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14. ABSTRACT

The phosphatidylinositol 3- kinase (PI3K)-pathway is frequently activated in breast cancer; therefore, considerable effort is focused on identifying compounds that can inhibit specific pathway components, in particular the hallmark oncogene PIK3CA. While targeted inhibition of a cancer survival gene holds significant promise, there are concerns that drug resistance may emerge within the cancerous cells, thus limiting clinical efficacy. Using genetically defined human mammary epithelial cells, we evolved resistance to the PI3K/mTOR-inhibitor NVP-BEZ235 and by genome-wide copy-number analyses identified MYC and eIF4E amplification within the resistant cells. Importantly, either MYC or eIF4E were required to bypass pharmacological PI3K/mTOR inhibition in resistant cells. Furthermore, these cells displayed elevated 5'cap-dependent protein translation. Collectively, these findings suggest that analysis of drivers of protein translation could facilitate the identification of cancer lesions that confer resistance to PI3K-pathway targeted drugs.

15. SUBJECT TERMS

cancer; PI3K; drug resistance; MYC; eIF4E; translation

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INTRODUCTION

Various components of the PI3K pathway are frequently deregulated in human cancer. Notably, phosphatidylinositol 3-kinase alpha (PI3Ka) bears frequent activating mutations in multiple major tumor types: breast, endometrial, ovarian, prostate, colorectal, pancreatic, liver, and lung cancer. Due to the high frequency of oncogenic activation of the PI3K-pathway, there is considerable interest in developing effective pharmacological inhibitors for cancer therapy. Currently, several PI3K inhibitors, including GDC0941 (Genentech) and BEZ235 (Novartis Pharmaceuticals), have entered Phase I clinical trials and new isoform-specific compounds are being developed. Despite the promise of targeted therapies, an emerging clinical obstacle is the acquisition of drug resistance within the tumor cells. Here, we explore acquired resistance to PI3K inhibitors and propose the prevailing mechanism of such resistance. Using genetically defined human mammary epithelial cells (HMECs), a model system which has previously been used for PI3K-pathway driven transformation due to its dependence on oncogenic PI3K signaling, we screened for emergence of BEZ235-resistance and identified genetic lesions involved.

BODY

We will present below the development of the project with regard to our original ***Statement of Work***:

> The proposed research will be conducted as stated in three **Specific Aims**:
> **1 – Assessment of kinase function by drug-resistant mutations in PIK3CA(H1047R)**
> **2 – Investigating the mechanisms of PI3K-inhibitor evasion by PIK3CA**
> **3 – Generation and assessment of PI3K drug-resistance expression signatures**

In brief, the oncogenic allele of PIK3CA (H1047R) was randomly mutagenized in XL1-Red bacteria and the mutagenized library was used to retrovirally transduce HMECs. These engineered cells were grown in the presence of BEZ235 (in parallel with the non-mutagenized controls) for 2 months, at which time a population of cells that could grow rapidly in the presence of the drug was detected. This "*Enriched*" population was expanded and the genomic DNA isolated. Here, we utilized SOLiD deep sequencing analysis to identify mutations present in the PCR-amplified ectopic *PIK3CA* cDNA. This analysis revealed the preponderance of two mutant alleles of PIK3CA. Surprisingly, both of the alleles contained mutations outside of the catalytic domain. One of the alleles contained a point mutation G320E in the linker region close to the start of the C2 domain of PIK3CA; the second mutant allele contained a point mutation F486S found in the linker region directly after the C2 domain. Notably, as this method cannot decipher between *cis* and *trans* positioning of the mutations, we employed standard Sanger sequencing of individual TOPO clones from the ectopic alleles. Notably, this method revealed that the two mutations are present in the drug-resistant cells positioned in *trans* with respect to each other. In addition, the latter method revealed an additional mutation positioned in cis with the F486S mutation – a 10-nucleotide deletion of residues 1030-1039, found within the C2 domain (**Appendix 2, Figure S1**).

As stated in **Aim 1A**, both mutant alleles of PIK3CA were generated and expressed in the HMEC system in order to asses if they can indeed render cells insensitive to PI3K inhibition. Surprisingly, when challenged with the PI3K-inhibitor BEZ235, neither of the two mutant alleles allowed for better growth of the infected cells as compared to the controls, suggesting potentially an alternative mechanism of resistance of the *Enriched* population. In addition, the two alleles seem to be no longer expressed in the *Enriched* cells (**Appendix 2, Figure S1**).

As described in **Aim 1B**, we measured the inherent PI3K activity in the two identified mutations using lipid kinase assay. In terms of their catalytic activity, the G320E point mutant (in the background of H1047R mutation) retained lipid kinase activity comparable to the H1047R-allele. On the other hand, the double-mutant, del;F486S allele, seems to be non-expressed or unstable, hence absence of lipid kinase activity is associated with its

overexpression. Furthermore, the *Enriched* population's decreased lipid-kinase activity is reflected in reduced downstream PI3K-pathway activation, as measured by phosphorylation of the downstream AKT (**Appendix 2, Figure S1**).

The two identified mutant alleles do not confer resistance to BEZ235 and therefore the efficiency of association with PIK3R1 (**Aim 2B**) was not prioritized. Consequently, our subsequent experiments were aimed at investigating the mechanism of BEZ235-resistance in the *Enriched* population. Given the fact that the *Enriched* population retains its BEZ235-resistance even when propagated in the absence of the drug for a substantial period of time, we chose to explore the possibility of the occurrence of additional acquired genetic changes. To this end, we have performed 250K SNP-array analysis to derive genome-wide gene copy numbers and have identified a genomic region of amplification. Specifically, the *Enriched* population contains amplification of a region within chromosome 8, which differentiate it from the drug-sensitive HMECs (**Appendix 2, Figure 2A**). Notably, there are a number of genes in this amplified region (more than sixty), thus we employed expression array analysis of the Enriched cells (**Appendix 2, Figure 2B**). Here, we integrated the two approached (SNP- and expression-array data) to determine which of the genes from the amplified regions in the Enriched cells are highly expressed on level of mRNA (**Appendix 2, Figure 2C**). Notably, this approach generated a significantly reduced list of candidate genes (**Appendix 2, Table S1**). Among them, we chose to focus on *MYC*, as it is already described as a human oncogene and pertinent to breast cancer pathogenesis. We verified the genomic amplification of *MYC* by genomic Q-PCR (**Appendix 2, Figure 2D**) and could furthermore show that MYC protein levels are 2-fold higher than within control cells (**Appendix 2, Figure 2F and 2G**). Notably, overexpression of MYC in HMECs is sufficient to mediate resistance to BEZ235 (**Appendix 2, Figure 3A**), yet renders the cells more sensitive to Taxol (**Appendix 2, Figure 4D**). In addition, using lentiviral shRNA targeted knock-down, we have verified the role of MYC in mediating drug resistance in the *Enriched* cells (**Appendix 2, Figure 3C and 3D**).

Towards **Aim 3**, we investigated the sensitivity to BEZ235 within a panel of breast cancer cell lines. Specifically, we attempted to address if *MYC* amplification correlates with resistance to BEZ235. To this end, we observed that upon overexpression of MYC, cell lines without pre-existing *MYC* amplification gain resistance to BEZ235 (**Appendix 2, Figure 5A**). On the other hand, cell lines with pre-existing *MYC* amplification do not gain additional insensitivity upon MYC overexpression (**Appendix 2, Figure 5A**). We addressed if breast cancer cell lines with *MYC* amplification become more sensitive to BEZ235 after genetic depletion of MYC using lentiviral shRNA (**Appendix 2, Figure 5B and 5C**). Here, we can show that reduction of MYC levels in these cell lines results in increased sensitivity to BEZ235 **(Appendix 2, Figure 5B and 5C**).

In addition, we expanded this study to characterize the mechanism of BEZ235 resistance in a second drug-resistant cell line, *HMECres* (**Appendix 2, Figure 1E**). Here, we performed genome-wide copy number analysis and determined that these cells contained a region of genomic amplification at chromosome 4 (**Appendix 2, Figure 6A**). Among the genes contained within this region of amplification (**Appendix 2, Table S2**), we

tested the eukaryotic translation initiation factor 4E (*eIF4E*) as a likely candidate that could mediate resistance to BEZ235. Notably, eIF4E is a critical translation initiation factor downstream of the PI3K/mTOR pathway mediating cap-dependent protein translation. We were able to confirm its amplification by genomic Q-PCR (**Appendix 2, Figure 6B**), and in addition, we found that its levels were increased in *HMECres* cells both on the mRNA (**Appendix 2, Figure 6C**) and protein levels (**Appendix 2, Figure 6D**). Importantly, we found that overexpression of eIF4E can confer resistance to BEZ235 (**Appendix 2, Figure 6E and 6F**), and that in addition, eIF4E is necessary for the drug resistance phenotype observed in the *HMECres* cells (**Appendix 2, Figure 6G and 6H**).

We further investigated if there was a mechanistic link between the two independently evolved types of resistance, mediated by amplification of *MYC* and *eIF4E*. To that end, we found that eIF4E was required for drug resistance mediated by MYC-overexpressing cells (**Appendix 2, Figure 6I and 6J**), and in addition, that eIF4E, as well as another translation factor, eIF2A, are upregulated upon MYC overexpression (**Appendix 2, Figure 7A**). Since eIF4E is a translation initiation factor, we tested if levels of cap-dependent translation were altered in drug-resistant cells relative to the control sensitive ones (**Appendix 2, Figure 7B**). Here, we found that cap-dependent translation is upregulated in all drug-resistant cell states studies, including the two evolved lines, *Enriched* and *HMECres*, as well as the engineered lines overexpressing MYC or eIF4E (**Appendix 2, Figure 7B**). Hence, we propose a model (**Appendix 2, Figure 7C**) where resistance to targeted PI3K/mTOR inhibition can be mediated by lesions downstream of these pathways which maintain the levels of cap-dependent translation in spite of upstream inhibition of the signaling pathways. Here, while PI3K and/or mTOR are pharmacologically inhibited which in effect suppresses cap-dependent translation, amplification of MYC or eIF4E restores the levels of translation, resulting in resistance to this type of drugs.

KEY RESEARCH ACCOMPLISHMENTS

- Evolved cell lines resistant to the PI3K-inhibitor BEZ235
- Performed 250K SNP-array analysis of the BEZ235-resistant cells; the analysis revealed regions of genomic amplification in chromosomes 8 in one cell line (*Enriched*), and in chromosome 4 (*HMECres*) in the other cell line
- Performed expression array analysis of the BEZ235-resistant *Enriched* cells
- Integration of the SNP- and expression-array analysis reveals a list of candidate drug resistance genes that are amplified and highly expressed
- Likely candidate gene from chromosome 8 amplicon is *MYC*; overexpression of *MYC* can recapitulate the resistance phenotype in HMECs.
- MYC is necessary for resistance in the *Enriched* cells
- MYC confers BEZ235-insensitivity in breast cancer cell lines without pre-existing *MYC* amplification
- Likely candidate from chromosome 4 amplicon is eIF4E
- eIF4E is necessary for resistance in the *HMECres cells*; eIF4E is also necessary for resistance in MYC-expressing cells
- Overexpression of eIF4E is sufficient for resistance in HMEC cells
- MYC or eIF4E overexpression confer resistance to other individual PI3K or mTOR inhibitors
- Cell lines resistant to BEZ235 exhibit elevated cap-dependent translation

REPORTABLE OUTCOMES

Degree obtained

Nina Ilic was awarded a **PhD degree from Harvard University**
Subject: Cell and Developmental Biology
Thesis title: **The PI3K-pathway in tumorigenesis and cancer drug resistance**
Thesis defense date: October 25, 2010

Book chapter (Appendix 1)

Nina Ilic and Thomas M. Roberts (2010) *Comparing the roles of the p110α and p110β isoforms of PI3K in signaling and cancer.* Current Topics in Microbiology and Immunology. Editors Peter Vogt and Bart Vanhaesebroeck

Research article (Appendix 2)

Nina Ilic, Tamara Utermark, Hans R. Widlund, and Thomas M. Roberts (2011) *PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis*. PNAS

Presentations

Nina Ilic presented the work funded by this award at two conferences:

- Dana-Farber/Harvard Cancer Center Gynecologic Cancer Researchers' retreat, March 25, 2011, Boston, MA

- Era of Hope Conference 2011 (Department of Defense), August 2-5, 2011, Orlando, FL (**poster competition finalist**)

Employment/research opportunities based on experience supported by this award

Nina Ilic is now a postdoctoral fellow at Dana-Farber Cancer Institute, Boston, MA, in the laboratory of Dr. William C. Hahn, conducting research on genetic vulnerabilities in breast cancer.

CONCLUSION

We report that genomic amplification of either of two proto-oncogenes, *MYC* and *eIF4E*, is able to provide resistance to BEZ235, apparently bypassing the inhibition by acting downstream of the pharmacologically inhibited targets to upregulate cap-dependent translation. Since both MYC and eIF4E amplification and overexpression are found in human tumor cells, it may be advantageous to assess MYC and eIF4E levels when considering PI3K inhibitors as a therapeutic strategy. Thanks to the support of this fellowship, we have been able to publish our results (see **Appendix 2**), which we hope will stimulate further research into the importance of deregulation of these genes when considering PI3K-targeted inhibition for therapeutic purposes in breast cancer.

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APPENDICES

Appendix 1

Nina Ilic and Thomas M. Roberts (2010) *Comparing the roles of the p110α and p110β isoforms of PI3K in signaling and cancer*. Current Topics in Microbiology and Immunology. Editors Peter Vogt and Bart Vanhaesebroeck

Appendix 2

Nina Ilic, Tamara Utermark, Hans R. Widlund, and Thomas M. Roberts (2011) *PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis*. PNAS

SUPPORTING DATA

The data figures are included in the published research article (Ilic et al. 2011) included in **Appendix 2**.

Comparing the Roles of the p110α and p110β Isoforms of PI3K in Signaling and Cancer

Nina Ilić and Thomas M. Roberts

Contents

Abstract Phosphatidylinositol-3-kinases (PI3K) are a family of enzymes that act downstream of cell surface receptors leading to activation of multiple signaling pathways regulating cellular growth, proliferation, motility, and survival. To date, most research efforts have focused on a group of PI3K-family enzymes termed class I, of which the most studied member is PI3Kα. PI3Kα is an oncogene frequently mutated in human cancer, as is the chief negative regulator of the pathway,

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the tumor suppressor PTEN. Recently, it has been suggested that tumors deficient for PTEN might depend on the function of another class I member, PI3Kβ, to sustain their transformed phenotype. Taken together, these findings provide a significant medical rationale to study the signaling cascades regulated by PI3Kα and PI3Kβ particularly in the context of their role in the development and maintenance of human cancer. Here, we summarize the current understanding of the upstream receptor regulation of the two PI3K isoforms and their roles in cancer as well as their functional requirements in downstream signaling cascades.

1 Introduction

The phosphatidylinositol 3-kinases (PI3Ks) constitute a family of intracellular lipid kinases, which phosphorylate the 3′-hydroxyl group (D-3) of phosphatidylinositol lipids in cellular membranes either acting constitutively or in response to extracellular stimuli such as growth factors and hormones. The D-3 phosphorylated phosphoinositides serve multiple functions in the cell, regulating cellular membrane trafficking and acting as second messengers, which serve to attract cytosolic signaling proteins containing pleckstrin-homology domains (PH-domains), unique binding domains for these lipids. Once recruited to the membrane, these cytosolic PH-domain containing signaling proteins activate diverse signal transduction pathways involved in the regulation of cellular growth, survival, metabolism, migration, and vesicular trafficking (Cantley 2002; Engelman et al. 2006).

The PI3Ks are divided into three classes (I–III) based on their substrate preferences and subunit composition. *In vivo* class I PI3Ks utilize phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3), while class II members generate both phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) from phosphatidylinositol-4-phosphate (PI-4-P) and PI-3-P from phosphatidylinositol (PI) (Engelman et al. 2006; Katso et al. 2001). Class III enzymes generate only PI-3-P from PI (reviewed in Vanhaesebroeck et al. 2001; Fruman et al. 1998).

Notably, class I PI3Ks are activated by various cell surface receptors, leading to further subdivision of this class into subfamilies IA and IB based on the classical notion that the IA members are activated upon receptor tyrosine kinase (RTK) stimulation, whereas IB are activated by G-protein coupled receptors (GPCRs) (Engelman et al. 2006; Katso et al. 2001; Vanhaesebroeck et al. 2001). In addition, the two classes exhibit structural differences: class IA members are heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit, whereas class IB has only one member consisting of a p110γ catalytic subunit and a p101 regulatory subunit (Vanhaesebroeck et al. 2001). There are three class IA catalytic subunit isoforms in mammals, encoded by three genes – PIK3CA (p110α), PIK3CB (p110β), and PIK3CD (p110δ). In addition, three genes – PIK3R1, PIK3R2, and PIK3R3 encode the class IA regulatory subunits p85α, p85β, and p55γ (Vanhaesebroeck et al. 1997a).

