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Oncogenic *ERBB3* Mutations in Human Cancers

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SUMMARY

The human epidermal growth factor receptor (HER) family of tyrosine kinases is deregulated in multiple cancers either through amplification, overexpression, or mutation. ERBB3/HER3, the only member with an impaired kinase domain, although amplified or overexpressed in some cancers, has not been reported to carry oncogenic mutations. Here, we report the identification of *ERBB3* somatic mutations in ~11% of colon and gastric cancers. We found that the ERBB3 mutants transformed colonic and breast epithelial cells in a ligand-independent manner. However, the mutant ERBB3 oncogenic activity was dependent on kinase-active ERBB2. Furthermore, we found that anti-ERBB antibodies and small molecule inhibitors effectively blocked mutant ERBB3-mediated oncogenic signaling and disease progression in vivo.

INTRODUCTION

The HER family of receptor tyrosine kinases (RTK), also known as ERBB receptors, consists of four members—EGFR/ERBB1/HER1, ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4 (Baselga and Swain, 2009; Hynes and Lane, 2005). The ERBB family members contain an extracellular domain (ECD), a single-span transmembrane region, an intracellular tyrosine kinase domain, and a C-terminal signaling tail (Burgess et al., 2003; Ferguson, 2008). The ECD is a four domain structure consisting of two L domains (I and III) and two cysteine-rich domains (II and IV) (Burgess et al., 2003; Ferguson, 2008). The ERBB receptors are activated by multiple ligands that include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and neuregulins (Yarden and Sliwkowski, 2001). Activation of the receptor involves a single ligand molecule binding simultaneously to domains I and III, leading to heterodimerization or homodimerization through a dimerization arm in domain II (Burgess et al., 2003; Cho and Leahy, 2002; Dawson et al., 2005; Lemmon and Schlessinger, 2010; Ogiso et al., 2002). In the absence of ligand, the domain II dimerization arm is tucked away via an intramolecular interaction with domain IV, leading to a "tethered," autoinhibited configuration (Burgess et al., 2003; Cho and Leahy, 2002; Ferguson et al., 2003; Lemmon and Schlessinger, 2010).

Significance

ERBB3, a kinase-impaired HER receptor tyrosine kinase family member, heterodimerizes with ERBB2 upon ligand binding to promote signaling. In particular, it is known to play a role in ERBB2-mediated oncogenic signaling. Whereas amplification and overexpression of ERBB3 are observed in some cancers, the occurrence and relevance of ERBB3 somatic mutations in oncogenesis is not established. Here, we report the identification of ERBB3 somatic mutations in ~11% of colon and gastric cancers. Functional characterization of the ERBB3 mutants show that, with ERBB2, they promote oncogenic signaling in a ligand-independent manner. We show that multiple targeted therapeutics that act on ERBB3, ERBB2, or their downstream proteins are effective in blocking ERBB3 mutant-mediated oncogenic signaling.

Although the four ERBB receptors share a similar domain organization, functional and structural studies have shown that the ERBB2 does not bind any of the known ERBB family ligands and is constitutively in an "untethered" (open) conformation suitable for dimerization (Garrett et al., 2003). In contrast, ERBB3, although capable of ligand binding, heterodimerzation, and signaling, has an impaired kinase domain (Baselga and Swain, 2009; Jura et al., 2009; Shi et al., 2010). Although, ERBB2 and ERBB3 are functionally incomplete on their own, their heterodimers are potent activators of cellular signaling (Holbro et al., 2003; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996).

Whereas the ERBB receptors are critical regulators of normal growth and development, their deregulation has also been implicated in the development and progression of cancers (Baselga and Swain, 2009; Hynes and MacDonald, 2009; Sithanandam and Anderson, 2008). In particular, gene amplification leading to receptor overexpression and activating somatic mutations are known to occur in ERBB2 and EGFR in various cancers (Hynes and MacDonald, 2009; Sithanandam and Anderson, 2008; Wang et al., 2006; Yamauchi and Gotoh, 2009). This has led to the development of multiple small molecule and antibody-based therapeutics that target EGFR and ERBB2 (Alvarez et al., 2010; Baselga and Swain, 2009). Although the precise role of ERBB4 in oncogenesis is not well established (Koutras et al., 2010), transforming somatic mutations in ERBB4 have been reported in melanoma (Prickett et al., 2009). Recently, ERBB3 has emerged as a potential cancer therapeutic target, given that it plays an important role in ERBB2 signaling and acquired resistance to existing therapeutics (Amin et al., 2010; Baselga and Swain, 2009). While ERBB3 amplification and/or overexpression is known in some cancers, only sporadic occurrence of ERBB3 somatic mutations has been reported (Ding et al., 2008; Greenman et al., 2007; Jeong et al., 2006; Kan et al., 2010; Stransky et al., 2011; TCGA, 2008, 2011). Furthermore, the functional relevance of these mutations has not been studied. Given the importance of ERBB3 in human cancers, we systematically surveyed human cancers and identified recurrent somatic mutations and show that these mutations are transforming. Further, we evaluated targeted therapeutics in ERBB3 mutant-driven animal models of cancer and show that a majority of them are effective in blocking ERBB3 mutant-driven oncogenesis.

RESULTS

Identification of *ERBB3* Mutations

In performing whole exome sequencing of 70 primary colon tumors along with their matched normal samples, we identified somatic mutations in *ERBB3* (Seshagiri et al., 2012). To further understand the prevalence of *ERBB3* mutation in human solid tumors, we sequenced all the coding exons of *ERBB3* in a total of 507 human primary tumor samples consisting of 100 colorectal (70 samples from the whole exome screen [Seshagiri et al., 2012], and 30 additional colon samples), 92 gastric, 71 non-small-cell lung (NSCLC) adenocarcinoma (adeno), 67 NSCLC (squamous), 45 renal carcinoma, 37 melanoma, 32 ovarian, 16 lung large cell, 15 esophageal, 12 small-cell lung cancer, 11 hepatocellular (HCC), and 9 other cancers (4 lung cancer [other], 2 cecum, 1 lung [neuroendocrine], 1 pancreatic and 1 rectal cancer) (Table S1 available online). We found protein-altering *ERBB3* mutations in 12% of gastric (11/92), 11% of colon (11/100), 1% of NSCLC (adeno; 1/71), and 1% of NSCLC (squamous; 1/67) cancers (Table S1; Figure 1A). Although previous studies reported sporadic protein-altering *ERBB3* mutations in NSCLC (squamous; 0.5% [3/188]; TCGA, 2008), glioblastoma (1% [1/91]; TCGA, 2008), hormone-positive breast cancer (4% [6/144]; Kan et al., 2010; Stephens et al., 2012), colon (1% [1/100]; Jeong et al., 2006), ovarian (1% [3/339]; Greenman et al., 2007; TCGA, 2011), gastric (10% [2/22]; Wang et al., 2011), and head and neck cancer (1% [1/74]; Stransky et al., 2011), none have evaluated their functional relevance in cancer (Figure 1A; Tables S2 and S3). Additionally, recent large-scale genomics studies reported ERBB3 mutations in colon (7% [14/212]; TCGA, 2012a) and breast (2% [8/484]; TCGA, 2012b) cancers. We confirmed all the mutations reported in this study to be somatic by testing for their presence in the original tumor DNA and absence in the matched adjacent normal tissue through additional sequencing and/or mass spectrometric analysis. Besides the nonsynonymous mutations, we also found three synonymous (nonprotein altering) mutations, one each in colon, gastric, and ovarian cancers.

A majority of the mutations identified in the human tumors clustered mainly in the ECD region, although some mapped to the kinase domain and the intracellular tail of ERBB3. Interestingly, among the ECD mutants were seven positions, V104, A232, P262 G284, D297, G325, and T355, that contained recurrent substitutions across multiple samples, indicating that these are mutational hot spots. Interestingly, the codon 104 mutation was the most frequent and was observed across multiple studies (Stephens et al., 2012; TCGA, 2012a, 2012b), indicating that it is functionally relevant. Furthermore, a majority of the recurrent missense substitutions at each of the hot spot positions resulted in the same amino acid change, indicating a potential driver role for these mutations. In addition to the ECD hot spots, our mutation data meta-analysis identified two recurring mutations, S846I and E928G, in the kinase domain (Jeong et al., 2006; TCGA, 2012a; Wang et al., 2011).

It is interesting to note that a majority of the mutated residues identified were conserved across ERBB3 orthologs (Figure S1A), indicating that these mutations likely have a functional effect.

