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Multipathway Model Enables Prediction of Kinase Inhibitor Cross-Talk Effects on Migration of Her2-Overexpressing Mammary Epithelial Cells[S]

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ABSTRACT

Small-molecule kinase inhibitors often modulate signaling pathways other than the one targeted, whether by direct "off-target" effects or by indirect "pathway cross-talk" effects. The presence of either or both of these classes of complicating factors impedes the predictive understanding of kinase inhibitor consequences for cell phenotypic behaviors involved in drug efficacy responses. To address this problem, we offer an avenue toward comprehending how kinase inhibitor modulations of cell signaling networks lead to altered cell phenotypic responses by applying a quantitative, multipathway computational modeling approach. We show that integrating measurements of signals across three key kinase pathways involved in regulating migration of human mammary epithelial cells, downstream of ErbB system receptor activation by epidermal growth factor (EGF) or heregulin (HRG), significantly improves prediction of cell migration changes resulting from treatment with the small-molecule inhibitors 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) and 2′-amino-3′-methoxyflavone (PD98059) for both normal and HER2-overexpressing cells. These inhibitors are primarily directed toward inhibition of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase kinase (MEK) but are known to exhibit off-target effects; moreover, complex cross-talk interactions between the PI3K/Akt and MEK/extracellular signal-regulated kinase (Erk) pathways are also appreciated. We observe here that treatment with LY294002 reduces migration of HRG-stimulated cells but not EGF-stimulated cells, despite comparable levels of reduction of Akt phosphorylation under both conditions, demonstrating that the target inhibition effect is not unilaterally predictive of efficacy against cell phenotypic response. Consequent measurement of levels of Erk and p38 phosphorylation, along with those for EGF receptor phosphorylation, after LY294002 treatment revealed unintended modulation of these nontargeted pathways. However, when these measurements were incorporated into a partial least-squares regression model, the cell migration responses to treatment were successfully predicted. Similar success was found for the same multipathway model in analogously predicting PD98059 treatment effects on cell migration. We conclude that a quantitative, multipathway modeling approach can provide a significant advance toward comprehending kinase inhibitor efficacy in the face of off-target and pathway cross-talk effects.

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Effects of small-molecule drugs such as kinase inhibitors on pathways other than those specifically targeted may detract from or contribute to efficacy (by which we mean the ability to alter a given cell behavioral phenotype in desired fashion), underscoring the contribution of multiple kinase pathways toward the control of cell functions (Kung and Shokat, 2005; Sevecka and Macbeath, 2006; Lazo et al., 2007). These unintended effects can arise from direct "off-target" interactions of the drug with additional proteins (Davies et al., 2000; Fabian et al., 2005) as well as by indirect "cross-talk" between the targeted pathway and other pathways in the signaling network regulating the cell behavioral response of interest (Natarajan et al., 2006). Although substantial progress is being made in improving kinase inhibitor specificities to reduce off-target effects, pathway cross-talk effects will—because of the fundamental interconnectedness of signaling networks—almost always be significant. An im-

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ABBREVIATIONS: LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PI3K, phosphatidylinositol 3-kinase; PD98059, 2′-amino-3′-methoxyflavone; MEK, mitogen-activated protein kinase kinase; EGF, epidermal growth factor; HRG, heregulin; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; hMEC, human mammary epithelial cell; Akt, protein kinase B; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PLSR, partial least-squares regression.

portant implication is that the effects of kinase inhibitors on downstream cell phenotypic behaviors are not easily predictable based on the degree of their direct target activity reduction. Accordingly, predictive understanding of drug efficacy requires a more holistic, multipathway perspective on consequent network perturbation and its ensuing influence on cell behavioral responses. Moreover, the notion of multitarget drugs, such as sorafenib and sunitinib (Hiles and Kolesar, 2008; Zhu, 2008), is arising, in which multiple pathways will be concomitantly modulated by design; this exciting approach will obviously require a network-wide view.

In recent publications, we have demonstrated that computational models employing a partial least-squares regression framework can generate successful a priori predictions of various cell behaviors (including apoptosis and cytokine release) based on quantitative dynamic measurement of signals representing approximately 10 key signaling pathways (Janes et al., 2005; Kemp et al., 2007; Miller-Jensen et al., 2007). On this foundation, we test here whether unintended effects resulting from treatment of human mammary epithelial cells with one of two commonly studied compounds—LY294002 [a phosphatidylinositol 3-kinase (PI3K) inhibitor] and PD98059 [a mitogen-activated protein kinase kinase (MEK) inhibitor]—can be predicted in terms of an analogous multipathway computational model. In particular, we examine the effects of these compounds in an in vitro cell assay characterizing the role of overexpression of human epidermal growth factor receptor 2 (HER2) in migration of mammary epithelial cells.