2 Class IA PI3Ks

Due to their causal involvement in the genesis of human disease, class IA has attracted the most attention of all PI3Ks. Their p85/p55 regulatory subunits bind tyrosine phosphorylated residues of activated receptor proteins or specific adaptor molecules (Engelman et al. 2006; Vanhaesebroeck et al. 2001), and thereby recruit the catalytic p110 subunits to the plasma membrane, where p110s then phosphorylate available membrane lipid PI-4,5-P_2 substrates. Although p85/p55 binding is required for recruitment of p110s to the receptor, it is generally considered that its binding has an inhibitory effect on the catalytic activity of p110 in the cytoplasm, which is relieved upon membrane recruitment (Yu et al. 1998). Additionally, p110 can bind activated Ras and Ras family members, allowing its localization to the membrane and activation of its lipid kinase activity (Katso et al. 2001). As a signal attenuating mechanism, the reverse dephosphorylation reaction converting PIP_3 to PI-4,5-P_2 is catalyzed by the phosphatase PTEN (phosphatase and tensin homologue), a tumor suppressor protein.

Among the three class IA catalytic subunits, p110α and p110β are ubiquitously expressed among tissues, while p110δ is found predominantly in leukocytes (Vanhaesebroeck et al. 1997a,b; Chantry et al. 1997). Even though the primary structures of p110β and p110δ are more homologous to each other than to that of p110α, the overall domain structures of all three isoforms are quite similar (Vanhaesebroeck and Waterfield 1999). Specifically, they all contain an N-terminal adaptor binding domain (ABD), followed by a Ras-binding domain (RBD), a C2 domain, helical domain, and the catalytic kinase domain. In addition, all the regulatory subunits contain two Src-homology 2 (SH2) domains, which bind the tyrosine-phosphorylated receptors and adaptor molecules, and also an inter-SH2 domain, which is necessary for interaction with the catalytic p110 subunit. The longer adapter isoform, p85, also contains an N-terminal Src-homology 3 (SH3) domain and two proline-rich regions flanking a BCR homology domain, which is thought to interact with proline rich proteins and small GTPases, respectively (Engelman et al. 2006; Katso et al. 2001).

3 Mechanisms of Activation of Class IA p110 Isoforms

3.1 Early Studies on In Vitro p110α/β Activation

Due to the structural and catalytic similarities among the class IA members, there has been considerable interest in elucidating any differences in their regulation and function, particularly with regard to the two ubiquitously expressed isoforms p110α and p110β. Although the primary structures of p110α and p110β are highly

homologous, the divergence among them is found in the Ras-binding domain, which has suggested altered specificities towards different small GTP-binding proteins (Deora et al. 1998). While it remains unclear whether the non-redundant functions of these isoforms can solely be explained by differential preferences towards various Ras-family members, there are several lines of evidence indicating that the two isoforms are differentially regulated by membrane receptors (Fig. 1). Classically, both p110α and p110β were thought to respond to RTK activation and a plethora of work has shown that p110α and p110β can indeed be recruited by various RTKs in response to numerous ligands including insulin (InsR), EGF (EGFR), and PDGF (PDGFR) (Roche et al. 1998; Hooshmand-Rad et al. 2000; Park et al. 2003). Notably, there have been indications of differential preference between the two isoforms by particular RTKs and to that end, *in vitro* experiments utilizing neutralizing anti-p110α and -p110β antibodies have suggested that p110α is more important for PDGF-signaling than p110β, whereas the opposite has been demonstrated for insulin-induced actin reorganization and signaling, suggesting a potential role for p110β in RTK signaling (Roche et al. 1998; Hooshmand-Rad et al. 2000; Asano et al. 2000). On the other hand, additional *in vitro* work suggested that both p110α and p110β are required for insulin response (Roche et al. 1998).

Early studies suggested that, while p110β (like p110α) can be activated by a synthetic phosphotyrosyl peptide (containing the YMXM motif found in RTKs), it could also be activated by the βγ subunit of G-proteins, thus indicating an additional mechanism of activation by GPCRs (Kurosu et al. 1997; Maier et al. 1999; Hazeki et al. 1998). Consistent with this notion, injection of p110β-specific neutralizing antibodies into fibroblasts inhibited DNA synthesis induced by lysophosphatidic acid (LPA), a GPCR ligand, but did not significantly affect PDGFR signaling (Roche et al. 1998). On the other hand, p110α-specific neutralizing antibodies did not significantly affect LPA-signaling, but did inhibit PDGFR signaling (Roche et al. 1994, 1998). Additional *in vitro* work also suggested that in contrast to p110β, p110α could not be activated by GPCR ligands (LPA and bombesin) (Roche et al. 1994, 1998; Murga et al. 2000) and a recent study using small-molecule inhibitors of p110β showed that it is not a major effector downstream of RTKs, but rather couples to GPCRs (Guillermet-Guibert et al. 2008). Here, inhibition of p110β did not block downstream signaling in response to RTK ligands – PDGF, insulin, and IGF-1, but did affect signaling stimulated by GPCR-ligands – stromal cell-derived factor (SDF-1α), sphingosine-1-phosphate (S1P), and LPA (Table 1).

With the development of pharmacological inhibitors of PI3K, the use of isoform-specific inhibitors suggests, in contrast to earlier studies (Roche et al. 1998; Hooshmand-Rad et al. 2000), that p110β only plays a minor role in insulin signaling (Foukas et al. 2006). Similarly, a pharmacological study utilizing a panel of isoform-specific inhibitors indicated that p110α is the primary insulin-responsive isoform in culture (in adipocytes and myotubes), whereas p110β remained dispensable under these conditions (Knight et al. 2006).

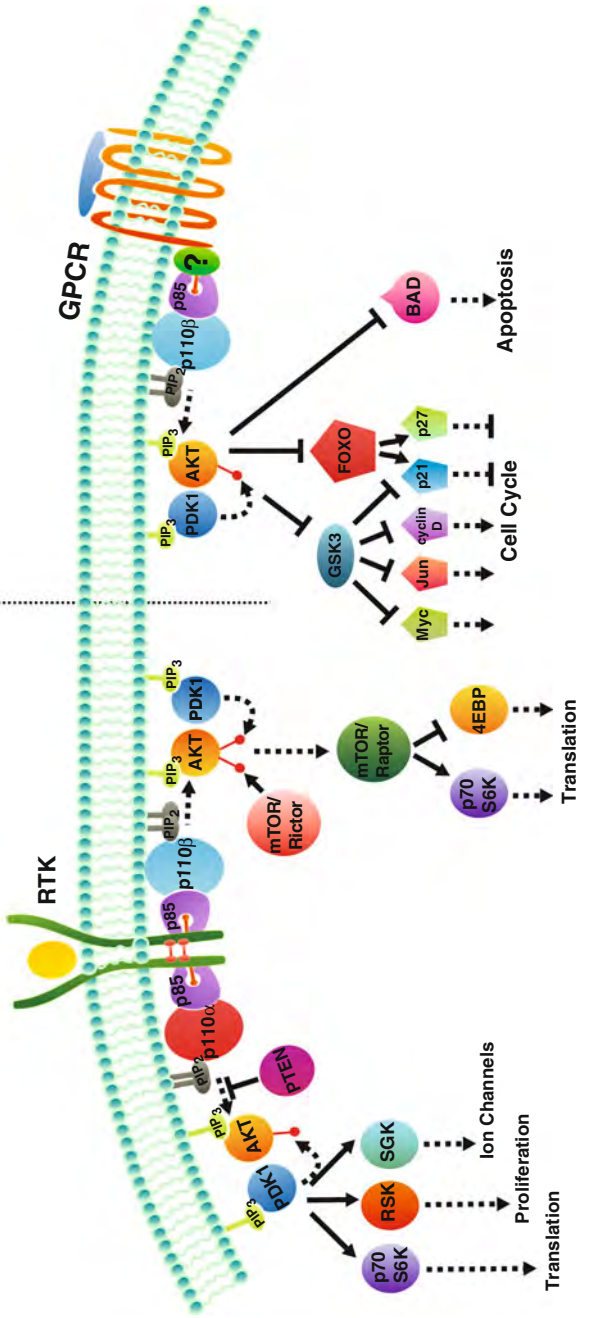

Fig. 1 *Schematic view of the PI3K-pathway.* Upstream activation of p110α and p110β isoforms by receptor tyrosine kinase (RTK) or p110β isoform by G-protein coupled receptor (GPCR) leading to downstream PI3K lipid kinase activity towards PIP_2. Membrane bound PIP_3 recruits AKT and PDK1, which leads to AKT phosphorylation and in turn suppression of GSK3 and FOXO-activities as well as mTOR pathway activation. In addition to activating AKT, PDK1 also directly phosphorylates and activates p70S6K, RSK, and SGK

Table 1 p110 isoform requirements for receptor signaling

p110α		p110β	
In vitro	*In vivo*	*In vitro*	*In vivo*
RTK activation by:			
Insulin PDGF (+/−) IGF-1 EGF FGFR	Insulin Leptin	Insulin	Insulin (+/−)
GPCR activation by:			
		LPA S1P SDF-1α Bombesin	

3.2 Studies on p110 Activation Using Engineered Mice

Gene-targeting experiments of the PI3K catalytic subunits in mice have shed additional light on upstream ligand and receptor activation requirements for p110α and p110β. Homozygous deletion of p110α is embryonic lethal between E9.5 and E10.5 as a result of severe proliferative defects, and *Pik3ca*-nullizygous embryonic mouse fibroblasts fail to proliferate in culture even in the presence of growth factors (Bi et al. 1999; Foukas and Okkenhaug 2003). Similarly, mice carrying a kinase-dead knock-in mutation in p110α (D933A) die early in embryonic development, but mice heterozygous for this allele are fertile and viable, although smaller than their wild type siblings (Foukas et al. 2006). In the heterozygous mice ($p110\alpha^{D933A/WT}$) the relative insulin-stimulated PI3K activity (associated with the InsR and IRS-1/2 in skeletal muscle, liver, and fat tissue) is reduced by approximately 50% (as well as phosphorylation of Akt upon insulin stimulation), suggesting a critical role of p110α in insulin signaling *in vivo*. Notably, these effects on insulin signaling are measurable despite the fact that the expression of insulin receptor (InsR) and its associated adaptor molecules IRS-1 and IRS-2, as well as the recruitment of p85 to the receptor complex remain unchanged. Similarly, in the central nervous system, specifically the hypothalamus, heterozygosity for the p110α(D933A) allele resulted in markedly reduced IRS-1/2-associated PI3K activity in response to both insulin and leptin treatment. Importantly, insulin stimulation seemed to significantly enrich the IRS-1/2 complexes with p110α compared to p110β, thus resulting in the majority of the total PI3K activity at these complexes being contributed by p110α. To bypass the issue of embryonic lethality of systemic p110α loss-of-function, mice with a conditionally targeted *Pik3ca* allele have been generated (Zhao et al. 2006). Here, ablation of p110α in MEFs severely reduced the response to insulin and insulin-like growth factor (IGF-1), as well as the epidermal growth factor (EGF). However, signaling response to PDGF was more moderately affected, and only at later time points than for the other RTK-ligands tested (Table 1).

Similarly, p110β knock-out mice have also been generated, and the homozygous embryos were found to die early in embryonic development, like p110α knockouts, while the heterozygous counterparts were viable (Bi et al. 2002; Brachmann et al. 2005). More recently, mice carrying a conditional *Pik3cb* allele were generated (Jia et al. 2008). Here, as in the p110α conditional mouse knock-in study (Foukas et al. 2006) and the pharmacological studies (Knight et al. 2006), p110β loss did not seem to have a major effect on signaling downstream of the InsR in the liver or in the isolated mouse embryonic fibroblasts (MEFs). Nevertheless, these animals displayed signs of impaired insulin metabolism demonstrated as increased levels of blood insulin, lower tolerance of glucose, and lower sensitivity to insulin as compared to control animals. In agreement with earlier *in vitro* work, deletion of p110β had no negative effect on PDGF or EGF response, but did block the response to LPA stimulation (Roche et al. 1998). Knock-in mice expressing a catalytically inactive Pik3cb(K805R) allele (Ciraolo et al. 2008), also exhibited mild insulin resistance and partially impaired Akt activation, but showed no impairment of signaling downstream of RTK ligands (insulin, IGF-1, EGF, or PDGF) *in vitro*. In agreement with preceding work (Maier et al. 1999; Guillermet-Guibert et al. 2008; Knight et al. 2006; Jia et al. 2008), this study also demonstrated a requirement for p110β for signaling downstream of GPCRs for LPA and S1P *in vitro* (Table 1).

While heterozygous loss of either p110α or p110β had no effect on insulin sensitivity, the double-heterozygous loss caused a significant impairment in the glucose tolerance test, which suggests that both p110α and p110β contribute to insulin response *in vivo* (Brachmann et al. 2005). However, the interpretation of these results is made difficult by the fact that the levels of the p85 regulatory subunit change dramatically in this setting, which may significantly affect insulin signaling (Ueki et al. 2003; Terauchi et al. 1999).

3.3 Unresolved Issues

Various studies to date have shown convincing evidence that p110β mediates signaling through GPCRs (Roche et al. 1994, 1998; Kurosu et al. 1997; Maier et al. 1999; Hazeki et al. 1998; Murga et al. 2000; Guillermet-Guibert et al. 2008; Jia et al. 2008; Ciraolo et al. 2008), the precise mechanism of which still remains unclear. Since free p110 catalytic subunits have not been detected (Geering et al. 2007), it is likely that the p85 regulatory subunit participates in the heterodimer recruitment to the receptor complex. Analogous to class IB PI3K, which has previously been shown to medicate GPCR-signaling in leukocytes (Brock et al. 2003; al-Aoukaty et al. 1999; Naccache et al. 2000; Stoyanov et al. 1995; Stephens et al. 1997), it is conceivable that the regulatory subunit p85 mediates the interaction with the receptor complex by direct binding to the intracellular receptor domains. However, due to the poor homology between the class IA PI3K regulatory subunit (p85) and the class IB regulatory subunit (p101), this model seems less likely. The affinity of p85 for tyrosine-phosphorylated residues provides the

attractive alternative explanation that a cellular tyrosine-kinase mediates this interaction by providing tyrosine-phosphorylated residues that could serve as docking motifs for the regulatory subunit p85. Indeed, several members of the Src family of nonreceptor tyrosine kinases have been shown to localize to and become activated by GPCR-complexes, including receptors for LPA and bombesin (Luttrell et al. 1996; Rodriguez-Fernandez and Rozengurt 1996). Several mechanisms have been suggested as to how the Src family may be activated by GPCRs, including direct interaction of the intracellular SH3-domain binding motifs of the GPCRs with the SH3 domain of Src itself (Liu et al. 2004). Since p85 can directly interact with Src (Gentili et al. 2002; Burnham et al. 1999), this is a potential explanation of how p110β is recruited to the GPCR complex. Additionally, Src has been shown to associate with GPCRs via its interaction with Gα subunits of heteromeric G proteins (Ma et al. 2000), which Src can directly phosphorylate (Hausdorff et al. 1992), suggesting another possible mechanism of p85-p110β recruitment to these receptors. It has also been shown that Src can directly bind β-arrestins, molecules associated with GPCRs that are involved in attenuation of the signal from the receptor and targeting it to clathrin-coated pits (Krupnick and Benovic 1998; Luttrell et al. 1999), providing yet another explanation of how p110β may be recruited to the GPCR complex by p85-Src interaction.

Meanwhile, it remains puzzling why p110β is found in RTK-complexes, when its ablation does not seem to significantly affect the downstream signaling (Guillermet-Guibert et al. 2008; Foukas et al. 2006; Knight et al. 2006; Jia et al. 2008; Ciraolo et al. 2008). One potential explanation is that in RTK-complexes this isoform only plays a scaffolding function. In this regard, it is worth noting, however, that three independent studies have reported that p110β loss does impair insulin metabolism on the organismal level, even though Akt phosphorylation downstream of InsR does not seem to be affected (Brachmann et al. 2005; Jia et al. 2008; Ciraolo et al. 2008).

In addition, assuming that p110α is the predominant isoform signaling downstream of RTKs, it is unclear why its ablation does not seem to significantly affect signaling downstream of particular RTKs, such as PDGFR (Zhao et al. 2006) or colony-stimulating growth factor receptor (CSF-1R, closely related to PDGFR) (Roche et al. 1994). Interestingly, it has been reported that p110β can also be recruited to PDGFR (Roche et al. 1998; Hooshmand-Rad et al. 2000), where (unlike p110α) it does not seem to affect actin reorganization upon PDGF stimulation (Roche et al. 1998; Hooshmand-Rad et al. 2000; Park et al. 2003). Several possible explanations present themselves for this apparent paradox, perhaps the simplest being that in the absence of p110α, p110β plays a compensatory signaling role on the level of PDGFR by taking over the signaling. This scenario could occur if approximately equal amounts of p110α and p110β bound to a given RTK. In this case, most of the PIP_3 would be produced by p110α, which has a roughly tenfold higher specific activity than p110β, and deletion of p110β would have little effect on receptor signaling. Consequently, in the absence of p110α, signaling through p110β would be relatively weak for non-abundant receptors such as EGFR present in MEFs, while for abundant receptors such as PDGFR, the amount of p110β bound might still be adequate to saturate downstream signaling.