To further understand the mutations, we mapped them to published ERBB3 ECD (Cho and Leahy, 2002) and kinase domain (Jura et al., 2009; Shi et al., 2010) crystal structures (Figures 1B–1D; Figure S1B). Interestingly, the hot spot mutations at V104, A232, and G284 cluster in the domain's I/II interface. The clustering of these three sites at the interface between domains I and II suggests that they may act via a common mechanism. Domain II comprises several cystine-rich modules arranged like vertebrae. Small changes in the relationship among these semi-independent features have been assigned functional importance among family members (Alvarado et al., 2009). Perhaps the V104/A232/G284 mutations shift one or more of these modules and cause an altered phenotype. The mutation at P262 is at the base of domain II, close to Q271 involved in the domain II/IV interaction required for the tethered, closed conformation. D297 is adjacent to the long arm of domain II and plays a role in heterodimerization under the influence of bound ligand. The large conformational difference seen in family members with and without ligand requires a hinge action at the

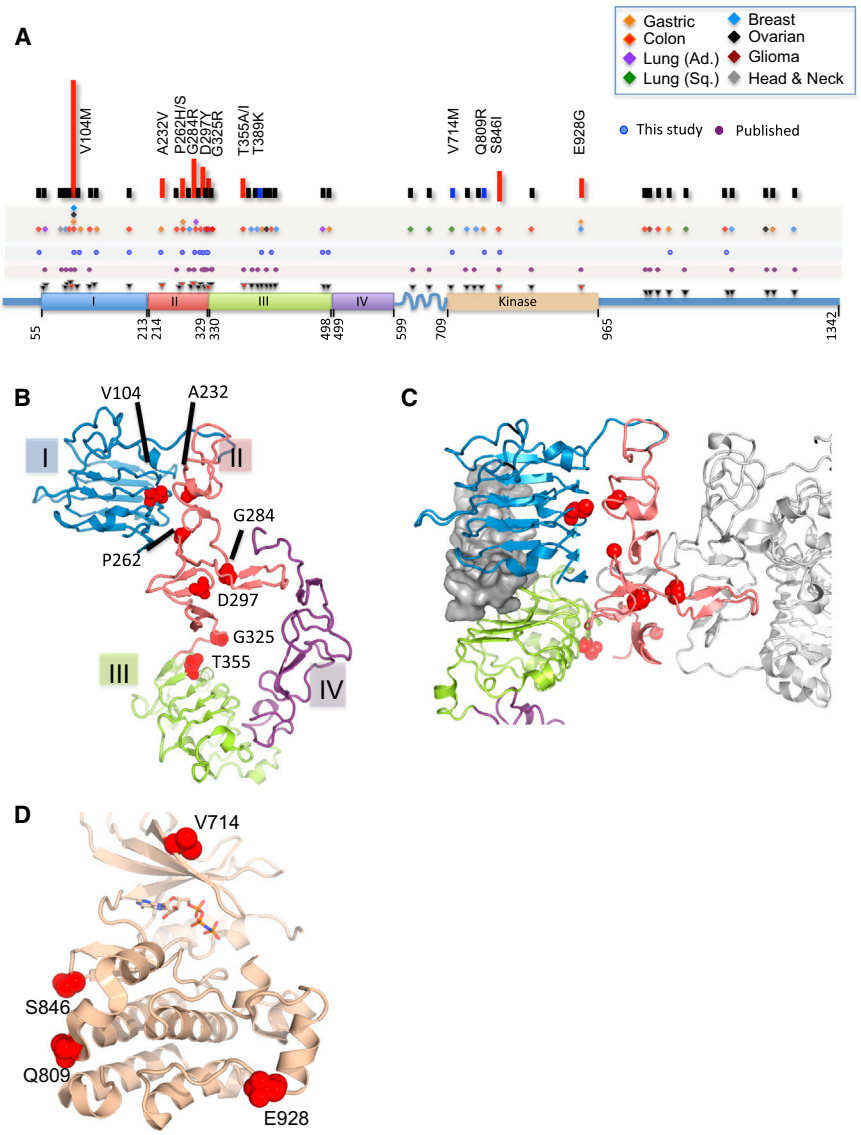


Figure 1. ERBB3 Somatic Mutations

(A) ERBB3 nonsynonymous somatic mutations (inverted triangles; red triangles depict hot spots; for mutation details see Tables S3 and S4) depicted over ERBB3 protein domains. The histogram on the top represents a count of mutations at each position observed in samples across studies (Tables S3 and S4; red bars indicate hot spot mutations and blue bars represent additional non-hot spot mutants tested for activity).

(B and C) Hot spot somatic ECD mutations mapped onto a crystal structure of "tethered" ERBB3 ECD (Protein Data Bank [PDB]: 1M6B) (B), or onto a model of "untethered" ERBB3/ERBB2 ECD heterodimer based on EGFR ECD dimer (PDB 1IVO) (C), using ERBB3 (PDB 1M6B) and ERBB2 (PDB 1N8Z). The ERBB3 ligand shown in (C) as a gray surface is based on EGF (PDB 1IVO).

(D) ERBB3 kinase domain somatic mutations mapped on to a structure of the ERBB3 kinase domain (PDB: 3LMG).

See also Figures S1 and Tables S1–S4.

border between domains II and III, where G325 is found. Similarly, T355 is also at the domain II/III border where large conformational transitions are likely to occur. Kinase domain mutations at residues 809 and 846 are homologous to positions proximal to the path taken by the C-terminal tail in the EGFR kinase structure, a segment that has been assigned a role in endocytosis. Residues at or near E928 are part of the protein/protein interface observed in the asymmetric kinase dimer seen among X-ray structures of ERBB family kinase domains. Most, but not all, of the mutations described here are located at or near sites of functional significance that affect ligand binding, heterodimer interactions, large conformational transitions, or possible signaling from subtle shifts among the modules in domain II. Details of the exact nature of changes at these sites and their phenotypic readout are beyond the scope of our current analysis. Sites of other mutations observed in ERBB3 mapped onto its structure are depicted in Figure S1B.

In an effort to understand the occurrence of mutations in select genes such as *KRAS*, *HRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *AKT1-3* in colon and gastric samples with *ERBB3* mutations, we sequenced and analyzed an additional set of genes in these samples. In ~30% of instances, we found that *ERBB3* mutations were independent of mutations in *KRAS*, *BRAF*, or *PIK3CA* in colon cancers (Figure S1C; Table S4). In gastric cancers, *ERBB3* mutations were independent of mutations in the receptor tyrosine kinase pathway genes in ~60% of the samples (Figure S1C).

ERBB3 Mutants Promote Anchorage-Independent Growth of Colonic and Breast Epithelial Cells

Immortalized mouse colonic epithelial (IMCE) cells can be transformed by expression of oncogenic Ras (D'Abaco et al., 1996; Whitehead et al., 1993). We used IMCE cells and tested ERBB3 mutants for anchorage-independent growth, signaling, and in vivo tumorigenesis by stably expressing the ERBB3 mutants either alone or in combination with ERBB2. We found that the ERBB3-wild type (WT) or the mutants when expressed on their own did not promote anchorage-independent growth (Figures 2A and 2B). However, a majority of the ERBB3 mutants, unlike the ERBB3-WT, when co-expressed with ERBB2 promoted anchorage-independent growth (Figures 2A and 2B). Consistent with the anchorage-independent growth observed, a majority of the IMCE cells expressing ERBB3 mutants along with ERBB2 showed elevated pERBB3 and/or pERBB2 and a concomitant increase in pAKT and/or pERK (Figures 2C and 2D). Although some of the ERBB3 mutants on their own showed elevated pERBB3 (Figure 2C), it did not promote anchorage-independent growth or downstream signaling. We tested if this was due to an increase in the autophosphorylation activity of the mutants in an in vitro kinase assay using purified recombinant ERBB3

