than coamplification may play a role in cancer. Here we are first to describe a new oncogenic role of an RNA binding protein FXR1 linking to PRKCI and ECT2 in NSCLC. We also demonstrate that although genomic amplification is a key mechanism of FXR1 gene overexpression, it is the gene expression level that drives tumor progression in vitro and in vivo.

In conclusion, our results present direct evidence that the RNA binding protein FXR1 is a novel candidate oncogene interacting with two other oncogenes ECT2 and PRKCI located on same 3q26-29 amplicon in lung cancer. Future investigations are warranted to explore whether FXR1 may have utility as a biomarker predictive poor outcomes of human cancers and may represent a novel therapeutic target.

## Materials and Methods

**Biospecimens.** Lung cancer tissues were collected from surgical specimens through the Specialized Program of Research Excellence (SPORE) in lung at Vanderbilt University Medical Center and the Department of Veterans Affairs

(VA) Medical Center in Nashville, Tennessee. All samples were reviewed by a pathologist (R.E). 292 lung cancer tissues contained in tumor tissue microarrays were used for the evaluation of FXR1 protein expression using immunohistochemistry. Clinical characteristics of these patients are described in *SI Appendix, Table S5*. All primary tumors were fresh-frozen, with efforts made to use samples with tumor content >70%. Studies using human biospecimens were approved by the Vanderbilt University Internal Review Board and the Nashville VA Internal Review Board.

**Gene Copy Number and Expression Data Analysis.** Array CGH and mRNA microarray expression data obtained ( $n = 24$ ) in our laboratory have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under the following accession numbers: GSE40048 and GSE40074. aCGH data were analyzed using Agilent DNA Analytics Software (version 4.0) with ADM-2 algorithm. The average log2 ratio of 0.8 was defined as the cutoff for amplification or 0.3 for low level gain. mRNA expression data were normalized and analyzed by Agilent GeneSpring11. The analysis on other types of human cancer cohorts publically available including 18 TCGA datasets was described in *SI Appendix*.

**Statistical Analysis.** The relevance of FXR1 protein or mRNA expression to the clinical parameters of cancer patients were compared using Student's *t* test, Wilcoxon two-sample test, or Kruskal-Wallis test. Pearson correlation was used for gene expression correlation analysis. The relationship of clinical outcomes such as overall survival, relapse free survival or distant recurrence free survival to FXR1 expression was assessed with the Cox proportional hazards regression and the Kaplan-Meier method. All statistics analysis was done using R package ([www.R-project.org](http://www.R-project.org)). Differences were considered statistically significant if the *P* value was <0.05.

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