4 Downstream Signaling: Acting Out Through AKT and PDK1

Signaling downstream of p110α and p110β involves recruitment of proteins that bind PIP_3, the shared second messenger of both isoforms. The current consensus is that proteins containing pleckstrin-homology (PH) domains directly bind PIP_3 generated in the membrane at the sites of PI3K activation. Here, the most well known of the PH-domain proteins are the serine-threonine kinases AKT (also known as protein kinase B (PKB)) and phosphoinositide-dependent protein kinase (PDK1) (Bellacosa et al. 1993; Coffer and Woodgett 1991; Jones et al. 1991) (Fig. 1). It is thought that binding of AKT and PDK1 to PIP_3 brings the two in close proximity to each other and allows for activating phosphorylation of AKT by PDK1 (Vanhaesebroeck and Alessi 2000; Alessi et al. 1997; Toker and Newton 2000). Subsequently AKT is phosphorylated by a second activating kinase termed PDK2, now believed to be a complex of mTOR (mammalian target of rapamycin) and RICTOR (TORC2 complex) (Sarbassov et al. 2005a). Activated AKT as well as PDK1 can phosphorylate a wide range of proteins, which impact cellular growth, proliferation, motility, and survival. PDK1 serves as the master regulator of the AGC-family of kinases, most of which require an additional phosphorylation event for complete activation through a parallel-acting pathway (reviewed in Mora et al. 2004; Bayascas 2008).

4.1 AKT Signaling

PI3K signaling via AKT controls the initiation of protein translation by regulating the mTOR signaling pathway. This protein signaling cascade proceeds through the tuberous sclerosis complex (TSC), which AKT inhibits via phosphorylation, followed by the continuation of signal transduction through the small G protein RheB (Ras homolog enriched in brain), and mTOR/RAPTOR (Regulatory associated protein of mTOR) complex (TORC1). The two critical downstream effectors of the mTOR pathway are S6K (p70 S6 kinase) and 4EBP (eukaryotic initiation factor 4E-binding protein), both of which when phosphorylated can promote protein synthesis and increase in cell volume (Zhao and Vogt 2008; Inoki et al. 2002, 2003; Garami et al. 2003; Tee et al. 2003; Zhang et al. 2003; Sarbassov et al. 2005b; Hanrahan and Blenis 2006; Fingar and Blenis 2004). There is also a negative feedback loop via which mTOR can attenuate the PI3K activity by phosphorylation of IRS1 (insulin-receptor substrate) by S6K (Manning 2004; Harrington et al. 2005). On the other hand, in a positive feedback loop, mTOR in a complex with RICTOR (rapamycin-insensitive companion of TOR) protein additionally activates AKT (Sarbassov et al. 2005a).

Another important target of AKT is glycogen synthase kinase 3 (GSK3) through which AKT regulates a range of different transcription factors and cell cycle entry. The phosphorylation of a constitutively active kinase GSK3 by AKT has also an

inhibitory effect. When active, GSK3 phosphorylates many transcription factors, including Myc and Jun, as well as cell cycle regulators, among which are cyclin D and p21, thereby keeping them in inactive states or promoting their proteasomal degradation (Rossig et al. 2002; Nikolakaki et al. 1993; de Groot et al. 1993; Sears et al. 2000; Gregory et al. 2003; Wei et al. 2005). However, when GSK3 activity is suppressed by AKT function, these downstream pathways are activated, underscored by their causal involvement as oncogenes.

Additionally, AKT can suppress apoptosis, working via inhibitory phosphorylation of its target, the FOXO (forkhead box O transcription factor) family of transcription factors, thereby mediating their retention in the cytosol by a formation of a complex with the 14-3-3 family of proteins and inhibiting transcription of anti-apoptotic genes normally stimulated by FOXOs, such as p27 and p21 (Brunet et al. 1999, 2001; Biggs et al. 1999; Kops et al. 1999; Takaishi et al. 1999; Tang et al. 1999; Medema et al. 2000; Seoane et al. 2004). Similarly, AKT negatively regulates the pro-apoptotic protein BAD (BCL2-antagonist of death) by generating a binding site for 14-3-3 proteins, thus barring BAD from an inhibitory interaction with BCL2 family members and allowing them to proceed with a cell survival response (Brunet et al. 2001; Zha et al. 1996; Franke and Cantley 1997).

Finally, signaling by PI3K is attenuated by dephosphorylation of PIP_3 by at least two types of phosphatases – the Src-homology 2 (SH2)-containing phosphatases (SHIP) and PTEN. Notably, these two classes of phosphatases differ by the position of the phosphate on the inositol ring that they can remove. SHIP1 and SHIP2 dephosphorylate the 5 position of PIP_3, thus generating PI-3,4-P_2; in contrast, PTEN dephosphorylates the 3 position to generate PI-4,5-P_2 (Clement et al. 2001; Lee et al. 1999; Maehama and Dixon 1999), which is the substrate for PI3K.

Since different isoforms of PI3K mediate signaling downstream of RTKs or GPCRs (or both, in case of p110β), it is intriguing to speculate that PI3K signaling effectors downstream of different receptor types might also be differentially regulated. Currently, there is almost no evidence available to suggest divergent signaling; however, the majority of studies to date rely on phosphorylation of total AKT as the major downstream read-out. Utilizing PIP_3 as a shared second messenger for all PI3K isoforms, it is possible that many of the effectors are shared as well. On the other hand, there are many PH-domain containing proteins including multiple AKT isoforms which could potentially mediate differential signaling by colocalizing to particular cellular microdomains with particular membrane receptors.

5 PI3K Isoforms in Cancer

Deregulation of the PI3K pathway is frequently found in human cancer where components of this pathway are mutated, amplified, or deleted in various tumors types. Importantly, PI3K itself frequently bears oncogenic mutations or amplifications. Specifically, the *PIK3CA*-gene, encoding p110α, undergoes activating mutations in breast (26%), endometrial (23%), ovarian (7%), urinary tract (17%),

colorectal (14%), pancreatic (8%), stomach (8%), liver (6%), and lung (3%) cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Here, the major missense mutations in *PIK3CA* are single point-mutations clustered in two regions of the gene, corresponding to the helical (E542K and E545K) and the kinase (H1047R) domain of the protein, all of which are thought to contribute to constitutive lipid kinase activity of p110α (Samuels et al. 2004, 2005; Samuels and Ericson 2006; Ikenoue et al. 2005; Kang et al. 2005). In addition to point mutations, amplification of the *PIK3CA* gene is found in a subset of head and neck, squamous cell lung carcinoma, cervical and gastric cancers (Engelman et al. 2006).

Mutant p110α expressed in cultured mammalian cells displays the oncogenic potential inferred from patient tumor material, demonstrated as its ability to transform primary cells as measured by anchorage-independent growth and tumor formation in xenograft experiments (Ikenoue et al. 2005; Kang et al. 2005; Zhao et al. 2005; Isakoff et al. 2005; Bader et al. 2006). Consistently, expression of oncogenic p110α in cells and tissues drives upregulation of signaling to many downstream PI3K effectors, such as AKT, S6K, GSK, FOXOs, etc.

Notably p110β has not been found mutant in human cancer as yet, even though it shares significant sequence homology and ubiquitous expression with p110α. Nonetheless, its potential as an oncogene has been studied in cell culture (Zhao et al. 2005; Kang et al. 2006). In immortalized human mammary epithelial cells (HMECs), p110β with an amino-terminal myristylation signal (for constitutive membrane localization) drives anchorage independent growth, xenograft tumor growth, and induces potent activation of downstream AKT signaling (Zhao et al. 2005). Contrary to synthetic activation by myristylation, an engineered p110β−allele mutant at E522K, the position analogous to E545K in p110α, fails to induce strong AKT phosphorylation or to promote anchorage independent growth in the same HMEC system (Zhao et al. 2005). In addition, when expressed in chicken embryonic fibroblasts (CEFs), wild-type p110β induces transformation which becomes more pronounced when a myristylation sequence is added; however, downstream AKT phosphorylation was only induced by myristylation but not by overexpression alone (Kang et al. 2006; Denley et al. 2008). Interestingly, the transforming potential of wild-type p110β is inhibited by a Ras-binding domain mutation (K230E) or by pharmacological inhibition of the MAPK pathway, suggesting the necessity of Ras binding and MAPK-activation for p110β-induced transformation. Notably, addition of a myristylation sequence to p110β was able to transform the cells even in the presence of the Ras-binding domain mutation (Kang et al. 2006; Denley et al. 2008), suggesting that interaction with Ras or a Ras-family member enables membrane recruitment.

5.1 Deregulated PI3K Pathway Components

In addition to p110α mutations, many other pathway components, both upstream and downstream of PI3K, are altered in cancer. Among these are various upstream

RTKs necessary for PI3K membrane recruitment and activation. Some of the most frequently deregulated RTKs are HER2, overexpressed or amplified in a large fraction of breast and ovarian cancers; epidermal growth factor receptor (EGFR), amplified or constitutively activated by mutation in gliomas and lung cancers; KIT and PDGFRα, bearing activating mutations in GISTs (gastrointestinal stromal tumors) (Yuan and Cantley 2008; Moasser 2007; Sauter et al. 1996; Narita et al. 2002; Arteaga 2006; Tornillo and Terracciano 2006). Notably, the most frequently deregulated component of the pathway downstream of PI3K is the tumor suppressor PTEN, which can be found inactivated either via point mutation or deleted in human cancers (reviewed in Keniry and Parsons 2008). Loss of PTEN results in constitutive activation of the PI3K axis due to the loss of one of its major suppressors, an effect which is exacerbated by a gain of an activating mutation in an RTK or p110α with concomitant loss of PTEN.

5.2 *Targeting PI3K in Cancer*

Considering the frequent oncogenic alterations in various PI3K-pathway components, this pathway has attracted substantial interest from pharmaceutical companies for therapeutic intervention with targeted therapy solutions. The rationale here is that PI3K-signaling attenuation would result in a cytotoxic or at least cytostatic effect on tumors that have acquired dependence on this pathway. Towards this goal, several class I PI3K inhibitors have entered phase I clinical trials and, in addition, compounds that exhibit isoform-specificity are emerging (reviewed in Garcia-Echeverria and Sellers 2008). However, it is not clear which PI3K-pathway component inhibition would give the most profound results when treating different types of tumors. It is likely that tumors bearing oncogenic *PIK3CA* mutations would indeed be sensitive to direct p110α inhibition. Nonetheless, it is not yet fully substantiated if p110α-specific inhibition would suppress tumors carrying other genetic alterations in the pathway, such as PTEN tumor-suppressor loss or conversely upstream RTK oncogene activation. To address these issues, several recent genetic studies have shed some light onto the question of which PI3K isoforms are responsible for driving several different tumor types.

5.3 *p110α as a Viable Tumor Target*

Generation of conditional *PIK3CA* knock-out (Zhao et al. 2006) and kinase-dead PIK3CA (D933A) knock-in mice (Foukas et al. 2006) has provided valuable genetic tools for studying the role of p110α in many different genetic tumor contexts. *In vitro* studies of immortalized MEFs from conditional *PIK3CA* knock-out mice suggest that p110α is necessary for transformation of cells driven by RTKs – such as insulin-like growth factor 1 receptor (IGF-1R), EGFR, and HER2, but

Table 2 p110 isoform ablation in cancer

Lesion\isoform	p110α	p110β
IGF-1R	√ *(in vitro)*	Not tested
EGFR	√ *(in vitro)*	√ (*in vitro,* level dependent)
HER-2	√ *(in vitro)*	√ (*in vivo,* kinase-dead)
vSRC	X *(in vitro)*	Not tested
HRAS	Not tested	√ (*in vitro*, kinase independent)
PTEN−/−	X (*in vivo*)	√ (*in vivo*)
KRAS	√ (*in vivo*, RBS[a] mutant)	Not tested

[a]*RBS* RAS binding site

interestingly v-Src induced transformation was not affected (Zhao et al. 2006) (Table 2). Although not yet corroborated in a genetic mouse model, this data suggests that specific targeting of p110α with small molecules might be a therapeutic choice for tumors with RTK alterations upstream of p110α. However, when the p110α knock-out mice were crossed to a prostate cancer-prone mouse model driven by *Pten* loss (Jia et al. 2008), surprisingly tumor formation was found to be independent of p110α function.

In certain tumor types, *PIK3CA* mutations are coexistent with RAS (KRAS in particular) mutations, whereas in other types they have been suggested to be mutually exclusive (reviewed in Yuan and Cantley 2008). As discussed earlier, the p110 subunits of PI3K contain a RAS-binding domain and therefore, it is important to determine if PI3K is necessary for RAS-induced tumorigenesis. To this end, it would be beneficial to determine if PI3K-inhibition would be a suitable therapy for RAS-driven tumors. To address the importance of RAS-PI3K molecular interaction, knock-in mice were generated bearing two *Pik3ca* point mutations (T280D and K227A) which prevent its interaction with endogenous RAS (Gupta et al. 2007). The homozygous MEFs isolated from these mice had significantly attenuated growth factor signaling towards the PI3K axis, particularly evident in response to EGF and fibroblast growth factor 2 (FGF2) stimulation. Furthermore, the mutant MEFs had a markedly decreased number of cells in S-phase, implying a proliferative defect induced by the loss of PI3K-RAS interaction. *In vitro*, the MEFs could not be efficiently transformed by oncogenic HRAS or EGFR, potentially highlighting the necessity for p110α inhibition in tumors driven by these oncogenes. In addition, when these mice were crossed to mice expressing an oncogenic Kras allele (which normally develop lung adenocarcinoma), tumor formation was virtually completely abrogated, suggesting importance of this interaction for the initiation of KRAS-induced tumorigenesis. As a complementary approach, it would be interesting to investigate tumor formation in mice that co-express an oncogenic allele of Pik3ca (E545K or H1047R) and the RAS-effector mutant that does not interact with p110. Recently, the question of RAS-PI3K interaction in tumorigenesis was addressed by the use of a small molecule inhibitor NVP-BEZ235, a dual pan-PI3K and mTOR inhibitor in clinical trials (Engelman et al. 2008). In this study, a mouse model for lung adenocarcinoma was generated by expression of Pik3ca(H1047R) in the lung. As expected, the mice that developed tumors responded well to PI3K/mTOR inhibition, measured as very potent reduction in

lung tumor burden. Strikingly, the lung-targeted oncogenic Kras mutant mice did not show substantial tumor effects when treated with the PI3K/mTOR inhibitor. However, significant tumor shrinkage could be achieved by the combination therapy including the PI3K/mTOR inhibitor and a MEK inhibitor. Notably, the results of this pharmacological study (in contrast to the previously discussed genetic study) suggest that PI3K inhibition may not be sufficient for the treatment of *KRAS* tumors once established, which in addition may require the inhibition of the RAS-RAF-MAPK signaling pathway.

5.4 p110β as a Drug Target

As most PI3K inhibitors which have therapeutic potential inhibit all class I PI3K isoforms (and not p110α specifically) (Garcia-Echeverria and Sellers 2008), it has become exceedingly important to investigate if inhibition of isoforms other that p110α would be beneficial in cancer therapy (as these have not been found mutant in human tumors). Since p110β is the only other ubiquitously expressed class I isoform, p110β mouse knock-out studies have provided useful clues towards answering this question. Notably, *in vitro* results suggest that targeted knock-out of p110β in MEFs prevents transformation driven by mutant HRAS and mutant EGFR, suggesting necessity for p110β in this setting (Jia et al. 2008) (Table 2). Surprisingly, molecular replacement with a kinase-dead allele of p110β rescued most or all of the observed phenotypes by allowing for MEF transformation, suggesting a potential scaffolding role for p110β. A possible explanation for this data is that lack of p110β makes cells sensitive to oncogene induced stress, a condition that can largely be rescued by kinase dead allele of p110β. In the case of the breast-targeted Her2 mouse model for breast cancer, mice with a knock-in of a kinase dead allele of p110β showed slower tumor development than did controls (Ciraolo et al. 2008). In addition, p110β-loss in the *Pten*-null tumor setting, in contrast to p110α-loss, seems to prevent tumor formation in the anterior prostate, suggesting that specific inhibition of p110β would be a reasonable therapeutic approach in PTEN-null prostate cancers (Jia et al. 2008) and that tumor formation driven by PTEN in the prostate might depend on p110β and not p110α. Consistent with this notion, it has been shown using shRNA approaches that knock-down of p110α does not affect growth and colony formation of three PTEN-null human cancer cell lines (originating form prostate, brain, and breast tumors), whereas knock-down of p110β profoundly affected their growth and signaling to the downstream AKT/mTOR pathway (Wee et al. 2008). Here, the growth retardation and signaling could not be rescued by the expression of the kinase-inactive p110β allele. Moreover, p110β also seemed to be required for tumor formation by these cells in nude mice, thus highlighting the role of p110β in PTEN-deficient tumors. Nonetheless, due to the limited number of cancer cell-lines used in this study and our lack of understanding of all genetic lesions that these cancer cell lines bear, it will be important to determine if p110β is important for tumorigenic state in all

PTEN-null tumors, or only within a subset of them where PTEN-loss co-occurs with particular other lesions. Collectively, this suggests that additional genetic mouse models need to be generated in order to obtain more definitive data on the role of p110β in tumor formation and maintenance.