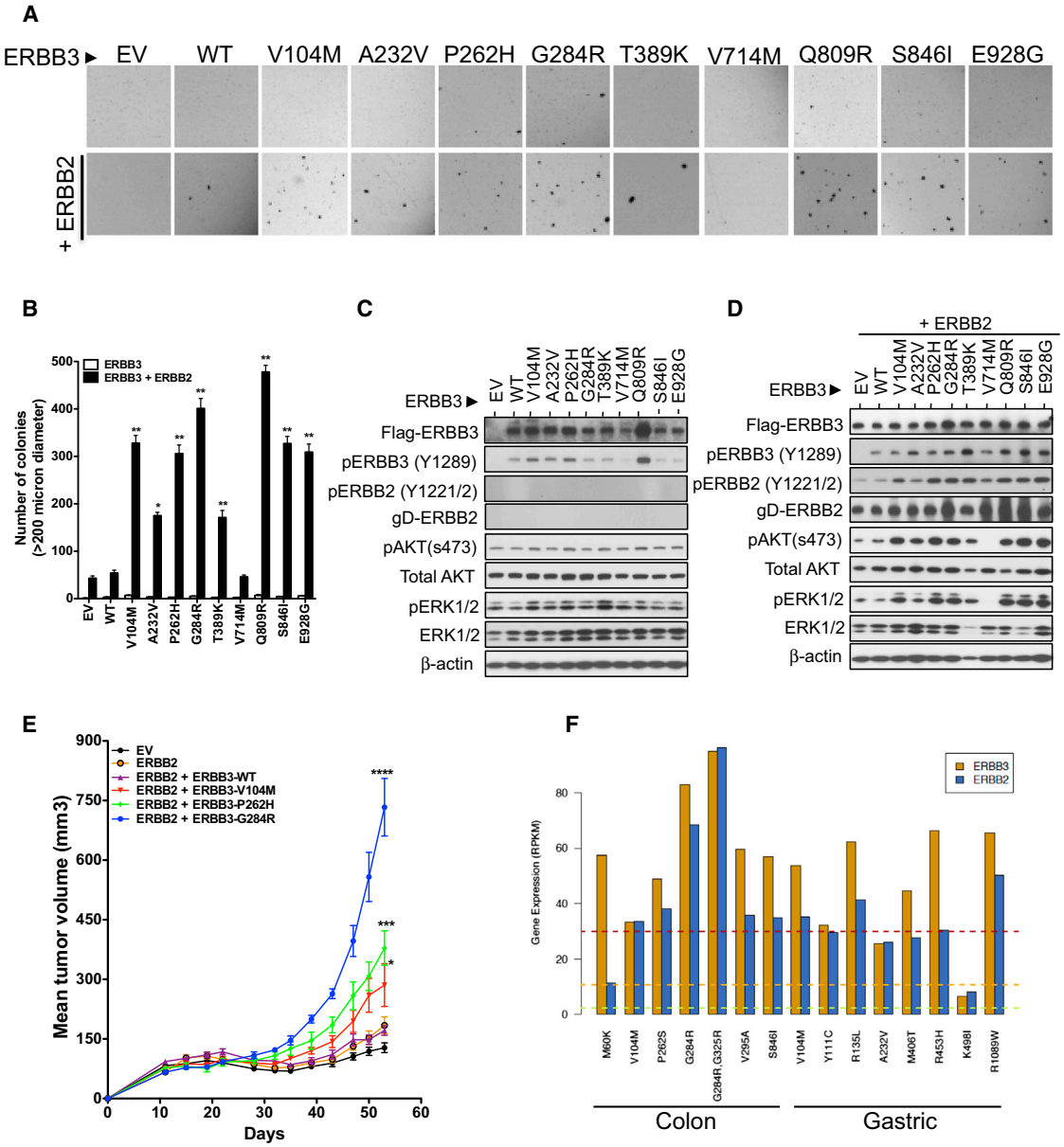


Figure 2. ERBB3 Mutants Support Transformation and In Vivo Tumor Growth of IMCE Colonic Epithelial Cells

(A–E) Representative images of anchorage-independent growth of IMCE colonic epithelial cells expressing either ERBB3-WT or mutants by themselves or combined with ERBB2 (A), colony counts (mean ± SEM) (B), phosphor-signaling (C and D), and in vivo tumor growth (E); mean ± SEM (n = 10 animals for each arm) compared to ERBB3-WT/ERBB2-expressing IMCE cells. EV, empty vector.

(F) Expression of *ERBB2* and *ERBB3* in *ERBB3*-mutant colon and gastric tumors as assessed by RNA-seq is expressed as reads per kilobase of exon model per million mapped reads (RPKM). The dashed red, orange, and green lines mark the average upper bound value below which lies the expression of 90%, 75%, and 50%, respectively, of the protein coding genes expressed in these samples.

See also Figure S2.

proteins. As reported recently (Shi et al., 2010), we found that the wild-type ERBB3 showed kinase activity (Figure S2A). However, under the same in vitro assay conditions, we did not detect an increase in the activity for the ERBB3 kinase mutants relative to the ERBB3-WT kinase protein (Figure S2A). It is likely that the observed increased pERBB3 level in cells expressing ERBB3 mutants alone is due to interactions with other endogenous ERBB family members in IMCE cells. To further confirm the oncogenic activity of the ERBB3 mutants, we tested several hot spot ECD mutant expressing cells for their ability to promote tumor growth in vivo. Consistent with their ability to support anchorage-independent growth and signaling, IMCE cells co-expressing ERBB3 V104M, P262H, or G284R, along with ERBB2 showed an increase in tumor growth (Figure 2E) compared to ERBB3-WT or ERBB2 alone or ERBB3-WT and ERBB2 combined. Consistent with the requirement for ERBB2

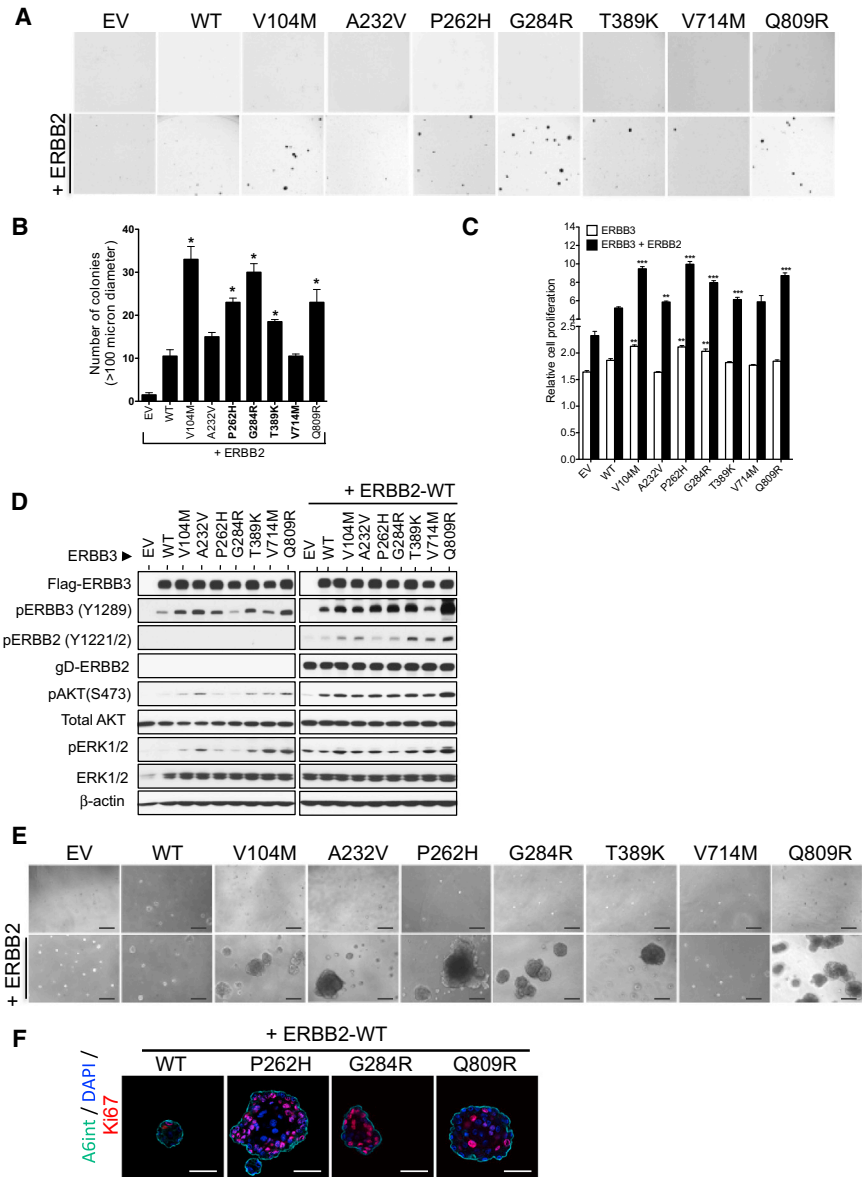


Figure 3. ERBB3 Mutants Promote Anchorage-Independent Growth, Proliferation, and Acinar Disruption

(A) Representative images depicting colonies formed by MCF10A cells expressing ERBB3 mutants either alone or combined with ERBB2.

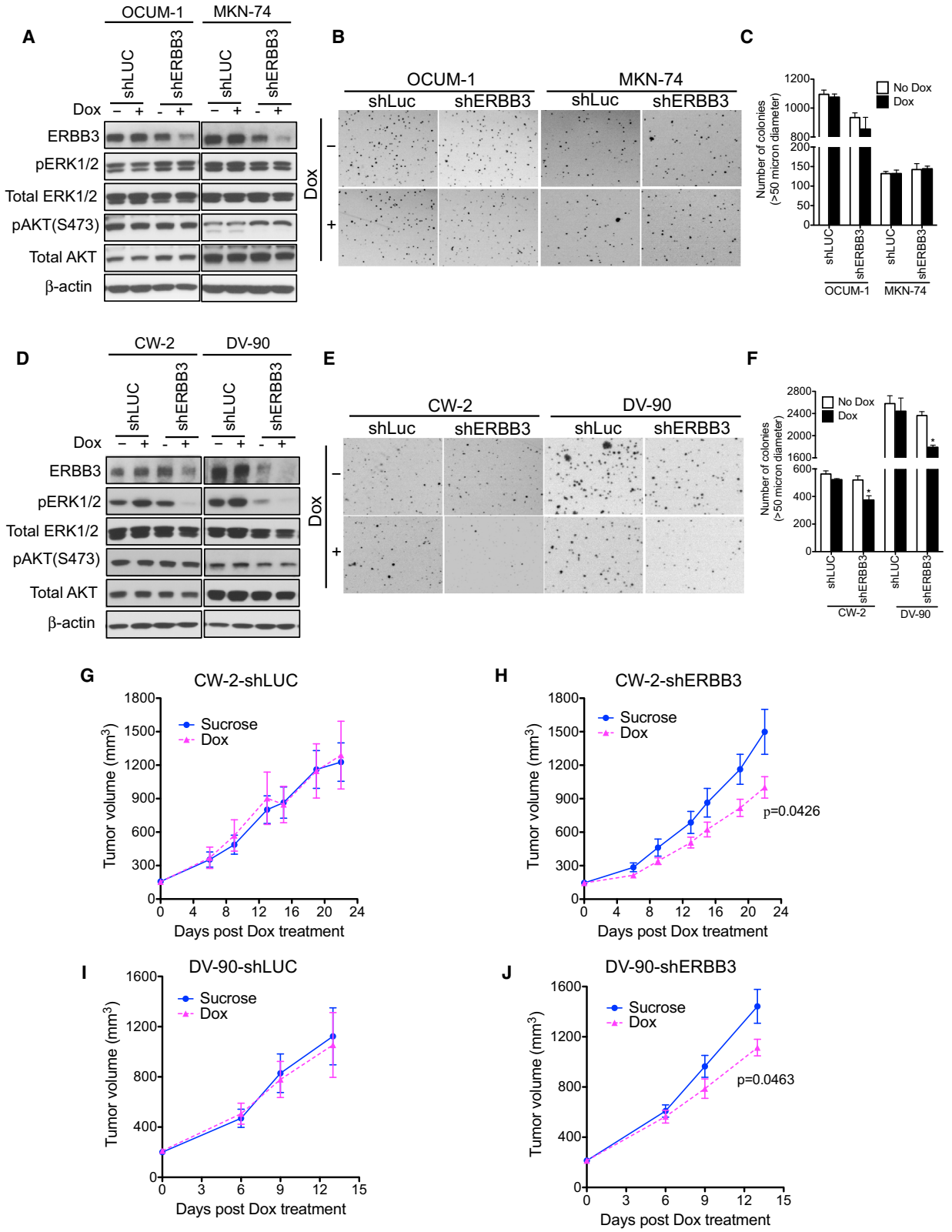
(B) Quantitation of the colonies from the assay depicted in (A) is shown for ERBB3 mutants coexpressed with ERBB2.