5.5 What Are the Take-Home Messages from p110-Isoform Knock-Out Studies In Vivo?

As targeted therapies for various tumor lesions are developed, genetic knock-out mouse models have become important tools in determining the use of these therapies in particular cancer settings. Thus, as discussed above, genetic ablation of p110-isoforms serves as a tool in helping to elucidate whether PI3K (and which particular PI3K isoform) should be targeted in various tumor types. However, one issue with these genetic approaches is that the ablation of p110-isoforms (i.e., conditional tissue-specific knock-out) usually takes place before a tumor driven by gain of a particular oncogene or loss of a tumor suppressor (e.g., gain of oncogenic Kras, loss of Pten, etc.) is formed (Jia et al. 2008; Ciraolo et al. 2008; Gupta et al. 2007). Thus, the question that can readily be answered from these types of studies is whether a particular isoform is involved in the process of tumor nucleation or initiation. Based on the *in vivo* studies available to date, it seems likely that one or both of the p110-isoforms are required for tumor initiation. However, it remains unclear if ablation of these isoforms in an already formed tumor would have an effect, or in other words, if p110-isoforms are necessary for tumor maintenance. Currently, the way to answer this question *in vivo* is either by using targeted therapies to treat the already formed tumors (provided that these therapies are available, as described in Engelman et al. (2008)) or by designing animal models where the expression of the Cre recombinase used for p110 ablation can be regulated by use of externally administered chemicals (such as doxycycline, Chin et al. 1999) independently from the mutation driving tumor formation. In addition, *in vitro* studies utilizing cancer cell lines with isoform-specific shRNA knock-down (such as Wei et al. 2005) may provide significant information as to which isoform should be inhibited in a particular setting of tumor lesions. However, in order to obtain firm conclusions from these studies, it is likely that large numbers of human cancer cell lines need to be used as this may bypass the recurring issue of poor annotation of genetic lesions present in them and provide significant statistical cohort analysis.

5.6 Kinase-Independent Roles of p110-Isoforms

Due to the suggested scaffolding (kinase-independent) role of p110β for transformation *in vitro*, it was of considerable interest to investigate the functions of kinase-dead

alleles *in vivo*. Mice carrying a germline knock-in mutation of kinase-dead allele of p110α (D933A) die during embryonic development (Foukas et al. 2006), thus no *in vivo* studies using these animals have been possible. In contrast, mice bearing kinase-inactive *Pik3cb*(K805R) allele survive to adulthood, however with growth retardation features (Ciraolo et al. 2008). Since classical knockout of *Pik3cb* is early embryonic lethal this data argues strongly for an important kinase-independent function for p110β during development. When crossed to breast cancer prone Her2 knock-in mice, the compound homozygous mice develop breast tumors much more slowly than control mice, implying that the kinase activity of p110β could be functionally necessary downstream of HER2 in human breast cancer. However, due to the lack of the corresponding p110α kinase-dead knock-in parallel experiments (such as the model described in Foukas et al. 2006), it is yet uncertain if p110α is necessary in Her2 tumorigenesis.

Correspondingly, it remains to be experimentally addressed if kinase activity of p110β contributes to Pten-induced transformation *in vivo* (Jia et al. 2008) or if prostate neoplasia can be attributed to its scaffolding role alone. Since ablation of p110β blocked the appearance of phosphorylated Akt in the prostate, it is possible that p110β kinase activity is required for tumorigenesis. In any event, it is currently unclear how the inferred scaffolding function of p110β may be carried out at a molecular level. One possible explanation is that p110β functions as an adaptor protein, serving to recruit a subset of signaling molecules to RTKs. Notably, p110β found in RTK-associated complexes exhibits low lipid kinase activity, measured as its contribution relative to co-precipitating p110α (Guillermet-Guibert et al. 2008; Foukas et al. 2006) and hence, it may be possible that its scaffolding function is more significant than its inherent kinase function at these receptors. Results showing that transformation of p110β-null MEFs by activated EGFR was rescued by the exogenous expression of the kinase-dead allele of p110β despite its negligible contribution to Akt signaling downstream of EGFR are in line with this notion (Jia et al. 2008). An alternative mechanism could be that the scaffolding role of p110β is exhibited at the level of cellular endocytosis. Specifically, p85α-p110β heterodimers have been found to directly bind the small GTPase Rab5 which regulates docking and fusion of early clathrin-coated endosomes during endocytosis (Christoforidis et al. 1999; Shin et al. 2005). In agreement, p110β-null MEFs exhibit a defect in the uptake of transferrin (marker for receptor-mediated endocytosis) which could be functionally rescued by overexpression of the kinase-dead p110β allele (Jia et al. 2008). Similarly, a defect in EGFR internalization and formation of clathrin-coated vesicles beneath the plasma membrane observed in knock-in MEFs expressing low levels of kinase-dead p110β allele was rescued by high levels of expression of the same allele (Ciraolo et al. 2008), also suggesting a scaffolding role during endocytosis. Although not yet studied in detail, it is possible that p110β can exhibit its scaffolding function at the level of GPCRs as well. Most studies to date have used phosphorylation of downstream AKT as a read-out of GPCR stimulation by various ligands (Guillermet-Guibert et al. 2008; Jia et al. 2008; Ciraolo et al. 2008). Notably, this particular process seems to depend on the lipid kinase activity of p110β. Nonetheless, it would be interesting to identify an

alternative experimental read-out that could enable the assessment of p110β kinase-independent role in GPCR-associated complexes.

6 Conclusions

Although PI3Ks are well studied enzymes, still many important questions remain to be answered as we have tried to illustrate herein. The recent animal models (Foukas et al. 2006; Jia et al. 2008; Ciraolo et al. 2008) and an ever-increasing number of sophisticated and highly isoform-specific PI3K inhibitors will serve as invaluable tools in the elucidation of the roles of PI3K isoforms during development and in disease. Of central mechanistic importance, it remains unanswered whether different PI3K isoforms (catalyzing the same chemical reaction) mediate differential downstream signaling, to what extent this may be exhibited in the context of different upstream receptor types (RTKs and GPCRs) and how signaling differs between cell-types. Pertaining to human cancer, we await conclusive determination of which PI3K isoforms are most valuable for therapeutic inhibition in different stages and types of tumors. Moreover, particular genetic lesions (such as KRAS-mutation or HER2-amplification) in combination with PI3K-mutation could lead to oncogene-dependence (tumor cell-addiction) whereby pharmacological inhibition would result in induction of apoptosis in the targeted tumor cells. Notably, several recent studies have already begun to answer this question (Jia et al. 2008; Ciraolo et al. 2008; Engelman et al. 2008; Wee et al. 2008), but still need to be complemented by reciprocal studies with remaining isoforms and performed in additional tumor genetic backgrounds relevant to human cancer.

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PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis

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The PI3K pathway is frequently activated in cancer; therefore, considerable effort is focused on identifying compounds that can inhibit specific pathway components, particularly the hallmark oncogene PIK3CA. Although targeted inhibition of a cancer survival gene holds significant promise, there are concerns that drug resistance may emerge within the cancerous cells, thus limiting clinical efficacy. Using genetically defined human mammary epithelial cells, we evolved resistance to the PI3K/mammalian target of rapamycin (mTOR) inhibitor BEZ235, and by genome-wide copy number analyses, we identified *MYC* and *eIF4E* amplification within the resistant cells. Importantly, either MYC or eukaryotic translation initiation factor 4E (eIF4E) was required to bypass pharmacological PI3K/mTOR inhibition in resistant cells. Furthermore, these cells displayed elevated 5′ cap-dependent protein translation. Collectively, these findings suggest that analysis of drivers of protein translation could facilitate the identification of cancer lesions that confer resistance to PI3K pathway-targeted drugs.

Various components of the PI3K pathway are frequently deregulated in human cancer through genetic alterations (point mutations and gene amplifications or deletions) or alternatively, epigenetic mechanisms [silencing of phosphatase and tensin homolog (PTEN) expression], resulting in constitutive pathway activation. Notably, PI3Kα is the only member of the PI3K pathway that bears frequent activating mutations in multiple major tumor types: breast, endometrial, ovarian, prostate, colorectal, pancreatic, liver, and lung cancers (1). The major oncogenic missense mutations in PI3K cluster in two separate regions of its p110α catalytic subunit—the helical (E542K and E545K) and the kinase (H1047R) domains—and both types yield constitutive lipid kinase activity (2, 3). In addition, the *PIK3CA* gene is amplified in a subset of head and neck, squamous cell lung, cervical, and gastric cancers (4).

Because of the high frequency of oncogenic activation of the PI3K pathway (1, 5–8), there is considerable interest in developing effective pharmacological inhibitors for cancer therapy. The expectation is that tumors bearing lesions along the PI3K pathway have acquired dependence on its activity and therefore, would exhibit augmented sensitivity to its inhibition, leading to growth arrest and/or induction of apoptosis. Currently, several PI3K inhibitors, including GDC0941 (Genentech) and BEZ235 (Novartis Pharmaceuticals), have entered phase I clinical trials, and in addition, isoform-specific compounds are being developed (9–11).

Despite the promise of targeted therapies, an emerging clinical obstacle is the acquisition of drug resistance within the tumor cells. To date, at least three distinct genetic mechanisms of resistance to kinase inhibitors have been described. First, the paradigm example for acquired drug resistance is the seminal observation that, during imatinib treatment of chronic myelogenous leukemia (CML), drug-resistant mutations arise in the drug target itself [breakpoint cluster region-Abelson leukemia homolog 1 (BCR-ABL)] (12, 13). Second, another documented mechanism of drug resistance derives from lesions in parallel pathways, which has been described for *MET* amplification leading to resistance to epidermal growth factor receptor (EGFR) inhibition by gefitinib and erlotinib in non-small cell lung carcinoma (NSCLC) (14, 15). Third, downstream lesions may occur in the same pathway, which has been described for trastuzumab resistance arising from PI3K pathway mutations (16, 17). Here, we explore acquired resistance to PI3K inhibitors and propose the prevailing mechanism of such resistance. Using genetically defined human mammary epithelial cells (HMECs), a model system that has previously been used for PI3K pathway-driven transformation because of its dependence on oncogenic PI3K signaling (18), we screened for emergence of BEZ235 resistance and identified genetic lesions involved.

Results

To develop a sensitive cell-based assay system to explore resistance to PI3K-targeted drugs, we chose to use immortalized HMECs, which had been modified to express both telomerase (hTERT) (19) and a synthetic dominant-negative p53 allele (p53DD) (20). Our overall goal was to explore whether mutations in the drug target itself or alternatively, acquisition of genetic alterations in downstream or parallel acting pathways, would yield drug resistance (12, 15, 21). Notably, these immortal HMECs are sensitive to PI3K inhibitors, as demonstrated by their response to treatment with the dual PI3K/mTOR inhibitor, BEZ235 (9), which significantly slows their growth and induces G1 cell cycle accumulation in a dose-dependent manner (Fig. 1 *A* and *B*).

Generation of BEZ235-Resistant Cells. To investigate whether point mutations within the coding region of *PIK3CA* may drive resistance to BEZ235, we chose to bias our approach by generating a randomly mutagenized plasmid library of second site mutants in the background of oncogenic PIK3CA(H1047R). We accomplished this by passaging a PIK3CA retroviral expression vector [pBABE-puro-PIK3CA(H1047R)] through the mutator bacteria strain, XL1-Red (Stratagene) (22), and then introducing this library of mutant PIK3CA constructs into immortalized HMECs by retroviral transduction (Fig. 1*C*). Cultures of immortalized HMECs expressing mutagenized PIK3CA(H1047R), nonmutagenized PIK3CA(H1047R), and parental immortalized HMECs (HMEC/hTERT/p53DD) were exposed to increasing concentrations of

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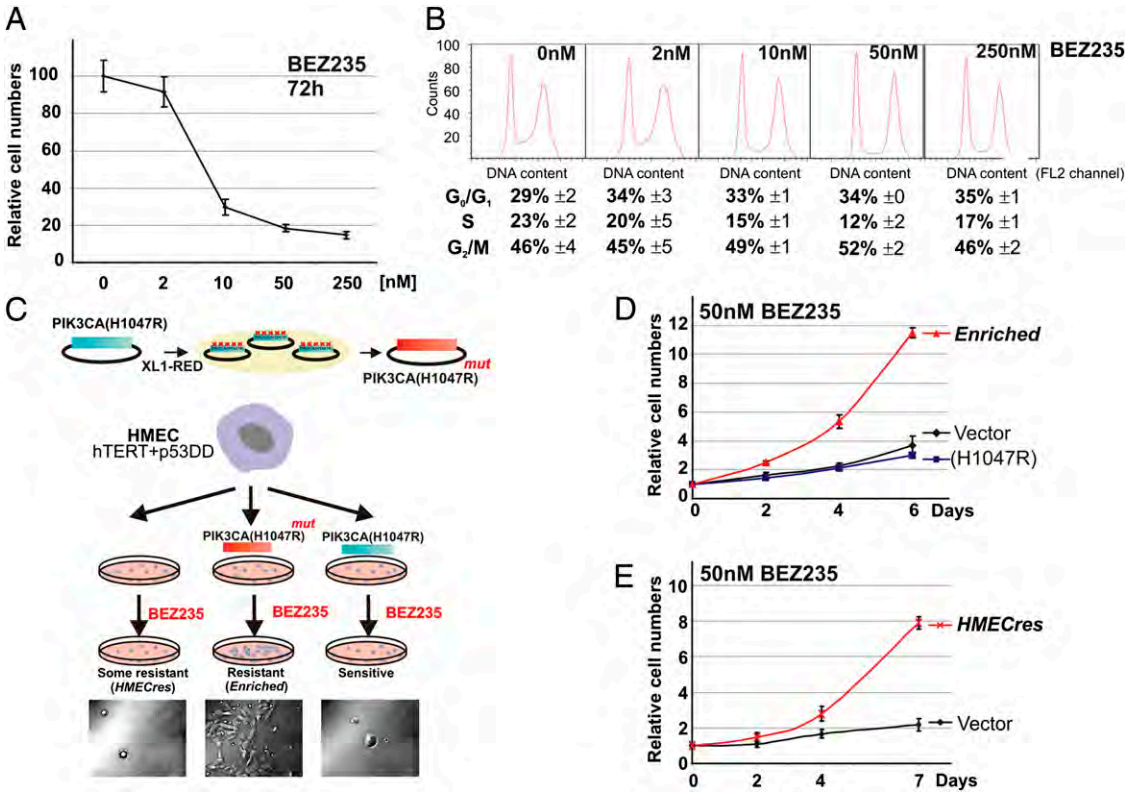

Fig. 1. Generation of BEZ235-resistant cells. (*A*) Growth curve of HMECs in the presence of BEZ235 (0–250 nM). (*B*) Cell cycle analysis by FACS counts of HMECs grown in indicated concentrations of BEZ235. (*C*) Schematic of the selection procedure used to generate BEZ235-resistant cells. (*D*) Growth curves of HMECs expressing vector control, PIK3CA(H1047R), and the *Enriched* cells in the presence of 50 nM BEZ235. (*E*) Growth curves of HMECs expressing vector control and the *HMECres* cells in the presence of 50 nM BEZ235. (Data are represented as mean ± SEM.)

BEZ235 in a stepwise fashion (*Methods*). Cells were maintained in the presence of BEZ235 for 2 mo until populations of proliferating cells emerged. In agreement with our expectations, this biased selection of HMECs expressing randomly mutagenized PIK3CA(H1047R) (Fig. 1*C*) produced a drug-resistant population of cells, which we will subsequently refer to as the *Enriched* population (BEZ235-resistant). Although PIK3CA(H1047R)-transformed cells failed to generate any cells resistant to BEZ235, rare cells originating from the parental HMECs emerged and could be propagated (referred to as *HMECres*). When these cultures were subsequently challenged with BEZ235, both the *Enriched* (Fig. 1*D*) and the *HMECres* (Fig. 1*E*) lines proliferated significantly faster than the parental (control) cells.

PIK3CA Point Mutations. Because the *Enriched* BEZ235-resistant population arose from cells expressing the randomly mutagenized PIK3CA(H1047R) library, it would be consistent with previous observations (22) that the resistant phenotype of these cells could be explained by mutations within the ectopic PIK3CA (H1047R) alleles. To this end, we used genomic DNA from the *Enriched* cells to amplify the integrated proviral DNA using proofreading PCR. In parallel, we amplified the ectopic PIK3CA allele from the naïve cells (i.e., cells expressing the randomly mutagenized PIK3CA library before selection with BEZ235). These two samples were analyzed by SOLiD sequencing (Applied Biosystems) and differential sequence analysis (Fig. S1*A*). Approximately 40% of the PIK3CA cDNA molecules from the drug-resistant cells contained a point mutation (G959A) resulting in a G320E amino acid substitution. Another 20% contained a point mutation (T1457C) resulting in an F486S amino acid substitution. Importantly, the mutagenized preselection PIK3CA alleles did not show any mutational prevalence. SOLiD sequencing is based on parallel sequencing of short DNA fragments that are 50 nt long; thus, it was unclear whether the mutations detected were positioned *in cis* or *in trans*. To determine this, we cloned the 3.2-kb proviral PCR fragments (containing the ectopic PIK3CA) and sequenced a cohort of them by conventional Sanger sequencing. We detected G320E in one-half (14 of 28) of the sequenced PIK3CA molecules, whereas 10 of 28 harbored the F486S mutation juxtaposed with a 10-bp deletion (nucleotides 1,030–1,039) (Fig. S1*B*). One clone harbored both G320E and F486S *in cis*. All sequenced clones retained the original H1047R mutation.

Owing to the fact that no point mutations were found within the catalytic domain, which would be indicative of altered drug binding, it was not immediately clear by what mechanism the identified mutations might contribute to the observed BEZ235 resistance. The G320E mutation is in the linker preceding the C2 domain, whereas the F486S mutation is in the linker after the C2 domain and co-occurs with an additional 10-nt frame-shift deletion inside the C2 domain (Fig. S1 *B* and *C*). To assess these alleles' inherent capacity to mediate BEZ235 resistance, we introduced these variants into the PIK3CA(H1047R) cDNA, transduced the parental HMECs with each allele individually (G320E or del; F486S), and measured their growth relative to controls in the presence of the drug (Fig. S1*D*). Using this approach, neither of the two variant alleles could recapitulate the drug resistance.

Although unable to yield BEZ235 resistance, we also examined these allele variants with regard to downstream signaling (Fig. S1*E*) and their inherent lipid kinase activity (Fig. S1*F*). Although overexpression of PIK3CA(G320E;H1047R) resulted in constitutive downstream signaling, measured as phospho-S473-AKT and heightened lipid kinase activity [similar to the signaling of the oncogenic PIK3CA(H1047R)], the PIK3CA(del;F486S;H1047R)

allele did not induce significant AKT phosphorylation, and there was no lipid kinase activity detectable (Fig. S1 *E* and *F*). Because we could not detect the ectopic PIK3CA alleles within the *Enriched* cells by Western analysis and the PIK3CA(del;F486S; H1047R) allele is expected to yield a truncated product, we measured the RNA expression of the ectopic virally encoded PIK3CA and the antibiotic marker puromycin. Notably, we could not detect even nominal RNA levels of the ectopic alleles within the *Enriched* cells, although expression of puromycin could be verified, indicating that these allele variants had lost expression during selection for drug resistance (Fig. S1 *G* and *H*).

Integrated Copy Number and Expression Analysis Indicates *MYC* Amplification in BEZ235-Resistant Cells. Because the identified PIK3CA point mutant alleles could not recapitulate the drug resistance phenotype (Fig. S1*D*), we hypothesized that alternative genetic changes might have occurred. Therefore, we used 250,000 genome-wide SNP-based copy number analysis to determine if any chromosomal gains or losses were discernable. Comparison of genomic DNA from the *Enriched* cells with the genomic DNA of the cells expressing vector or oncogenic PIK3CA(H1047R) revealed a discrete region of genomic amplification on chromosome 8q24, which became more focal upon additional selection in BEZ235 (Fig. 2*A*). We chose to use gene expression analysis to limit the number of amplified candidate genes mediating the observed drug resistance (Table S1). Using total RNA extracted from immortalized HMECs expressing vector (control), PIK3CA(H1047R), and the *Enriched* cells (in biological triplicates), which had been grown in the presence of 50 nM BEZ235 for 72 h, we performed a comparative gene expression microarray analysis. Cluster analysis revealed that triplicates of vector control and PIK3CA(H1047R) cells cluster closer together than *Enriched* cells (Fig. 2*B*), indicating that the latter population has a gene expression signature that is significantly different from its preselection counterparts. By reducing the analysis to genes contained within regions (*X*) of chromosomal gain (>3.4) or loss (<0.6) in the *Enriched* genome (Fig. 2*A*), we generated a narrow list of 12 genes, which are differentially expressed and could potentially be responsible for the observed BEZ235 resistance (Fig. 2*C*). This integrated analysis

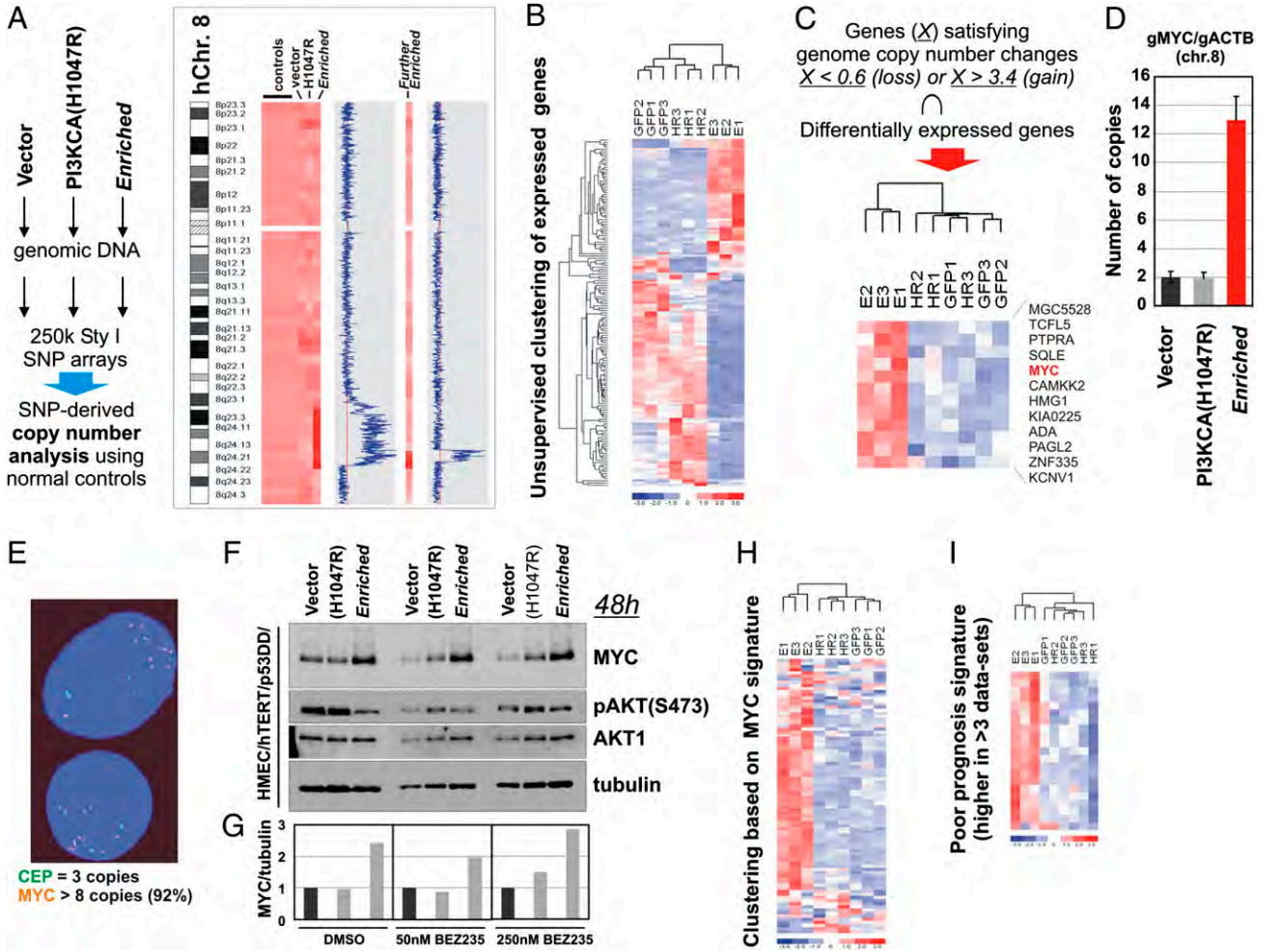

Fig. 2. Genome-wide copy number and expression analyses of BEZ235-resistant cells. (*A*) SNP array analysis of HMECs expressing vector control (GFP), PIK3CA (H1047R) (HR), or the *Enriched* (E) cells indicating a region of amplification at chromosome 8 present in the BEZ235-resistant *Enriched* cells that becomes narrowed on additional drug selection (Further Enriched). (*B*, *C*, *H*, and *I*) Expression array analysis of triplicate samples of HMECs expressing vector control (GFP), PIK3CA(H1047R) (HR), or the *Enriched* (E) cells. (*B*) Unsupervised clustering of expressed genes. (*C*) Differential expression of genes within the amplified regions in *A*. (*D*) Genomic QPCR analysis of the lines in *A* confirming *MYC* amplification. (*E*) FISH analysis of the *Enriched* cells using the genomic probe for *MYC* (orange) and centromeric 8 control probe (green). (*F*) Western analysis in the absence or presence of BEZ235 (50 and 250 nM) of the cell lines in *A*. (*G*) Quantification of MYC protein levels normalized to tubulin from the Western assay in *F*. (*H*) Clustering based on published MYC signature (25). (*I*) Clustering based on the published poor prognosis signature (26).

identified *MYC*, a well-known human oncogene present in the 8q24 amplicon. Notably, MYC is implicated as a downstream target of the PI3K/mTOR pathway (23) and frequently deregulated in breast cancer (24); thus, we considered it a likely candidate for mediating BEZ235 resistance. We verified that *MYC* was amplified in the *Enriched* cells by measuring *MYC* genomic copy numbers by quantitative real-time PCR (QPCR) relative to controls (Fig. 2*D*). In addition, FISH confirmed *MYC* amplification in the *Enriched* cells (Fig. 2*E*). Furthermore, we could detect a twofold increase in MYC protein levels in the *Enriched* cells relative to the controls, independent of BEZ235 treatment (Fig. 2 *F* and *G*).

Enrichment of a MYC Signature in the BEZ235-Resistant Cells. If *MYC* amplification were indeed the genetic event that mediated the observed BEZ235 resistance, it would be expected to be traceable by changes in expression of MYC-regulated genes. To test the hypothesis that MYC may be the driver of resistance to BEZ235 in our system, we applied a previously derived MYC signature (25), which we curated for higher stringency to contain only those probe sets (genes) that are up-regulated. When this signature was applied to our dataset, the *Enriched* cells exhibited a close relationship with the MYC signature, whereas the controls [vector and PIK3CA(H1047R)] did not (Fig. 2*H*). In addition, it was recently reported that induction of MYC-regulated genes is associated with poor outcome in human cancers (26). Comparison of the *Enriched* profile with the most common poor prognosis genes (curated to contain probe sets that were associated with higher expression in at least four datasets) revealed that the *Enriched* cells display this signature (Fig. 2*I*). Collectively, the expression array analysis suggested that the *Enriched* cells exhibit expression patterns resembling those patterns of human tumors with deregulated MYC.

MYC Overexpression Is Sufficient to Confer Resistance to PI3K and mTOR Inhibition. We next wanted to test whether overexpression of MYC could functionally recapitulate BEZ235 resistance. Immortalized HMECs transduced to express MYC were exposed to BEZ235 in growth assays (Fig. 3*A*) and found to generate a BEZ235 resistance phenotype similar to the resistance of the *Enriched* cells. MYC overexpression on its own seems to be dominant in rendering resistance to BEZ235, because we did not observe a synergistic effect of MYC overexpression in combination with PIK3CA(H1047R). Importantly, overexpression of Cyclin D1, a documented downstream effector of MYC (27), or overexpression of a suggested gatekeeper mutant PIK3CA allele (I848V) (28) did not parallel the resistance conferred by MYC (Fig. S2). Notably, introduction of ectopic MYC in HMECs expressing oncogenic PIK3CA(H1047R) suppressed the expression of the ectopic PIK3CA allele and resulted in reduced downstream PI3K signaling measured as pS473-AKT (Fig. 3*B*),

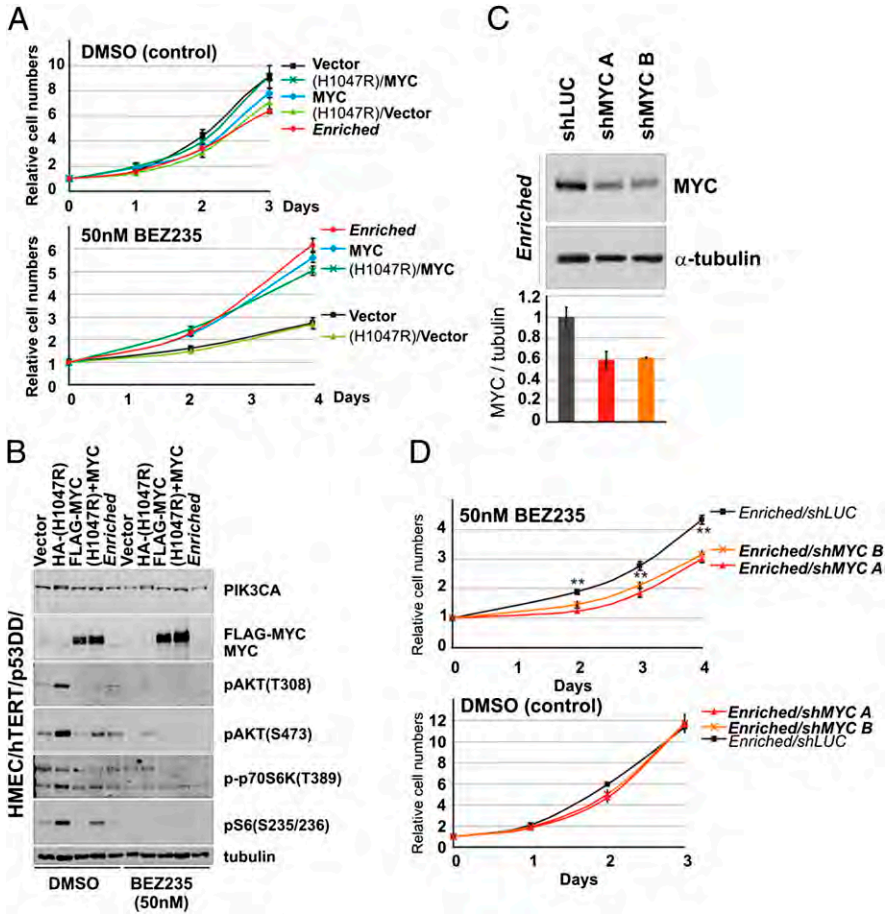

Fig. 3. MYC is sufficient and necessary for BEZ235 resistance. (*A*) Growth curves in the absence (DMSO) or presence of 50 nM BEZ235 for HMECs expressing vector control, PIK3CA(H1047R), MYC, PIK3CA(H1047R)+MYC, or the *Enriched* cells. (*B*) Western analysis of the cell lines in *A* grown in the absence or presence of 50 nM BEZ235. (*C*) Western assay of *Enriched* cells with shLUC, shMYC A, or shMYC B accompanied by quantification of MYC levels normalized to tubulin. (*D*) Growth curves of the cell lines from *C* in the absence or presence of 50 nM BEZ235 (**$P < 0.005$; Student two-tailed *t* test for paired samples). (Data are represented as mean ± SEM.)

and thus, it may explain why the *Enriched* cells evolved to attenuate expression of ectopic PIK3CA.

Having established that MYC overexpression is sufficient to recreate the BEZ235 resistance observed in the *Enriched* cells, we wanted to examine if MYC was functionally necessary. Therefore, we used lentiviral shRNA knockdown of MYC in the drug-resistant cells (Fig. 3*C*). Notably, we achieved ~40% MYC protein depletion in the *Enriched* cells, which taking into account the twofold increase caused by *MYC* amplification, effectively reverted MYC expression to nominal endogenous levels. When grown in the presence of BEZ235, *Enriched* cells expressing shRNA against MYC showed a decreased drug resistance phenotype relative to the shRNA against luciferase (shLUC) -infected control (Fig. 3*D*).

MYC Confers Resistance to Pan-PI3K and TORC1/2 Inhibition. To determine if the drug resistance phenotype resulting from MYC overexpression and amplification was specific for BEZ235, we tested response of these cells to other kinase inhibitors that share targets with BEZ235–PI3K and/or mTOR (Fig. 4*A*). First, we examined if MYC could confer resistance to GDC0941 (29), a pan-PI3K inhibitor that does not inhibit mTOR (Fig. 4*B*), and found that, indeed, MYC overexpression results in resistance to GDC0941. Because BEZ235 is a dual PI3K/mTOR inhibitor, we also tested if MYC renders cells insensitive to TORC1 inhibition by Rapamycin as well as the catalytic inhibitor of mTOR (TORC1 and TORC2) and Ku-0063795 (30) (Fig. 4*C*). As was seen for dual PI3K/mTOR inhibition by BEZ235, we found that MYC overexpression was sufficient to render resistance to both Rapamycin and Ku-0063795. In addition to targeted inhibitors, we analyzed whether MYC overexpression may affect general drug resistance. MYC overexpression had no effect on cellular sensitivity to Doxorubicin (Fig. S3) but rendered cells even more sensitive to Paclitaxel (Fig. 4*D*), which are two classical chemotherapeutic agents.