(C–F) EGF-independent proliferation (C), downstream signaling assessed by western blot (D), acinar architecture (E), and Ki67 staining (F) of MCF10A cells stably expressing ERBB3 mutants together with ERBB2 compared to those expressing ERBB3-WT/ERBB2. Scale bars = 100 μm in (E) and 50 μm in (F). Data in (B and C) represent mean ± SEM of the three independent experiments. Studies involving MCF10A were performed in the absence of serum, EGF, and NRG1. EV, empty vector.

See also Figure S3.

in ERBB3 mutant signaling, we confirmed the expression of the ERBB3 mutants and the expression of ERBB2 (Figure S2) using RNA-seq data (Seshagiri et al., 2012) in both colon and gastric tumors. Furthermore, we found that the ERBB3 mutant human primary tumor samples expressed both ERBB2 and ERBB3 at high levels (Figures 2F and S2B–S2D). The expression of ERBB2 and ERBB3 is significantly higher (p values of 4.102×10^{-5} and 2.05×10^{-6} for ERBB2 and ERBB3, respectively) compared to the 75th percentile expression of all the protein coding genes expressed in the tumor samples (Figures 2F, S2C, and S2D). Also, we found that the expression of ERBB2 and ERBB3 was significantly high (p values of 1.143×10^{-3} and 1.096×10^{-5} to 4.102×10^{-5} for ERBB2 and ERBB3, respectively) in the recent TCGA colon tumor data set (TCGA, 2012a; Figure S2E). In addition, we found both ERBB3 and ERBB2 to be expressed in CW-2 and DV-90, two recently identified ERBB3 mutant cancer cell lines (Garnett et al., 2012), at a similar or higher level compared to ERBB3-mutated tumors (Figure S2F). Furthermore, we confirmed that levels of ectopically expressed ERBB3 and ERBB2 at the protein level in IMCE cells were comparable or lower than the levels (Figure S2G) observed in ERBB3 mutant cell lines, CW-2 and DV-90, further supporting the relevance of ERBB3 mutants in oncogenic signaling.

We further tested ERBB3 mutants for their oncogenic activity using MCF10A breast epithelial cells, given that ERBB3 mutations were also found in breast tumors. The MCF10A cells require EGF for proliferation (Petersen et al., 1992; Soule et al., 1990) and can be rendered EGF-independent upon expression of oncogenes (Debnath et al., 2003; Muthuswamy et al., 2001). Also, MCF10A has been used to assess the oncogenic potential of ERBB family members (Muthuswamy et al., 2001; Wang et al., 2006). To further confirm the transforming activity of the ERBB3 mutants, we tested a subset of the ERBB3 mutants for their ability to promote EGF-independent growth, acinar formation, signaling, anchorage-independent growth, and migration by stably expressing them alone or in combination with ERBB2 in MCF10A cells (Figures 3A–3F and S3). We found that when the ERBB3 mutants were expressed alone in MCF10A and in the absence of exogenous ERBB3 ligand NRG1 and EGF, there was no colony formation (Figure 3A). However, expression of ERBB3 mutants in combination with ERBB2 showed a significant increase in colony formation compared to ERBB3-WT/ERBB2 cells (Figures 3A and 3B). Similarly, while expression of ERBB3 mutant alone showed only a modest ligand-independent proliferation, in the presence of ERBB2 they showed a significant increase in proliferation compared to ERBB3-WT/ERBB2-expressing cells (Figure 3C). Additionally,



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we observe elevated pERBB3, pAKT, and pERK in ERBB mutant-expressing cells when compared to ERBB3-WT (Figure 3D). The increase in signaling in MCF10A cells expressing ERBB3 alone is modest when compared to cells expressing both ERBB3 and ERBB2 and is likely due to endogenous EGFR present in the MCF10A cells (Figure 3D). In addition, the ERBB3 mutants in combination with ERBB2 led to increased level of pERBB3, pERBB2, pAKT, or pERK (Figure 3D).

MCF10A cells form acinar-cell spheroids when cultured on reconstituted three-dimensional (3D) basement membrane gel cultures in the presence of EGF (Muthuswamy et al., 2001; Muthuswamy, 2011). However, expression of some oncogenes can render them EGF-independent and also result in complex multiacinar structures (Brummer et al., 2006; Bundy et al., 2005; Debnath et al., 2003). In 3D culture studies lacking serum, EGF, and NRG1, ectopic expression of ERBB3 mutants in combination with ERBB2 in MCF10A cells promoted large acinar structures compared to MCF10A cells that co-express ERBB3-WT with ERBB2 (Figure 3E). Although ERBB2 overexpression in MCF10A cells cultured in media containing serum and EGF is known to disrupt acinar formation (Muthuswamy et al., 2001), we did not observe this with ERBB2 because our studies were done in the absence of serum, EGF, and NRG1. Staining for Ki67, a marker for proliferation, in acini derived from ERBB3 mutant/ERBB2 co-expressing MCF10A cells showed increased proliferation in all the mutants tested (Figure 3F). In addition, the same MCF10A cells expressing a subset of the ERBB3 mutant and ERBB2 also showed increased migration (Figures S3A and S3B) compared to ERBB3-WT/ERBB2 cells. These results taken together further confirm that the ERBB3 mutants in the presence of ERBB2 are capable of oncogenic signaling.

shRNA-Mediated ERBB3-Knockdown Impairs In Vivo Tumor Growth

Having established the oncogenic activity of ERBB3 mutants in IMCE cells, we sought to test the effect of knocking down ERBB3 in tumor cell lines. A recent study reported ERBB3 E928G mutation in the CW-2 colon cell line, and ERBB3 V104M mutation in the DV-90 lung line (Garnett et al., 2012). We used these lines to further test the relevance of ERBB3 in tumor formation following targeted knockdown. In addition, we used ERBB3-WT gastric lines OCUM-1 and MKN-74 as controls. We generated stable CW-2, DV-90, OCUM-1, and MKN-74 cell lines that expressed a previously published doxycycline (Dox)-inducible shRNA that targets *ERBB3* (Lee-Hoeflich et al., 2008). As an additional control, we generated stable lines that expressed a Dox-inducible luciferace (LUC) targeting shRNA. We found that the induction of the ERBB3 or LUC targeting shRNA in ERBB3-WT cell lines did not affect downstream signaling (Figure 4A) or anchorage-independent growth (Figures 4B and 4C), although ERBB3 levels were decreased in these lines (Figure 4A). However, in ERBB3 mutant lines, upon induction of ERBB3 shRNA in contrast to LUC-shRNA, led to a decrease in *ERBB3* and a concomitant decrease in pERK (Figure 4D). Furthermore, we did not see a significant decrease in pAKT (Figure 4D) following ERBB3 knockdown in CW-2 and DV-90, and this likely is due to the mutations in *KRAS* and *PI3KCA* found in these lines (Garnett et al., 2012). Additionally, consistent with the loss of ERBB3 and pERK following Dox-induction, both DV-90 and CW-2 showed reduced anchorage-independent growth compared to LUC shRNA-expressing lines or the uninduced lines (Figures 4E and 4F). We next tested whether knockdown of ERBB3 in DV-90 and CW-2 cells might affect their ability to form tumors in vivo. Upon Dox-mediated induction of *ERBB3* targeting shRNA, we found that both DV-90 and CW-2 cells showed a significant decrease in tumor growth compared to animals bearing DV-90 or CW-2 cells that expressed LUC-shRNA or were not induced to express the *ERBB3* shRNA (Figures 4G–4J). These data are indicative of an oncogenic role for ERBB3 in ERBB3 mutant lines.