MYC Overexpression Renders Breast Cancer Cell Lines Resistant to BEZ235. We next explored if manipulation of MYC levels could affect the response to BEZ235 in established human breast cancer cell lines. To this end, we used five breast cancer cell lines, two of which (SKBR3 and HCC1954) bear amplification of the 8q24 genomic region (Cosmic; Sanger Wellcome Trust). The remaining three lines, CAMA-1, MDA-MB-453, and, MDA-MB-468, are diploid over the 8q24 region (Cosmic; Sanger Wellcome Trust) (Fig. 5*A*). Notably, overexpression of MYC in the lines without 8q24 amplification resulted in increased resistance to BEZ235. However, overexpression of MYC in lines where *MYC* was already amplified did not result in additional drug resistance. We next tested if MYC is necessary for growth in the presence of BEZ235 in the *MYC* genomic region-amplified cell lines SKBR3 and HCC1954 (Fig. 5*B*). Knockdown of MYC in both of these cell lines resulted in the impaired growth in the presence of BEZ235 relative to the controls (shLUC) (Fig. 5*C*).

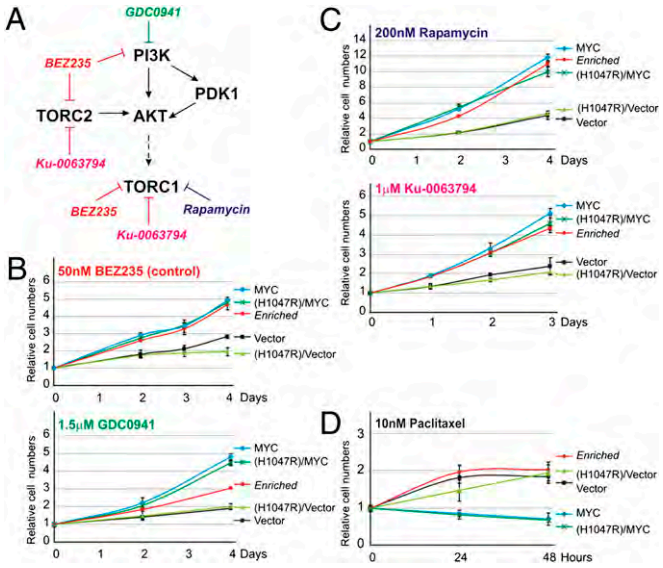

Fig. 4. MYC confers resistance to pharmacological PI3K or mTOR inhibitors. (*A*) Schematic representation of the PI3K-mTOR pathway with the inclusion of kinase inhibitors used in this study. (*B–D*) Growth curves of HMECs expressing empty vector, PIK3CA(H1047R), MYC, PIK3CA(H1047R)+MYC, or the *Enriched* cells grown in (*B*) 50 nM BEZ235 (control) or 1.5 μM GDC0941, (*C*) 200 nM rapamycin or 1 μM Ku-0063794, or (*D*) 10 nM Paclitaxel. (Data are represented as mean ± SEM.)

Amplification of *eIF4E* Can Mediate Resistance to BEZ235. Upon extended selection in BEZ235, we propagated an additional seemingly drug-resistant population of cells, referred to as *HMECres*, that originated from the original immortal HMEC cells. Compared with the *Enriched* population, *HMECres* emerged 1 mo later during the course of the selection procedure. Notably, when assayed, these cells proliferate significantly faster in the presence of BEZ235 relative to the parental cells in a manner paralleling the *Enriched* cells (Fig. 1*E*). Importantly, these cells retain their resistance phenotype upon drug recovery (i.e., prolonged propagation in the absence of inhibitor). Hence, the nature of their acquired BEZ235 resistance would likely be genetically mediated. Thus, we used genome-wide copy number analysis to investigate if any detectable chromosomal changes were identifiable. A comparison of the genomic DNA from the *HMECres* BEZ235-resistant cells to the genomic DNA of the parental HMEC line revealed a region of genomic amplification centered around chromosome position 4q22 (Fig. 6*A*). Among the genes contained within the chromosome 4 amplicon (Table S2), the eukaryotic translation initiation factor 4E, *eIF4E*, seemed to be a likely candidate mediating resistance to PI3K/mTOR inhibition because of its downstream position in translation control relative to the PI3K-mTOR axis (31). Interestingly, it also has a well-documented role as a MYC-regulated gene (32, 33). Genomic QPCR analysis of *HMECres* cells confirmed *eIF4E* amplification in these cells, with an indicated four-copy gain of this gene (threefold increase) (Fig. 6*B*). Concordantly, real-time mRNA quantification revealed a threefold increase in eIF4E mRNA levels in *HMECres* relative to HMEC control, hence paralleling the copy number increase (Fig. 6*C*). Similarly, quantitative Western blot analysis also confirmed these findings, showing a 3- to 4.5-fold increase in eIF4E protein levels in the *HMECres* cells relative to control (Fig. 6*D*).

To determine whether *eIF4E* amplification could be a causal event mediating BEZ235 resistance in *HMECres* cells, we overexpressed eIF4E in the parental HMECs by retroviral transduction (Fig. 6*E*). We were able to obtain only a twofold increase in eIF4E protein levels by ectopic expression, an amount lower than yielded by amplification in *HMECres* (3- to 4.5-fold) (Fig. 6*D*). Nevertheless, we proceeded to analyze whether eIF4E overexpression could yield resistance to BEZ235 by measuring growth in the presence of the drug. Indeed, a twofold increase in protein conferred resistance to BEZ235, although notably less pronounced compared with *HMECres* cells that bear higher eIF4E protein levels (Fig. 6*F*). Furthermore, eIF4E overexpression also improves growth in the presence of the PI3K inhibitor GDC0941 and mTOR inhibitor Ku-0063794 (Fig. S4*A*). To underscore the capacity of eIF4E and MYC to drive resistance to PI3K/mTOR inhibitors, we also increased the dosage of these inhibitors and could determine maintained ability to evade drug inhibition of growth (Fig. S4*B*). As expected, increased BEZ235 dosage resulted in potentiated suppression of downstream pathway signaling measured as phosphorylation of AKT and p70S6K (Fig. S4*C*).

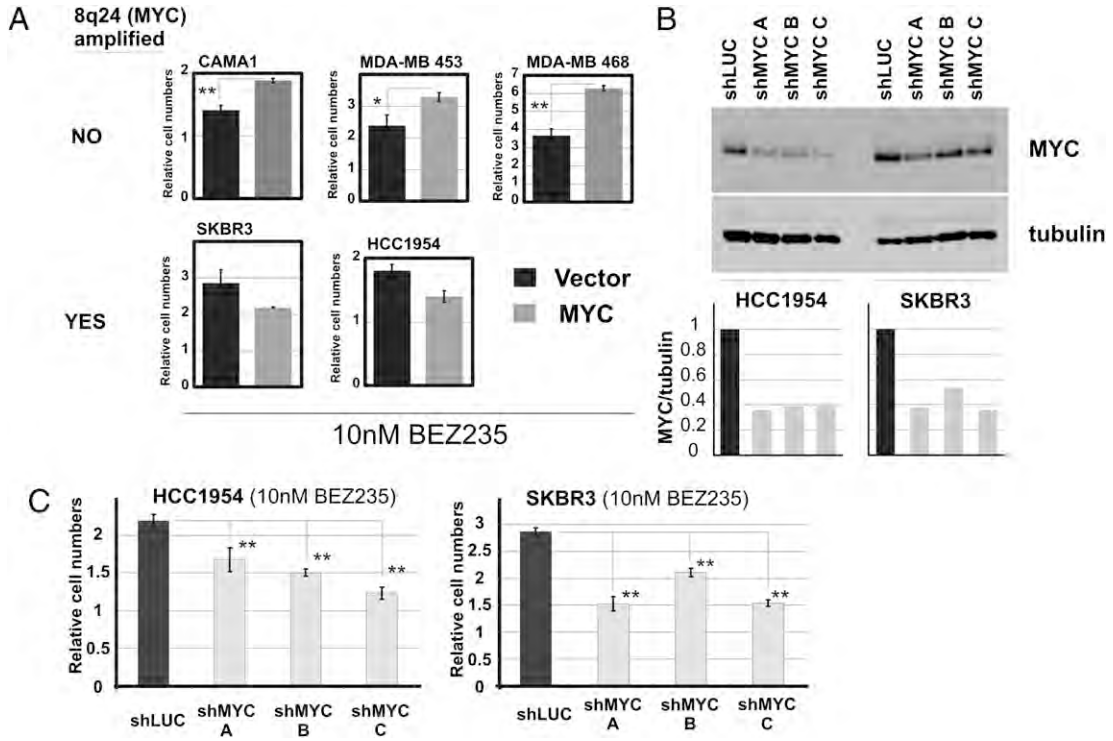

Fig. 5. MYC confers resistance to BEZ235 in breast cancer cell lines without *MYC* amplification. (*A*) Expression of vector control vs. MYC in breast cancer cell lines without (CAMA1, MDA-MB-453, and MDA-MB-468) or with (SKBR3 and HCC1954) 8q24 genomic amplification grown in 10 nM BEZ235. (*B*) Knockdown of MYC in HCC1954 and SKBR3. Western blot assay showing protein levels and quantification of MYC protein levels normalized to tubulin. (*C*) Growth in the presence of 10 nM BEZ235 of the cell lines in *B* (*$P < 0.02$, **$P < 0.005$; Student two-tailed *t* test for paired samples). (Data are represented as mean ± SEM.)

In addition, using a loss of function approach, we investigated if eIF4E is necessary for BEZ235 resistance in *HMECres* cells. Two short-hairpin eIF4E-targeting constructs (1426 and 1774) resulted in significant reduction of eIF4E protein levels relative to control (shLUC) (Fig. 6*G*). Although neither of the shRNA constructs affected normal cellular growth in the absence of the drug, the two that conferred significant protein-level reduction also revealed an increased sensitivity to BEZ235 relative to control (Fig. 6*H*). Collectively, these findings suggest that *eIF4E* amplification, resulting in increased eIF4E protein levels, may be an important factor mediating resistance to BEZ235.

We also investigated whether eIF4E expression is required for MYC-mediated BEZ235 resistance, because it has been suggested to be a downstream target of MYC function (32, 33). Using the two verified shRNA constructs against eIF4E (Fig. 6*G*), we could show reduced eIF4E protein levels in MYC-transduced cells (Fig. 6*I*). When grown in the presence of BEZ235, MYC-expressing cells with reduced eIF4E expression displayed increased sensitivity to BEZ235 relative to the shLUC control (Fig. 6*J*). Notably, this finding suggests that eIF4E expression is a required downstream factor enabling MYC-mediated resistance to PI3K/mTOR inhibition. Furthermore, we examined whether expression of canonical PI3K-mTOR pathway components was affected in drug-resistant cell lines (Fig. S4*D*). We could not detect any significant variations in the expressed amounts of these proteins, indicating that the effects that we document are likely driven by downstream lesions.

MYC- and eIF4E-Driven Resistance Is Indicative of Up-Regulated Cap-Dependent Translation. Because eIF4E is a known canonical effector of the PI3K/mTOR pathway and a critical translation initiation factor that regulates 5′ cap-dependent translation (32–34), we postulated that both eIF4E and MYC may up-regulate cap-dependent translation, which would otherwise be down-regulated by the dual inhibition of PI3K and mTOR. To test this hypothesis, we investigated if MYC affects eIF4E expression in our system. As shown in Fig. 7*A*, overexpression of MYC increases cellular levels of eIF4E. In addition, when overexpressed, MYC up-regulates translation initiation factor eIF2A RNA levels (34, 35), which is also observed in the *Enriched* cells (Fig. 7*A*). Given the increased expression of translation initiation factors in the BEZ235-resistant cells, we examined whether cap-dependent translation may be generally altered. Using a bicistronic luciferase reporter construct pRL-HCV-FL (36), we assessed the relative ratios of cap-dependent vs. -independent [internal ribosome entry site (IRES)-dependent] translation in the BEZ235-resistant cells. Both in the absence and presence of BEZ235, our derived resistant cell lines, as well as the MYC- and eIF4E-overexpressing cells, exhibit elevated levels of cap-dependent translation relative to control cells (Fig. 7*B*). Consequently, this finding suggests that both MYC- and eIF4E-mediated BEZ235 resistance display common effects leading to an increase in 5′ cap-dependent translation, a convergence point with the PI3K-mTOR pathway.

Discussion

Because many tumor types exhibit survival and/or growth dependence on a particular mutationally activated gene, commonly a kinase, through a process termed oncogene addiction, the principle of targeted kinase inhibition has provided clinical success in treating diverse cancer types (37–41). However, a major concern hampering the sustained clinical benefits of targeted therapies is the observed emergence of acquired drug resistance. As a result of inhibitor specificity as well as the inherent capacity of cancer cells to select for growth-promoting genetic variants, several resistance mechanisms have been documented involving either secondary mutations in the drug target gene (12, 13, 21) or alternatively, activation of parallel-acting or downstream pathway components (14). Here, we report that genomic amplification of either of two proto-

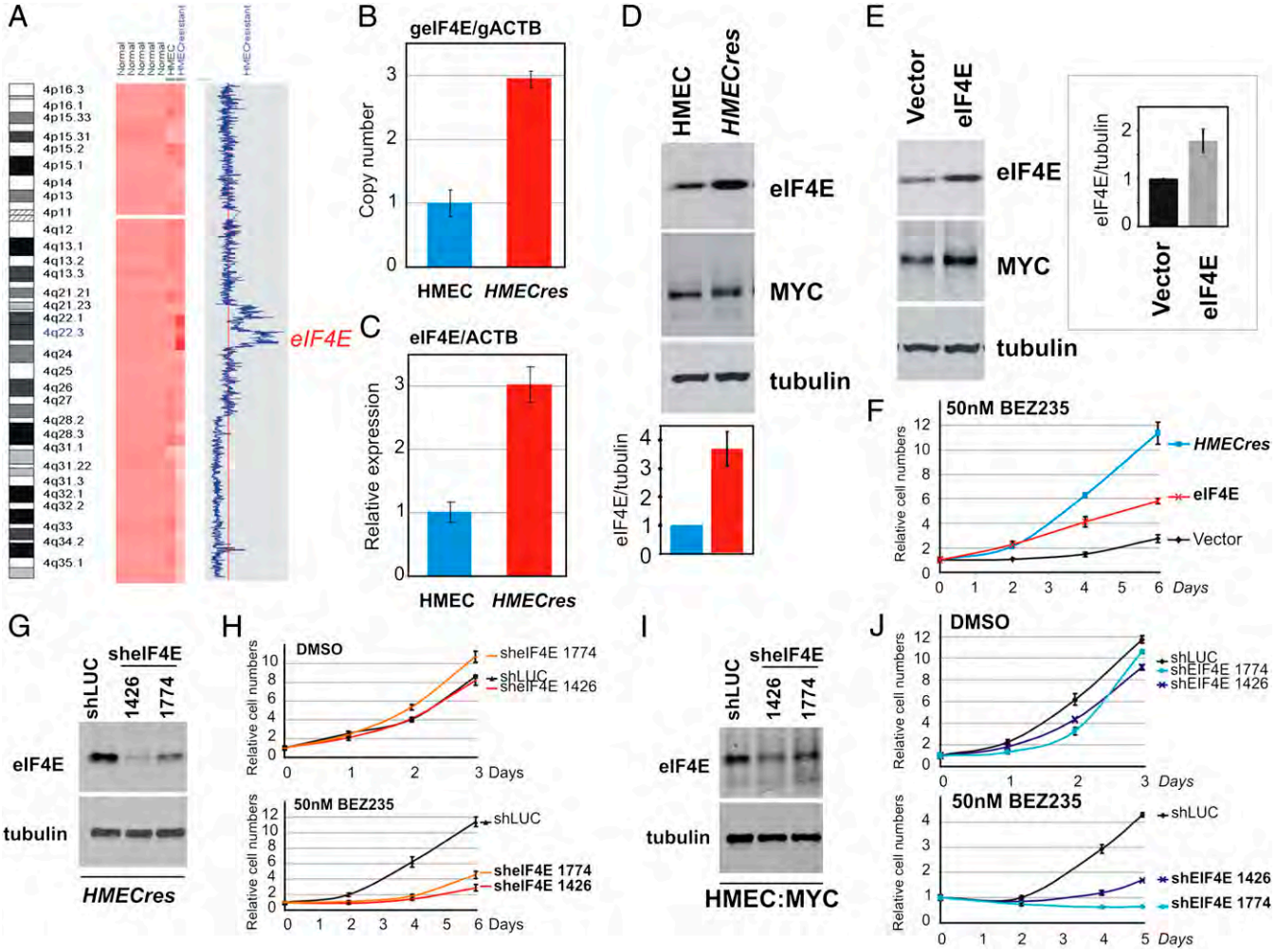

Fig. 6. *eIF4E* is amplified and overexpressed in *HMECres* and confers resistance to BEZ235. (*A*) SNP array analysis of *HMECres* vs. parental HMECs indicating a region of amplification at chromosome 4 present in the BEZ235-resistant *HMECres*. (*B*) Genomic QPCR confirming *eIF4E* amplification in *HMECres*. (*C*) QPCR analysis comparing eIF4E mRNA expression levels normalized to ACTB in *HMECres* with parental HMEC. (*D*) Western blot assay confirming eIF4E overexpression in *HMECres*. (*E*) Western blot analysis of HMECs transduced with vector control or eIF4E. (*F*) Growth assay in the presence of 50 nM BEZ235 comparing *HMECres* with HMECs transduced with vector control or eIF4E. (*G*) Western analysis of HMECs transduced with shLUC (control) or two sheIF4E constructs (1426 and 1774). (*H*) Growth curves of the cell lines in *G* in the absence or presence of 50 nM BEZ235. (*I*) Western analysis of HMECs overexpressing MYC transduced with shLUC (control) or two sheIF4E constructs (1426 and 1774). (*J*) Growth curves of the cell lines in *I* in the absence or presence of 50 nM BEZ235. (Data are represented as mean ± SEM.)

oncogenes, *MYC* and *eIF4E*, is able to provide resistance to PI3K/mTOR pathway inhibitors, apparently bypassing the inhibitors by acting downstream of the pharmacologically inhibited targets.