ERBB3 Mutants Promote Interleukin-3-Independent Cell Survival and Transformation

BaF3 is an interleukin (IL)-3 dependent pro-B cell line that has been widely used to study oncogenic activity of genes and development of drugs that target oncogenic drivers (Lee et al., 2006; Warmuth et al., 2007). We used this system to test several ERBB3 ECD mutants (V104M, A232V, P262H, G284R, and T389K) that included five ECD-hot spot mutants and four ERBB3 kinase domain mutants (V714M, Q809R, S846I, and E928G) for their effects on interleukin-3 (IL-3)-independent cell survival, signaling, and colony formation by stably expressing the ERBB3 mutants either alone or in combination with ERBB2. ERBB3 is kinase impaired and following ligand binding it preferentially forms heterodimers with ERBB2 to promote signaling (Holbro et al., 2003; Karunagaran et al., 1996; Lee-Hoeflich et al., 2008; Sliwkowski et al., 1994). Consistent with this, in the absence of exogenous ligand, ERBB3 WT and the ERBB3 mutants on their own did not promote IL-3-independent survival of BaF3 cells above background levels (Figure 5A). However, in the absence of exogenous ERBB3 ligand, the ERBB3 mutants, unlike ERBB3-WT, promoted (~10- to 300-fold) IL-3-independent BaF3 cell survival when co-expressed with ERBB2 (Figure 5A), indicating that the ERBB3 mutants may function in a ligand-independent fashion. The cell survival activity of ERBB3 mutants was abrogated when they were co-expressed with a kinase-dead (KD) ERBB2-K753M mutant, confirming the requirement for a kinase-active ERBB2 (Figure 5A). We further investigated ERBB3 mutants for their ability to promote IL-3-independent colony formation. The ERBB3 mutants, on their own, as observed in the survival assay, did not form colonies (Figure 5B). However, we found that a majority of the ERBB3 mutants tested in combination with ERBB2 show increased colony formation and growth when compared to

Figure 4. shRNA-Mediated *ERBB3* Knockdown Delays Tumor Growth in ERBB3 Mutant CW-2 and DV-90 Cells

(A and D) Western blot showing levels of ERBB3 and pERK upon ERBB3 knockdown.

(B and E) Representative images showing anchorage-independent growth of cells expressing a Dox-inducible shRNA targeting *ERBB3* or *LUC*.

(C and F) Colony count from experiments depicted in (B) and (E). Data represent mean ± SEM.

(G–J) In vivo growth of ERBB3 mutant lines following induction of shRNA targeting *ERBB3* (H and J) or luciferase (G and I). Data shown in (G)–(J) are mean ± SEM (n = 8 for each arm).

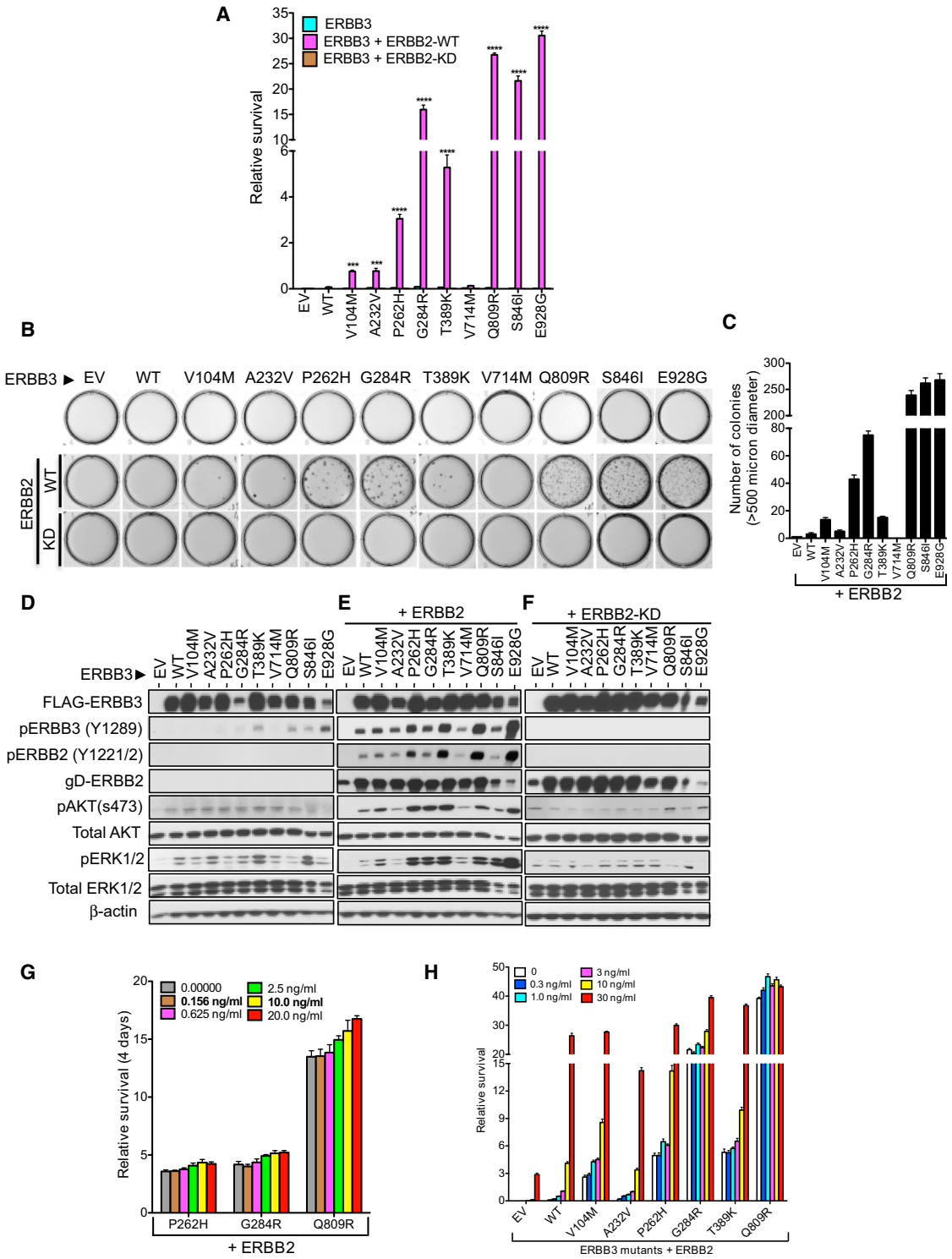

Figure 5. ERBB3 Mutants Transform and Promote IL-3-Independent Survival of BaF3 Cells

(A) IL-3-independent survival of BaF3 cells stably expressing ERBB3 mutants either alone or together with ERBB2 or ERBB2-KD.

(B) A representative image of colony formation by BaF3 cells stably expressing ERBB3 mutants either alone or in combination with either ERBB2 or ERBB2-KD.

(C) Bar graph (mean ± SEM) showing the number of colonies formed by BaF3 cells expressing the ERBB3 mutants along with ERBB2 depicted in (B).

(D–F) Western blot showing pERBB3, pERBB2, pAKT, and pERK status of BaF3 cells expressing ERBB3 mutants either alone (D) or in combination with ERBB2 (E) or ERBB2-KD (F).

(G) Effect of anti-NRG1, an NRG1 neutralizing antibody (Hegde et al., 2013; Figure S4A) on IL-3-independent survival of BaF3 cells promoted by ERBB3 mutants co-expressed with ERBB2.

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ERBB3-WT/ERBB2-expressing BaF3 cells (Figures 5B and 5C). As observed in the proliferation assay, ERBB3 mutants in the presence of ERBB2-K753M KD (ERBB2-KD) did not promote colony formation (Figure 5B), confirming the requirement for kinase-active ERBB2 in ERBB3 mutant-mediated oncogenic signaling. Western blot analysis of the BaF3 cells showed that the expression of ERBB3 mutants in combination with ERBB2 led to an increase in pERBB3, pERBB2, pAKT, and/or pERK compared to ERBB3-WT (Figures 5D–5F). Consistent with the lack of cell survival activity or colony formation, the ERBB3 mutants on their own or in combination with ERBB2-KD did not show elevated pERBB2 and/or pAKT/pERK (Figures 5D and 5F), although ERBB3 mutants on their own showed some elevated pERBB3 levels that were likely due to low levels of endogenous ERBBs expressed by BaF3 cells. However, this moderate increase of pERBB3 in ERBB3 mutants alone was not sufficient to increase pAKT and/or pERK and hence was unable to promote IL-3-independent cell survival or colony formation. In combination with ERBB2, the ERBB3 V714M kinase domain mutant consistent with its weak signaling showed only a modest cell survival activity and no colony formation (Figures 5A–5C). In contrast, Q809R, S846I, and E928G mutants in combination with ERBB2 showed robust downstream signaling compared to ERBB3-WT (Figures 5A–5C).

To further understand the mechanism by which the ERBB3 mutants promote oncogenic signaling, we tested the ligand dependency of the ERBB3 mutants in our BaF3 system by treating these cells with an increasing dose of an ERBB3-ligand neutralizing anti-NRG1 antibody (Hegde et al., 2013; Figure S4A). We found that the addition of a NRG1 neutralizing antibody had no effect on the ability of the ERBB3 mutants to promote IL-3-independent survival (Figure 5G), indicating that this increase in IL-3-independent survival of ERBB3 mutants is not due to the presence of residual ligand NRG1 or the secretion of NRG1 by the BaF3 cells. Consistent with this, in the absence of ligand, immunoprecipitation performed following cell surface receptor crosslinking showed increased levels of ERBB3 mutant/ERBB2 heterodimers when compared to cells co-expressing ERBB3-WT and ERBB2 (Figure S4B). This was further confirmed by the elevated levels of cell surface heterodimers in BaF3 cells expressing ERBB3 mutant/ERBB2 cultured in the absence of IL-3 and NRG1 using a proximity ligation assay (Söderberg et al., 2006; Figures S4C–S4E). Although further studies will be needed to understand the precise mechanism of oncogenic activation by ERBB3 mutants, these observations suggest that the ERBB3 mutants, combined with ERBB2, are capable of signaling in a NRG1-independent manner.

Having established that the ERBB3 mutants can signal independent of ligand, we tested if their activity could be augmented by ligand addition. We found that NRG1 was unable to support survival of BaF3 cells expressing ERBB3-WT or the mutants alone (Figure S4F). At the lower concentration range of NRG1 tested, unlike ERBB3-WT, most of the ERBB3 mutants except A232V in combination of ERBB2 did not increase IL-3-independent survival. However, at the highest concentration of exogenous NRG1 tested, a majority of the ERBB3 mutants, when co-expressed with ERBB2, increased the IL-3-independent survival of BaF3 cells (Figure 5H). Interestingly, the A232V ERBB3 mutant, like ERBB3-WT, showed a NRG1 dose-dependent, IL-3-independent survival response (Figure 5H). In contrast, the more active ERBB3 mutants, G284R and Q809R, did not show a significant increase in survival following ligand addition when compared to untreated cells expressing these mutants. The minimal response to ligand addition by G284R ECD and Q809R kinase domain mutants suggests a dominant role for the ligand-independent mode of signaling by these mutants (Figure 5H). These results show that while all the ERBB3 mutants are capable of ligand-independent signaling, some of them are still capable of responding to ligand stimulation.

ERBB3 Mutants Promote Oncogenesis In Vivo

We and others have shown that BaF3 cells, rendered IL-3-independent by ectopic expression of oncogenes, promote leukemia-like disease when implanted in mice and lead to reduced overall survival (Horn et al., 2008; Jaiswal et al., 2009a). We tested the ability of BaF3 cells expressing ERBB3-WT, two ECD mutants (P262H or G284R), or the kinase domain ERBB3 mutant (Q809R) together with ERBB2 for their ability to promote leukemia-like disease. BaF3 cells transduced with ERBB3-WT alone or ERBB2 together with empty vector were used as controls. We found that mice transplanted with BaF3 cells expressing ERBB3 mutants together with ERBB2 showed a median survival of 22–27 days (Figure 6A). In contrast, mice receiving BaF3 cells expressing either ERBB3-WT alone or ERBB2 with empty vector were all alive at the end of the 60-day study period. However, animals receiving BaF3 cells co-expressing ERBB3-WT and ERBB2 developed leukemia-like disease with a significantly longer latency (median survival, 39 days; Figure 6A). Although the ERBB3-WT/ERBB2 BaF3 cells in vitro did not show IL-3 independence, their activity in the animal model is likely due to the presence of growth factors and cytokines in vivo that can activate ERBB3-WT/ERBB2 dimers and in part due to ligand-independent signaling reported for ERBB3-ERBB2 heterodimers (Junttila et al., 2009). To follow disease progression, we conducted necropsies at 20 days on a cohort of three mice per treatment. Bone marrow, spleen, and liver samples from these animals were reviewed for pathologic abnormalities. Because the BaF3 cells were tagged with GFP, we examined isolated bone marrow and spleen for infiltrating cells with fluorescence-activated cell sorting (FACS). Consistent with the decreased survival, bone marrow and spleen from mice transplanted with cells expressing ERBB3 mutants/ERBB2 showed a significant increase in the proportion of infiltrating GFP-positive cells compared with bone marrow and spleen from mice receiving ERBB3-WT or ERBB2/empty vector control cells (Figures 6B–6D). Furthermore, concordant with the longer latency observed, a very low level of infiltrating

(H) Effect of increasing doses of exogenous NRG1 ligand on ERBB3 ECD mutants/ERBB2-expressing BaF3 cells following IL-3 withdrawal. BaF3 studies performed in the absence of IL-3 (A–H) and in the absence of NRG1 (A–F). Data shown in (A, G, and H) are mean ± SEM of at least three independent experiments. EV, empty vector.
See also Figure S4.

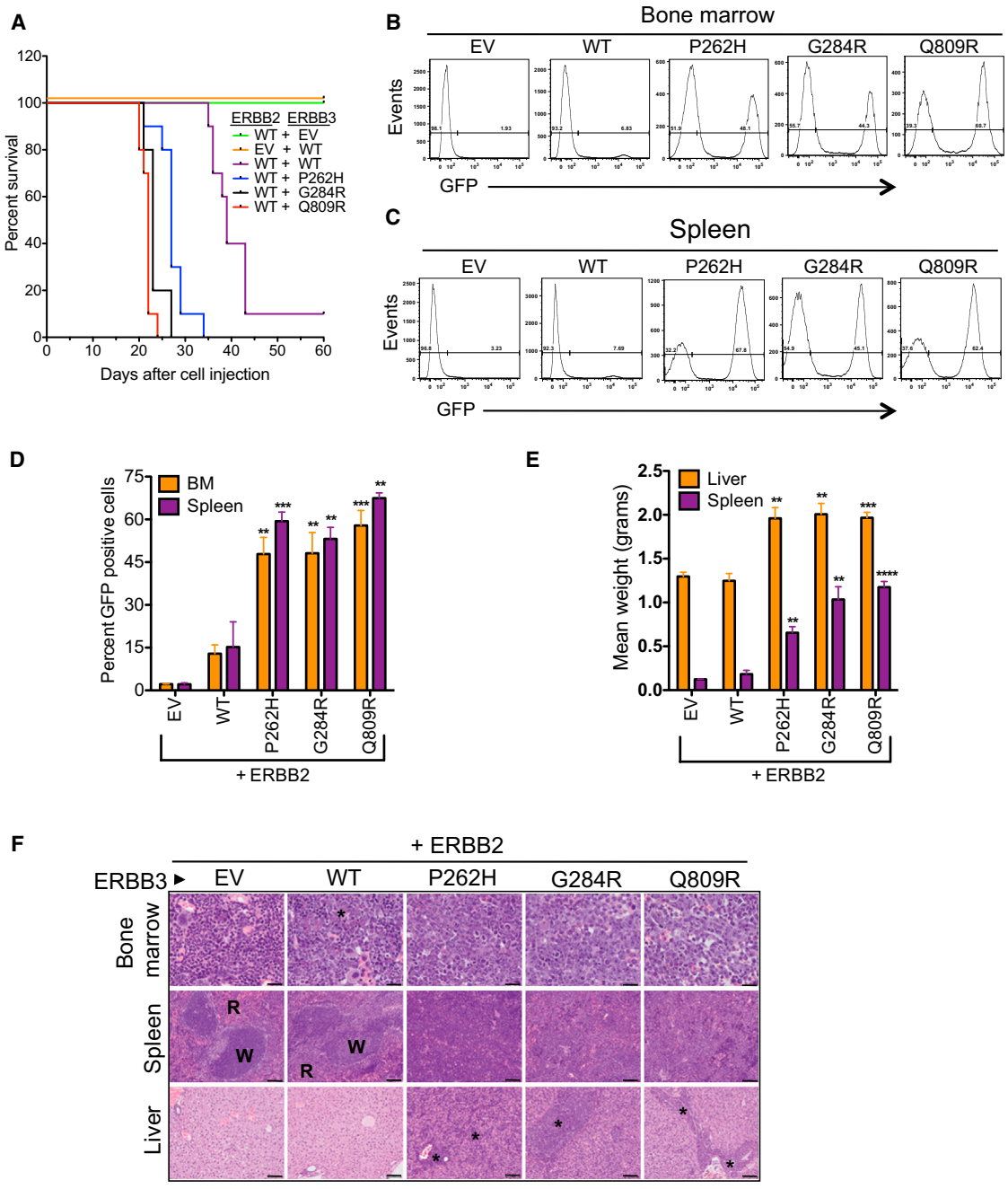

Figure 6. ERBB3 Mutants Promote Oncogenesis and Lead to Reduced Overall Survival

(A) Kaplan-Meier survival curves for cohorts of mice implanted with BaF3 cells expressing the indicated ERBB3 mutant/ERBB2 combination compared to control (EV) BaF3 cells (n = 10 for each arm; log-rank test $p < 0.0001$).

(B and C) Flow cytometric analysis of total bone marrow cells (B) and spleen cells (C) isolated from mice receiving GFP-tagged BaF3 cells expressing the various ERBB3 mutants/ERBB2.