Notably, our initial expectation was that mutations in the oncogenic PI3K, possibly in the kinase domain (inhibitor binding), would yield resistance to catalytic PI3K inhibition. It is possible that we could not achieve this mode of resistance with our approach, because the inhibitor that we chose for analysis (BEZ235) has since been found to exhibit dual specificity to both PI3K and mTOR. Although we biased our selection process to second site mutations within the *PIK3CA* gene, the emerging variant alleles in our *Enriched* population did not recapitulate the resistance to BEZ235 (Fig. S1). One intriguing possibility is that MYC seems to suppress the expression of ectopic PIK3CA alleles in our system (Fig. 3*B*), which may have resulted in selection of rare cells with preexisting *MYC* amplification during growth in the presence of the drug. This result would also consolidate the presence of two different acting variant alleles identified, G320E;H1047R and F486S:del;H1047R, exhibiting oncogenic and null lipid kinase activity, respectively (Fig. S1*F*).

After we eliminated the possibility that the selected PIK3CA alleles were contributing to the observed resistance (Fig. S1 *G* and *H*), we opted to search for genomic alterations in the host cells, which might be responsible for the observed acquired resistance. Our data firmly suggest that two independent selection processes have identified two oncogenes already closely tied to a common pathway. Using an integrated copy number and expression-based approach within the *Enriched* cells, we determined that MYC, a commonly deregulated breast cancer oncogene, was responsible for the acquired BEZ235 resistance that we had evolved (Fig. 3*D*). It was striking to us that the *Enriched* cells exhibit a gene expression signature like the one reported by Bild et al. (25) that was obtained by overexpression of MYC in HMECs (Fig. 2*H*). It was, therefore, not surprising that overexpression of MYC in immortal HMECs could confer BEZ235 resistance (Fig. 3*A*), indicating that MYC is not only necessary but also sufficient to drive this drug-resistant state. In addition, an independent selection of immortalized HMECs using BEZ235 resulted in the emergence of a second drug-resistant cell line, *HMECres*, containing a discrete region of genomic amplification on chromosome 4 (Fig. 6*A*). From among

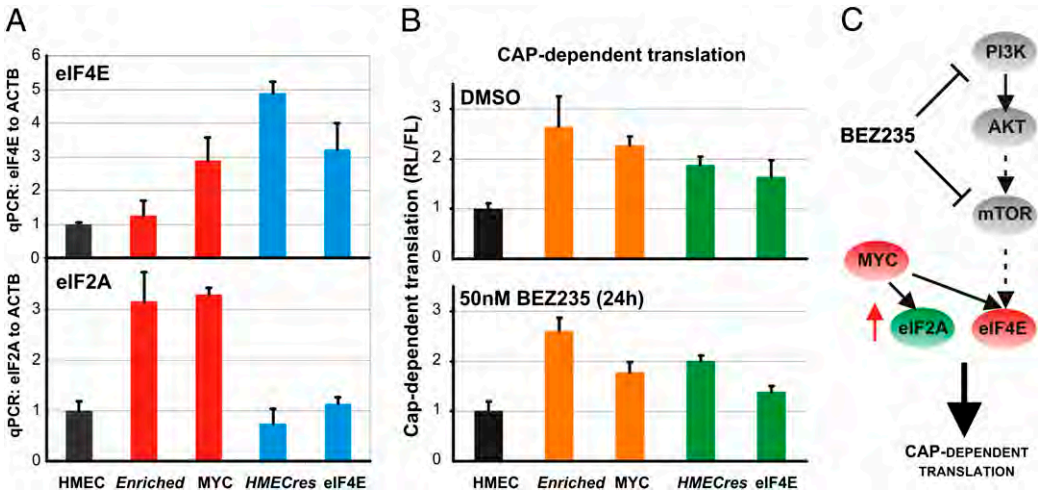

Fig. 7. MYC and eIF4E overexpression up-regulate cap-dependent translation. (*A*) QPCR analysis comparing eIF4E and eIF2A mRNA expression levels in parental HMECs or HMECs expressing MYC or eIF4E as well as the *Enriched* and *HMECres* cells. (*B*) Relative levels of cap-dependent translation. Cell lines from *A* transfected with the bicistronic luciferase reporter pRL-5′-IRES-FL are grown in the absence or presence of 50 nM BEZ235 and assayed 24 h posttransfection; ratios of RL to FL are represented and normalized to HMEC control, indicating changes in cap-dependent translation. (*C*) Schematic model of our results indicating that both MYC and eIF4E mediate resistance to BEZ235 through up-regulation of cap-dependent translation. (Data are represented as mean ± SEM.)

the genes in this region, we selected *eIF4E* as a likely BEZ235 resistance candidate gene, because eIF4E is already a known downstream effector of the PI3K-mTOR pathway (31). Moreover, eIF4E lies downstream of and is regulated by MYC (32–34). In addition, eIF4E expression is indeed elevated in MYC overexpressing HMECs (Fig. 7*A*), and this expression seems to be required for resistance to BEZ235 (Fig. 6*J*). Notably, MYC and eIF4E have been shown to collaborate to transform cells in vitro (42) and in vivo (43). However, we could not detect elevated expression of eIF4E in the *Enriched* cells, which may be because of lower MYC levels than in the ectopically expressing MYC cells (Fig. S4*C*).

It is intriguing how amplification of *MYC* and *eIF4E* might contribute to the observed PI3K/mTOR inhibitor resistance. By far, the simplest explanation of the MYC-eIF4E resistance axis is that, under the growth conditions used for selection, the critical element downstream of PI3K/mTOR signaling is initiation of 5′ cap-dependent translation (Fig. 7). Certainly, a great deal of literature shows the conserved role of MYC in driving cell growth and the requirement for eIF4E in controlling growth (44, 45). However, the PI3K pathway promotes the activity of a large number of proteins involved in driving key cellular processes. It is, therefore, somewhat surprising that acquired resistance to PI3K/mTOR pathway inhibitors provoked selection and elevation of the 5′ cap-dependent translation machinery. By allowing for cap-dependent translation to proceed despite upstream pathway blockage by BEZ235 (for example, by overexpressing eIF4E), mitogenic proteins with structure-rich 5′ UTRs like Cyclin D1 (46) and ornithine decarboxylase (47) can be produced. Forced expression of Cyclin D1 alone could not recapitulate resistance in our system (Fig. S2), arguing again for the global effects on the translation machinery that MYC and eIF4E exert downstream of the PI3K-mTOR pathway. If our selection had been done in a cell line that undergoes apoptosis in response to pharmacological PI3K inhibition, it is quite possible that the resistance genes identified would have been different, because it would not strictly involve growth promoting functions but rather, cell survival.

Because PI3K inhibitors have only recently entered phase I/II clinical trials, resistance to this class of targeted therapies has not yet been studied in clinical materials. However, it is of crucial importance to predict possible mechanisms of resistance to these inhibitors, especially if such mechanisms may preexist within the tumors, because this information will help guide clinicians in selecting patients who may benefit maximally from this class of therapy. In addition, identification of rare drug-resistant clones from a pretreatment specimen may help in targeting resistance before it is selected for by combining PI3K inhibition with inhibition of the lesion that leads to resistance. Since both MYC and eIF4E amplification and overexpression are found in human tumor cells (24, 48) and may coexist with mutations along the PI3K pathway, it may be advantageous to assess MYC and eIF4E levels before considering PI3K inhibitors as a therapeutic strategy and also, monitor changes during therapy, as our data implicate these changes as drivers of resistance. Furthermore, considering that both of these lesions seem to converge on up-regulation of cap-dependent translation, it is plausible that alternate genetic events that positively regulate the same functional cascade may also mediate resistance to PI3K/mTOR inhibitors.

Methods

Mammalian Cell Culture. Immortal HMECs expressing hTERT and p53DD (previously described in refs. 18 and 49) were cultured in DMEM/F-12 (1:1) with 0.5% FBS, hEGF (10 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), and cholera toxin (1 μg/mL). PIK3CA(H1047R) transductions were performed as described earlier (50). HMECs were treated with the drugs NVP-BEZ235 (Novartis), GDC0941 (Selleck Chemicals), Rapamycin (Cell Signaling), Ku-0063794 (Chemdea), Paclitaxel (Sigma), and Doxorubicin (Sigma) at the indicated doses and indicated time intervals. At various time points, cells grown on tissue culture dishes in the presence of drugs were fixed with 10% ethanol/10% acetic acid, subsequently stained with 0.4% crystal violet in 20% ethanol, and washed; after drying, the dye was extracted with 10% acetic acid. The absorbance, corresponding to cell numbers attached on the dishes, was measured at 595 nm on a multiwell plate reader (Bio-Rad).

Expression and shRNA Vectors. The pWZL-blast-FLAG-MYC retroviral construct was generated by cloning an FLAG-MYC cDNA from pCMV5-FLAG-MYC (Michael Cole, Dartmouth University, Hanover, NH) into the pWZL-blast backbone by cohesive EcoRI digests. The pBABE-puro-eIF4E retroviral construct was generated by excising an eIF4E cDNA from the pHA-eIF4E construct (Addgene plasmid 17343 from Dong-Er Zhang, University of California at San Diego, La Jolla, CA), blunting the ends with Klenow polymerase, and ligating into the pBABE-puro backbone cut with SnaBI. The retroviral construct pBABE-puro-HA-PIK3CA(H1047R) has been previously described (18). The shRNA constructs targeting MYC were obtained from the Broad Institute RNAi Consortium as pLKO.puro-shMYC vectors. Three reproducible and efficient shMYC target sequences (shMYC A: CAGTTGAAACACAAACTTGAA, shMYC B: CAGGAACTATGACCTCGACTA, and shMYC C: GAACTATGACCTCGACTACGA) were se-

lected, and the pLKO.puro-shMYC constructs were modified by replacing puromycin with blasticidin. The shRNA lentiviral constructs targeting eIF4E were purchased from Sigma-Aldrich, and two constructs were validated for efficient and reproducible knockdown (sheIF4E 1426, clone ID: NM_001968.2–1426s1c1; sheIF4E 1774, clone ID: NM_001968.2–1774s1c1).

Random Mutagenesis and Generation of BEZ235-Resistant Cells. XL1-RED bacteria (Stratagene), which induce random mutations into DNA, were transformed with the pBABE puro-HA-PIK3CA(H1047R) retroviral construct. Plasmid DNA was purified, and the resulting library of mutated PIK3CA constructs was retrovirally transduced into HMECs. HMECs expressing the mutagenized PIK3CA library were exposed to increasing concentrations of BEZ235. BEZ235 concentrations were increased stepwise from 10 to 250 nM for 2 mo total, at which point the cells acquired growth rates similar to the untreated control cells. The cells were allowed to grow in the absence of the drug for 2 wk after the drug selection before being tested for resistance. To confirm the phenotype of resistant cells (*Enriched* cells), crystal violet growth assays were performed at different drug concentrations. The *Enriched* cells were passaged in the absence of drug for 6 mo and still maintained BEZ235 resistance, which was confirmed by crystal violet growth assays.

Sequencing. Retrovirally integrated mutagenized ectopic PIK3CA cDNA was PCR-amplified from the naive cells (before BEZ235 selection) and the *Enriched* BEZ235-resistant cells. The primers used for PCR amplification spanned the vector backbone–cDNA boundary and were FWD-TCCAAGCTTCACCATGG-GGTACCCAT and REV-ATGCATTGAACTGAGTCGACCAGCTGTGG. Each PCR product was processed to generate a fragment library according the manufacturer's protocol (Applied Biosystems). The library was then sequenced on an SOLiD version 3 Plus instrument to generate sequences 50 bases long from one end of each fragment. Resulting sequence data were aligned with the PIK3CA cDNA sequence using Corona Lite software (Applied Biosystems). In addition, PCR fragments were cloned by pCR2.1-TOPO-TA (Invitrogen), and clones with inserts of expected size were analyzed by standard automated Sanger sequencing.

Cell Cycle Analysis. Cells were harvested by trypsin digestion at 48 h postdrug addition and fixed/permeabilized in 35% ethanol; after RNaseH treatment and propidium iodide staining, the samples were subsequently analyzed on a BD FACS Canto flow cytometer for DNA content. Experiments were conducted in triplicates.

Western Assays. Immunoblotting was performed using standard procedures. The commercial antibodies used were HA (clone 6E2), p110α, pAKT(S473), pAKT (T308), p-p70S6K (T389), pS6(S234/236), c-MYC (clone D84C12), AKT1, and pMAPK(Y42/44) from Cell Signaling and α-tubulin (clone DM1A) from Sigma.

Lipid Kinase Assay. Lipid kinase assays were performed as previously described (51). In brief, PI3K was purified from cells by nondenaturing immunoprecipitation and preincubated at room temperature with a lipid mix containing sonicated lipids phosphatidylserine (2 μg/μL; Avanti) and phosphatidylinositol (4 μg/μL; Avanti). Subsequently, ATP mix was added to the reaction [10 μCi 32P-γ-ATP, ATP (0.5 mM), $MgCl_2$ (100 mM), 67 mM Hepes, pH 7.4]. After 10 min, reactions were stopped with 4 N HCl. After extraction, lipids from the chloroform (organic) layer were spotted onto the TLC plate and separated overnight by TLC in *n*-propanol:2 M acetic acid (65:35). Results were visualized using a Typhoon phosphoimager, and image acquisition and analysis were performed with the accompanying software.

Microarray Analyses. Genomic DNA (DNeasy; Qiagen) and total RNA (RNeasy Plus; Qiagen) were extracted from logarithmically growing cultures of the indicated genotypes of HMECs. Genomic DNA was processed for interrogation on StyI 250k SNP arrays (Affymetrix) according to the manufacturer's recommendations. SNP-derived copy number analysis was performed using dChip (52) using normal controls set to diploid for standardization of calls and signal intensities. Qualifying genomic alterations were determined as regions $\underline{X}$ (containing more than 4 SNPs) outside of $0.6 > \underline{X}$ or $\underline{X} > 3.5$. Expression array analysis was conducted using Affymetrix HG-U133AV2.0 arrays (in triplicate) using RNA extracted from samples grown in the presence of BEZ235 for 72 h before harvesting. Resulting Affymetrix Cell Intensity (.CEL) files were normalized in intensity, and signal/calls were computed using dChip. Expression analysis was conducted on filtered variant probe sets (genes) that satisfy variation among samples: $0.5 < SD$, mean $<$ 1,000, and present calls in 33% of samples. Clustering was done by calculating average Euclidean distance as a metric. The data have been deposited to the Gene Expression Omnibus under accession number GSE25173.

QPCR. Expression levels of eIF4E and eIF2A mRNA were determined by QPCR using the amplicons eIF4E (FWD: CAATCCGGTTTGAATCTCAT and REV: AGTCCACTCTGCTTTTTGAAGA) and eIF2A (FWD: CTGGACCTCATGCAGCTT-TAGC and REV: CTCCATAGTAGGAAGCTCCTGTC) and normalized to ACTB (FWD: ATTGCCGACAGGATGCAGAA and REV: GCTGATCCACATCTGCTGGAA) using SYBR chemistry on an ABI StepONE plus instrument (94 °C, 2 min; 40 cycles: 94 °C, 20 s; 51 °C, 20 s).

Genomic QPCR. *MYC* amplification levels were confirmed by real-time genomic PCR using the primers qgMYC_FWD: CACCAGGCTTAGATGTGGCTCTTT and qgMYC_REV: CTTCCTCATCTTCTTGTTCCTCCTC and qgeIF4E_FWD: CTA-GGAAACCACCCCTACTCCTA and qgeIF4E_REV: TCCAAGTGAAAATAGAACC-CTCA compared with qgACTB_FWD: CTCCATCATGAAGTGTGACGTGGA and qgACTB_REV: CAGGAAAGACACCCACCTTGATCT in the sample measured relative to control normal DNA set to diploid (gene copy number ≡ 2) using an Applied Biosystems 7300 with SYBR green chemistry and ROX as a passive reference.