(D) Mean number (mean ± SEM) of GFP-positive cells in the bone marrow and spleen of mice (n = 3) from each study arm.

(E) Mean weights (mean ± SEM) of spleen and liver from the mice (n = 3) in the indicated study arms are depicted.

(F) Representative H&E-stained bone marrow, spleen, and liver sections from the same mice analyzed in (B). The bone marrow from EV animals consists of normal hematopoietic cells. *Infiltrating tumor cells. R, red pulp; W, lymphoid follicles of white pulp. In the unmarked spleen section, there is a loss of red/white pulp architecture due to disruption by infiltrating tumor cells. Scale bar = 100 μm. EV, empty vector.

See also Figure S5.

GFP-positive cells were detected in the liver and spleen from animals receiving ERBB3-WT/ERBB2 cells. Also, animals from the ERBB3 mutant/ERBB2 arm showed increased spleen and liver size and weight compared to empty vector control or ERBB3-WT/ERBB2 (Figures 6E and S5), further confirming the presence of infiltrating cells. Additionally, histologic evaluation of hematoxylin and eosin (H&E) stained bone marrow, spleen, and liver sections showed significant infiltration of blasts in animals with cells expressing ERBB3 mutant/ERBB2 when compared to control at day 20 (Figure 6F). These results demonstrate the in vivo oncogenic potential of the ERBB3 mutants.

Targeted Therapeutics Are Effective against ERBB3 Mutants

Multiple agents that target the ERBB receptors directly or their downstream components are approved for treating various cancers (Alvarez et al., 2010; Baselga and Swain, 2009). Several additional candidate drugs that target ERBB family members, including ERBB3, and their downstream components are in various stages of clinical testing and development (Alvarez et al., 2010). We tested trastuzumab (an anti-ERBB2 antibody that binds ERBB2 domain IV; Junttila et al., 2009), pertuzumab (an anti-ERBB2 antibody that binds ERBB2 domain II and prevents dimerization; Junttila et al., 2009), anti-ERBB3.1 (an anti-ERBB3 antibody that binds domain II; Schaefer et al., 2011), anti-ERBB3.2 (an anti-ERBB3 antibody that binds domain III and blocks ligand binding; Wilson et al., 2011), MEHD7945A (a dual ERBB3/EGFR antibody that binds to domain III of EGFR or ERBB3 and blocks ligand binding; Schaefer et al., 2011), cetuximab (an anti-EGFR antibody that binds to domain III and blocks ligand binding; Li et al., 2005), Lapatinib (a dual ERBB2/EGFR small molecule inhibitor; Medina and Goodin, 2008), and GDC-0941 (a PI3K inhibitor; Edgar et al., 2010) for their effects on blocking cell survival and colony formation using the BaF3 system (Figures 7 and S6A). We found that in both the survival and colony formation assays, the small molecule inhibitor Lapatinib was quite effective against all the mutants (Figures 7A and 7B), further confirming that the ERBB2 kinase activity was needed for the oncogenic activity of the ERBB3 mutants. However, while GDC-0941 was effective against all the mutants tested, it was less effective against Q809R at the dose tested (Figures 7A and 7B). We also tested the effect of PD0325901 (a MEK inhibitor; Thompson and Lyons, 2005) and a combination of GDC-0941 and PD0325901 on proliferation of BaF3 cells expressing the ERBB3 mutants. We found that combination of GDC-0941 and PD-0325901 was quite effective in blocking all the ERBB3 mutants, including the Q809R mutant (Figure S6B). Among the antibodies tested in the colony formation assay, trastuzumab, anti-ERBB3.2, and MEHD7945A were effective against all the mutants tested (Figure 7B). However, pertuzumab and anti-ERBB3.1, although very effective in blocking proliferation and colony formation induced by ERBB3 ECD mutants, were only modestly effective against the Q809R kinase domain ERBB3 mutant (Figures 7A and 7B). Consistent with this, in BaF3 cells co-expressing mutant ERBB3 and ERBB2 in vitro, these inhibitors, when efficacious, blocked or reduced pAKT and/or pERK levels, and also the levels of ERBB3 and/or pERBB3 (Figures 7C and S6C).

We also tested the efficacy of trastuzumab, anti-ERBB3.1, and anti-ERBB3.2 against G284R and Q809R ERBB3 mutants using the BaF3 system in vivo (Figure 8). As observed in vitro, trastuzumab was very effective in blocking leukemia-like disease in mice receiving BaF3 co-expressing G284R or Q809R ERBB3 mutant and ERBB2 (Figures 8A and 8B). Similarly, both anti-ERBB3.1 and anti-ERBB3.2 blocked the development of leukemia-like disease in mice receiving BaF3 co-expressing G284R ERBB3 ECD mutant and ERBB2 (Figure 8A). However, these anti-ERBB3 antibodies were only partially effective in blocking disease development in mice receiving BaF3 cells expressing Q809R ERBB3/ERBB2, although they significantly improved survival compared to control antibody-treated animals (Figure 8B). Consistent with the efficacy observed for the targeted therapeutics, we found a significant decrease in infiltrating BaF3 cells expressing the ERBB3 mutants in the spleen and bone marrow (Figures 8C, 8D, and S7). Concomitant with the observed reduced infiltration of BaF3 cells, the spleen and liver weights (Figures 8E and 8F) were within the normal range expected for Balb/C nude mice (Figures 6E, 8E, and 8F). These data indicate that multiple therapeutics, either in development or approved for human use, can be effective against ERBB3 mutant-driven tumors.

DISCUSSION

In this study, we report the identification of *ERBB3* somatic mutations, including several hot spot mutations, which promote oncogenic signaling in the presence of kinase-active ERBB2. While some of the ECD mutants, V104M, A232V, P262H, and T389K, were oncogenic in the absence of ERBB3 ligand NRG1, they can be further stimulated by the addition of NRG1. In contrast, the G284R (ECD) and Q809R (kinase domain) mutants appear to be less sensitive to ligand-mediated activation, indicating a distinct mode of activation for these mutants. We propose that the ECD mutations may shift the equilibrium between tethered and untethered ERBB3 ECD toward an untethered conformation relative to WT. Compared to the ECD mutants, our functional data, and the location of these mutations, suggest that the Q809R, S846I, and E928G ERBB3 kinase domain mutant functions differently. Although the kinase domain of ERBB3 is thought to be catalytically inactive, a recent study reported a low level of kinase activity (Shi et al., 2010). In light of this observation, one could surmise that the kinase domain mutant, Q809R, S846I, and E928G, may have acquired an elevated constitutive phosphotransferase activity and hence became ligand independent. However, our inability to see any increase in the phosphotransferase activity of ERBB3 Q809R and E928G, under the assay conditions tested, suggests that further experiments are required to support this possibility. Another possibility is that the kinase domain mutation may alter the conformation of ERBB3 so that it becomes more permissive to form ERBB3/ERBB2 dimers in a ligand-independent fashion. Determination of the precise mechanism of action of both the ECD and kinase domain mutants requires further cellular, biochemical, and structural studies.

Several targeted therapeutics that block signaling by ERBB family members, including ERBB3, are in development or are already used in the clinic to treat patients with cancer (Baselga

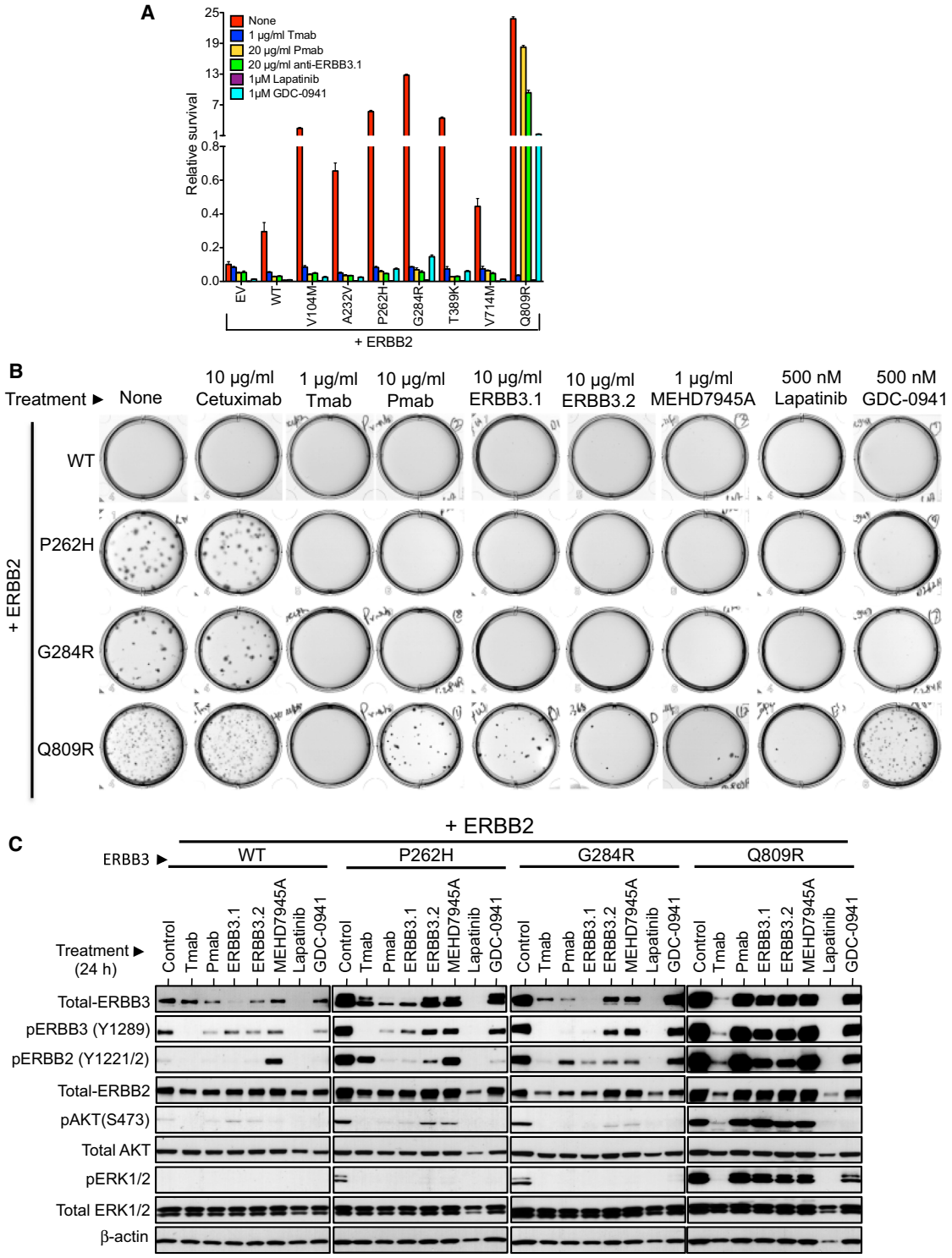

Figure 7. Efficacy of Anti-ERBB Antibodies and Small Molecule Inhibitors on Oncogenic Activity of ERBB3 Mutants

(A–C) Effect of targeted therapeutics on IL-3 independent proliferation (mean ± SEM) (A), representative images of colony formation assay (B), and signaling at 24 hr after treatment as assessed by western blot (C) of BaF3 cells stably expressing ERBB3 mutants together with ERBB2. The concentration of antibodies and small molecule inhibitors used for treatment in (C) is same as that in (B).

See also Figure S6.

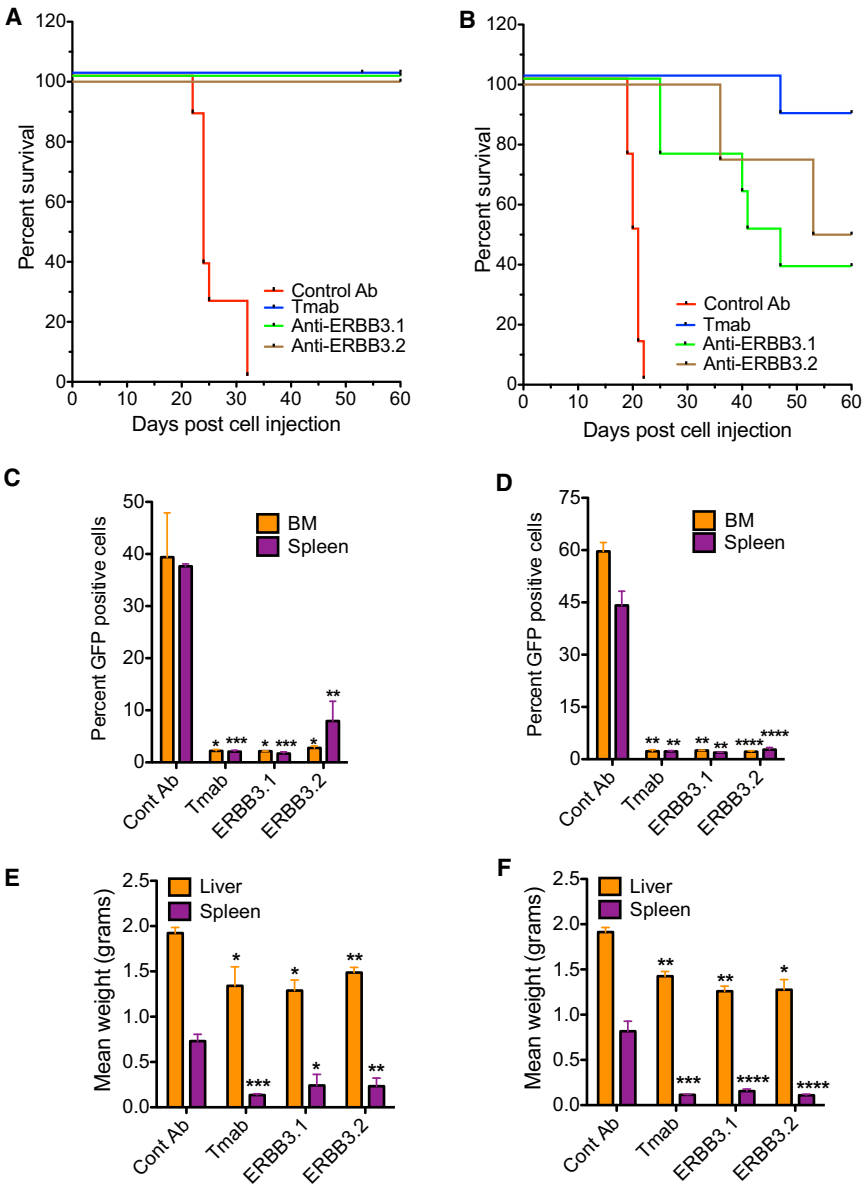

Figure 8. Anti-ERBB3 Antibodies Are Effective against ERBB3 Mutants In Vivo

(A and B) Efficacy of trastuzumab (Tmab), anti-ERBB3.1, and anti-ERBB3.2 antibodies in blocking leukemia-like disease induced by BaF3 cells expressing ERBB3 G284R (A) or Q809R mutant (B) in combination with ERBB2 (n = 8 for each arm). Anit-ragweed antibody was used as a control.

(C and D) Proportion of infiltrating BaF3 cells expressing ERBB3 G284R (C) or Q809R (D) mutant in bone marrow and spleen following treatment with the antibodies.

(E and F) Liver and spleen weight from animal implanted with ERBB3 G284R (E) or Q809R (F) mutant cells following treatment with the antibodies as indicated. Data in (C)–(F) are mean ± SEM (n = 3).

See also Figure S7.

and Swain, 2009). We found that multiple small molecule inhibitors, anti-ERBB2, and anti-ERBB3 ECD antibodies were quite effective in blocking oncogenic signaling by a majority of the ERBB3 mutants. Interestingly, pertuzumab, anti-ERBB3.1, and GDC-0941 were not as effective in blocking the kinase domain mutant Q809R signaling, indicating a distinct mode of action by this mutant. Previous studies have shown that while pertuzumab is quite effective in blocking ligand-mediated ERBB3/ERBB2 dimerization, trastuzumab is more effective in blocking ligand-independent ERBB2/ERBB3 dimer formation (Junttila et al., 2009). Consistent with this, the ligand nonresponsive Q809R ERBB3 kinase domain mutant is much more responsive to inhibition by trastuzumab compared to pertuzumab, suggesting a potential role for a nonliganded heterodimeric complex in Q809R ERBB3 signaling. Although the PI3K inhibitor GDC-0941 is quite active against most of the ERBB3 mutants tested, its reduced efficacy in blocking kinase domain mutant suggests the engagement of other downstream signaling molecules besides the PI3K.

The presence of activating *ERBB3* mutations increases the importance of ERBB3 in cancer. However, further studies are needed to fully elucidate the mechanism of action of the ERBB3 mutants, their predictive and prognostic values, and their contributions to acquired resistance.

EXPERIMENTAL PROCEDURES

Samples and Mutation Detection

Appropriately consented primary human tumor samples with institutional review board approval were obtained from commercial sources (Table S1). The human tissue samples used in the study were de-identified (double-coded) prior to their use and hence, the study using these samples is not considered human subject research under the US Department of Human and Health Services regulations and related guidance (45 CFR Part 46). Tumor content in all the tumors used was confirmed to be >70% by pathology review. Tumor DNA was extracted using the QIAGEN Tissue easy kit (QIAGEN, CA). All coding exons of *ERBB3* in tumor samples were amplified using primers in the Supplemental List. The nested PCR reaction products were sequenced using ABI3730xl sequencer (LifeTechnologies, CA). The sequencing data were analyzed for the presence of variants not present in the dbSNP database using Mutation Surveyor (Softgenetics, PA) and additional automated sequence alignment programs. To confirm the somatic nature of the mutations, the putative variants identified were confirmed by DNA sequencing or mass spectrometry analysis (Sequenom, CA) using the original tumor DNA followed by confirmation of its absence in the adjacent matched normal DNA. Additional genes indicated in Figure S1C were PCR amplified, sequenced, and analyzed in colon and gastric tumors with *ERBB3* mutations.

Animal Studies

All animal studies were conducted under protocols approved by Genentech's Institutional Animal Care and Use Committee guidelines. Further details on the animal studies can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four tables, Supplemental Experimental Procedures, and one list and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.04.012.

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