Luciferase Reporter Assays. HMEC cells were transiently transfected with a bicistronic luciferase reporter plasmid, pRL-5′-IRES-FL (36), using Polyfect (Qiagen). The reporter plasmid directs cap-dependent translation of the *Renilla* luciferase and hepatitis C virus (HCV) IRES-dependent translation of the firefly luciferase gene. Twenty-four hours posttransfection, dual luminescence was measured according to the manufacturer's instructions (Promega) using a Veritas Microplate Luminometer. Assays were performed in quadruplicate from lysed cells grown in the presence of 50 nM BEZ235 or vehicle (DMSO), and results were expressed as average ± SEM and normalized to controls.

FISH. Growing cultures of cells were treated with colcemid, harvested, and treated with hypotonic buffer before fixation onto slides. FISH analysis was performed using the CEP8 reference probe (green fluorescence) and the human c-Myc probe (orange fluorescence) to assess the c-Myc copy numbers. Fluorescent probes were generated by standard nick translation DNA labeling and hybridized to interphase cells followed by fluorescent microscopy analysis and statistical counting; 100 nuclei were scored for each cell line.

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Supporting Information

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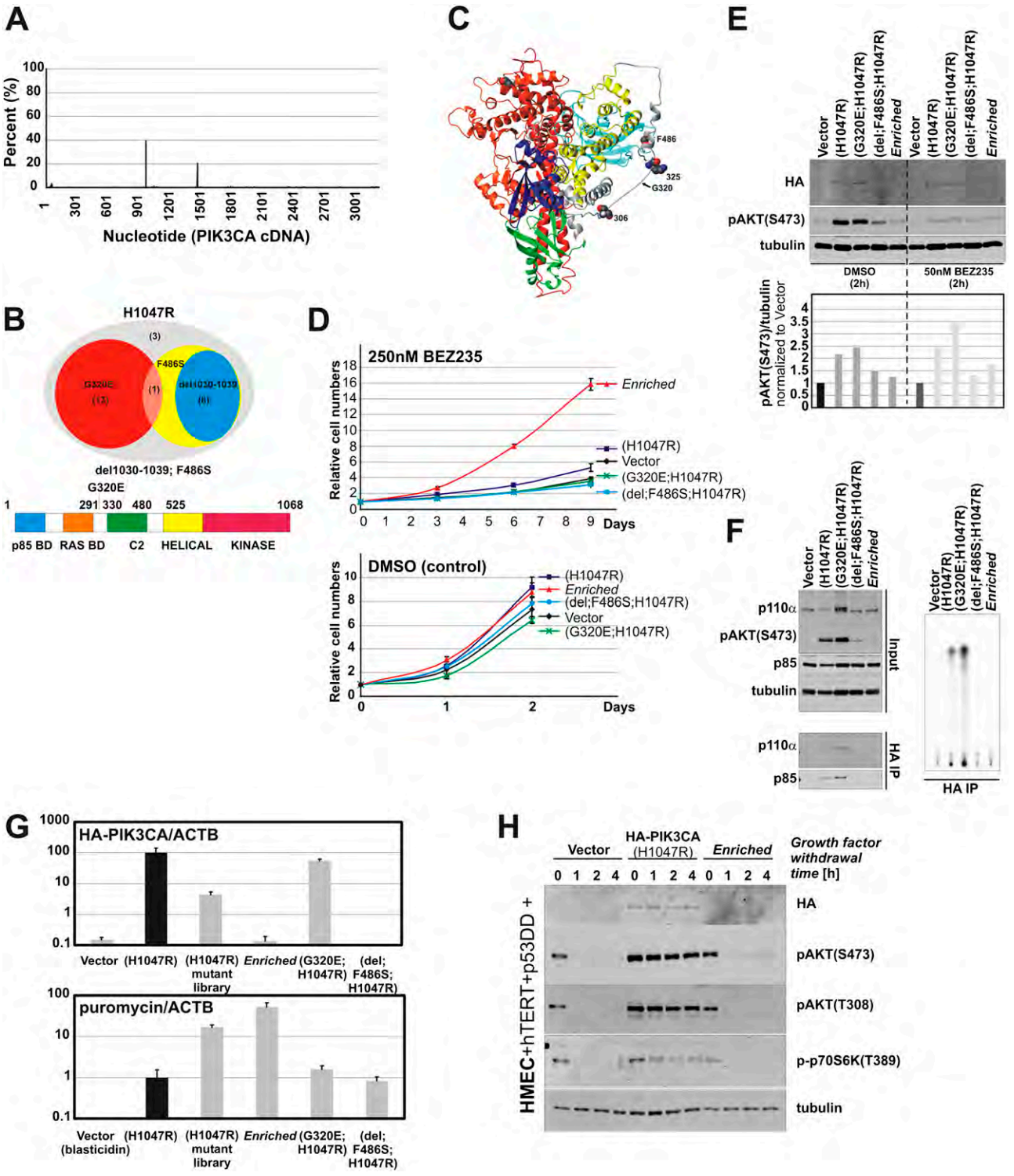

Fig. S1. Mutational analysis of BEZ235-resistant cells (related to Fig. 2). (*A*) SOLiD deep sequencing analysis of the exogenous PIK3CA(H1047R) alleles expressed in the BEZ235-resistant *Enriched* cells indicating the presence of two point mutations: G320E and F486S substitutions. (*B*) Results of manual sequencing of TOPO (Invitrogen) subclones of 28 individual exogenous PIK3CA(H1047R) alleles from the *Enriched* cells indicating preponderance of two alleles—G320E and

Legend continued on following page

del;F486S *in trans*. (*C*) Published crystal structure of p110α (1) with two positions of our two identified mutations positioned indicated. (*D*) Growth curves of human mammary epithelial cells (HMECs) expressing vector control, PIK3CA alleles—H1047R, G320E;H1047R, del;F486S;H1047R, or the *Enriched* cells in the presence of DMSO (vehicle) or 250 nM BEZ235. (*E*) Western blot analysis of the cell lines in *D* in the absence or presence of 50 nM BEZ235. (*F*) HA-tag immunoprecipitation (IP) and Western blot analysis of cell lines in *D*; matching IP samples were used for lipid kinase assays to measure the exogenous alleles' lipid kinase activity. (*G*) Real-time quantitative PCR for ectopic PIK3CA (HA-PIK3CA) and puromycin expression within the indicated genotypes of HMECs—vector (control), PIK3CA(H1047R), PIK3CA(H1047R) mutant library (preselection), *Enriched*, PIK3CA(G320E;H1047R), or PIK3CA(del;F486S;H1047R) normalized to beta-actin (ACTB). (*H*) Western blot analysis comparing HMECs expressing vector control, PIK3CA(H1047R), and *Enriched* cells after withdrawal from growth factors from the media for the indicated time periods. (Data are represented as mean ± SEM.)

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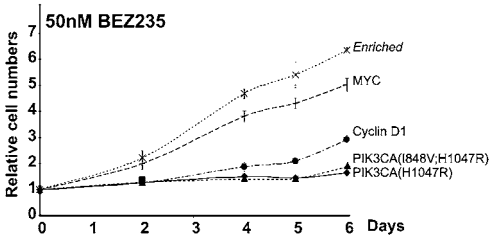

Fig. S2. Neither Cyclin D1 nor PIK3CA gatekeeper mutant I848V confers resistance to BEZ235 (related to Fig. 3). Growth curve in 50 nM BEZ235 of HMECs expressing PIK3CA alleles (H1047R or I848V;H1047R), Cyclin D1, MYC, and *Enriched* cells. (Data are represented as mean ± SEM.)

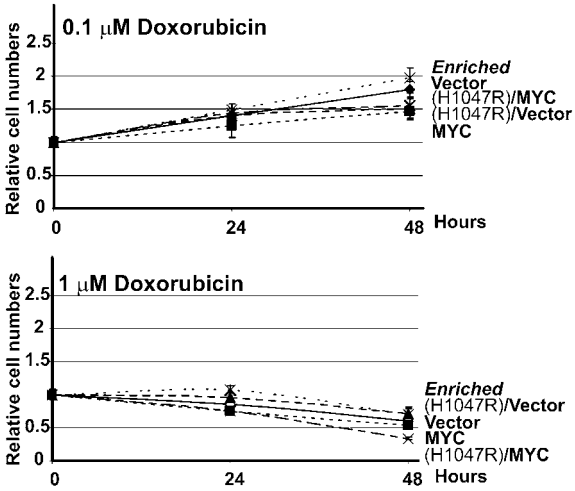

Fig. S3. MYC does not confer resistance to Doxorubicin (related to Fig. 4). Growth curves of HMECs expressing empty vector, PIK3CA(H1047R), MYC, PIK3CA (H1047R)+MYC, or the *Enriched* cells grown in 0.1 or 1 μM Doxorubicin. (Data are represented as mean ± SEM.)

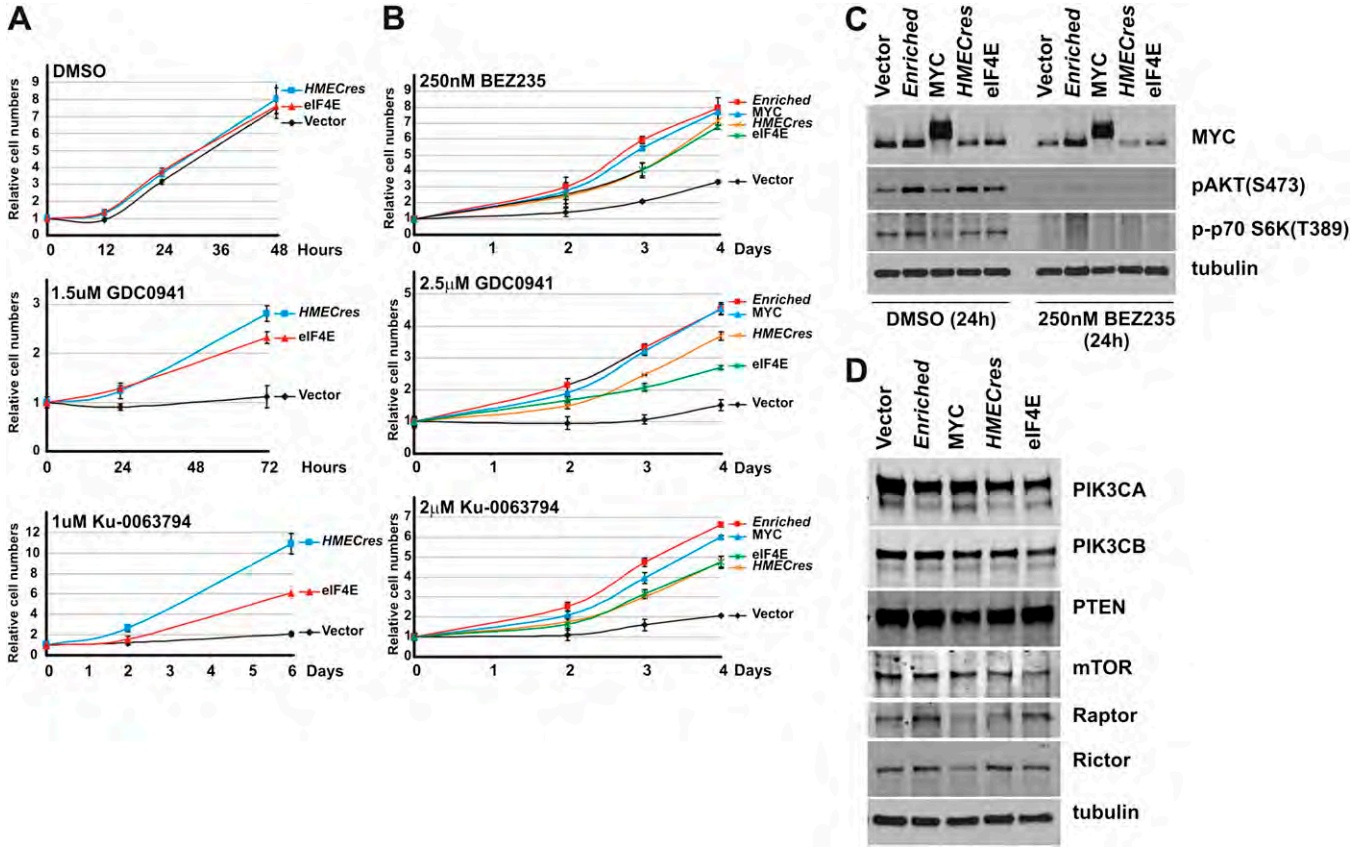

Fig. S4. eIF4E overexpression confers resistance to PI3K-mTOR inhibition (related to Fig. 6). (*A*) Growth assays in the presence of DMSO (control), 1.5 μM GDC0941, and 1 μM Ku-0063794 of parental HMECs transduced with vector (control), eIF4E, or *HMECres*. (*B*) Growth assays in the presence of 250 nM BEZ235, 2.5 μM GDC0941, or 2 μM Ku-0063794 of parental HMECs transduced with vector (control), *Enriched* cells, MYC, *HMECres*, or eIF4E. (*C*) Western assay of the cell lines in *B* in the absence or presence of 250 nM BEZ235. (*D*) Western assay of parental HMECs transduced with vector (control), *Enriched* cells, MYC, *HMECres*, or eIF4E.

Table S1. Genes contained within the chromosome 8 amplicon in the *Enriched* BEZ235-resistant cells (related to Fig. 2)

Abbreviation	Gene
RSPO2	R-spondin 2 homolog (*Xenopus laevis*)
EIF3E	Eukaryotic translation initiation factor 3, subunit E
TTC35	Tetratricopeptide repeat domain 35
TMEM74	Transmembrane protein 74
TRHR	TSH-releasing hormone receptor
NUDCD1	NudC domain containing 1
EBAG9	Estrogen receptor binding site-associated antigen 9
KCNV1	Potassium channel, subfamily V, member 1
CSMD3	CUB and Sushi multiple domains 3
TRPS1	Trichorhinophalangeal syndrome
EIF3H	Eukaryotic translation initiation factor 3, subunit H
RAD21	RAD21 homolog (*Schizosaccharomyces pombe*)
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
MED30	Mediator complex subunit 30
EXT1	Exostoses (multiple) 1
SAMD12	Sterile α-motif domain containing 12
TNFRSF11B	TNF receptor superfamily, member 11b
MAL2	Mal, T-cell differentiation protein 2
NOV	Nephroblastoma overexpressed gene
TAF2	RNA polymerase II, TATA box binding protein (TBP) -associated factor, 150 kDa
COL14A1	Collagen, type XIV, α1
MRPL13	Mitochondrial ribosomal protein L13
HAS2	Hyaluronan synthase 2
ZHX2	Zinc fingers and homeoboxes 2
DERL1	Der1-like domain family, member 1
FAM83A	Family with sequence similarity 83, member A
WDYHV1	WDYHV motif containing 1
ANXA13	Annexin A13
C8orf78	Chromosome 8 ORF 78
TMEM65	Transmembrane protein 65
RNF139	Ring finger protein 139
ZNF572	Zinc finger protein 572
TRIB1	Tribbles homolog 1 (*Drosophila*)
FAM84B	Family with sequence similarity 84, member B
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
CCDC26	Coiled-coil domain containing 26
GSDMC	Gasdermin C
ASAP1	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1
ADCY8	Adenylate cyclase 8 (brain)

Table S2. Genes contained within the chromosome 4 amplicon in the *HMECres* BEZ235-resistant cells (related to Fig. 6)

Abbreviation	Gene
BMPR1B	Bone morphogenetic protein receptor, type Ib
UNC5C	UNC5, *Caenorhabditis elegans*, homolog of c
PDHA2	Pyruvate dehydrogenase, e1-α polypeptide, testis-specific form
C4orf37	Chromosome 4 ORF 37
RAP1GDS	RAP1, GTP-GDP dissociation stimulator 1
TSPAN5	Tetraspanin 5
EIF4E	Eukaryotic translation initation factor 4E
METAP1	Methionyl aminopeptidase 1
ADH5	Alcohol dehydrogenase 5 (class III), χ-polypeptide
ADH4	Alcohol dehydrogenase 4 (class II), π-polypeptide
ADH6	Alcohol dehydrogenase 6 (class V)
ADH1A	Alcohol dehydrogenase 1A (class I), α-polypeptide
ADH1B	Alcohol dehydrogenase 1B (class I), β-polypeptide
ADH7	Alcohol dehydrogenase 7 (class IV), μ- or σ-polypeptide
C4orf17	Chromosome 4 ORF 17
RG9MTD2	RNA (guanine-9-) methyltransferase domain containing 2
MTTP	Microsomal triglyceride transfer protein
DAPP1	Dual adaptor of phosphotyrosine and 3-phosphoinositides
LAMTOR3	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 3
DNAJB14	DnaJ (Hsp40) homolog, subfamily B, member 14
H2AFZ	H2A histone family, member Z
DDIT4L	DNA damage-inducible transcript 4-like
EMCN	Endomucin