Increased migration, and particularly increased directional persistence of migration, is a hallmark of invasive breast cancer cells (Friedl et al., 1995; Deisboeck et al., 2005). HER2, a member of the ErbB or HER family of receptors, is overexpressed in ~30% of breast cancers and correlates with increased metastasis (Moasser, 2007), and enhanced motility and invasiveness of HER2-overexpressing breast carcinoma cells has been demonstrated (Spencer et al., 2000). Our laboratory has recently shown that effects of HER2 overexpression on cell migration transpire via enhancement of directional persistence (Kumar et al., 2006b). Induction of signaling in HER2 systems occurs through homodimerization or via ligand-driven heterodimerization with other HER family receptors. Epidermal growth factor (EGF) and heregulin (HRG), two HER family ligands implicated in tumor progression, bind HER1 (also called EGFR) and HER3, respectively, to induce the activation of HER2 through heterodimerization (Harari and Yarden, 2000). EGFR and HER3 can also form homodimers as well as heterodimers with each other upon ligand binding, although HER3 homo-dimers are expected to be inactive (Engelman and Cantley, 2006).

In this study, we measured inhibitor efficacy against persistent migration in the context of serum-free, EGF, or HRG treatment for normal (parental line) and HER2-overexpressing (24H subline) human mammary epithelial cells (hMECs). To quantify pathway cross-talk effects of the inhibitors and gain a further understanding of signaling giving rise to directional persistence, we measured phosphorylation of central actors in three key kinase pathways downstream of ErbB receptor activation: Akt (serine 473), Erk (threonine 202/tyrosine 204), and p38 (threonine 180/tyrosine 182). We also measured EGFR phosphorylation (tyrosine 1173 and tyrosine 1068) as a partial indicator of initiating receptor tyrosine kinase activity induced by ligand stimulation (Harari and Yarden, 2000). These kinases have been shown to regulate cell migration in breast cancer epithelial cells (Ho et al., 2001; Arboleda et al., 2003; Huang et al., 2004; Shin et al., 2005; Hirsch et al., 2006), have been implicated in the control of directional migration (Merlot and Firtel, 2003; Sawyer et al., 2003), and have been found for many cell behavioral functions to be highly informative integrators of multiple signaling pathways (Oda et al., 2005). Figure 1 offers a schematic illustration of these pathways, showing at least some appreciated loci of cross-talk along with downstream effectors of migration behavior. LY294002 and PD98059 each target one of the measured kinase pathways, do not directly interact with any of the other pathway signals measured here, but do have other off-target binding activities (Davies et al., 2000; Fabian et al., 2005; Bain et al., 2007). However, the presence of cross-talk leading to network modulation across their respective pathways has been indicated (Zimmermann and Moelling, 1999; Chaudhary et al., 2000; Mograbi et al., 2001; Moelling et al., 2002; Hausenloy et al., 2004) and might be further exacerbated by off-target activities of these fairly nonselective inhibitors as well as by convoluting feedback loops within the pathways (Asthagiri and Lauffenburger, 2001; Birtwistle et al., 2007).

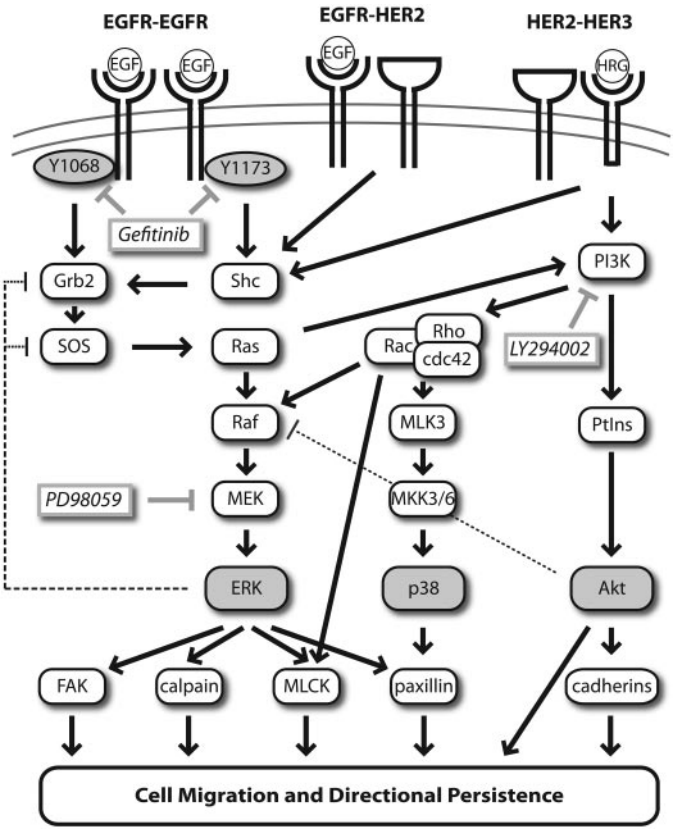

Fig. 1. Partial illustration of signaling network downstream of EGFR and HER2 leading to cell migration, featuring pathways studied herein. Upon binding of EGF to the EGF receptor, EGF receptors homo- and heterodimerize with HER2. HRG binds to HER3, which then dimerizes with HER2. Arrows depict potential direct and indirect influences and activations; negative feedback loops are indicated by dotted repressor lines. Gray boxes and circles indicate proteins and sites, respectively, the phosphorylation of which was measured. Focal adhesion kinase (FAK), calpain, myosin light chain kinase (MLCK), paxillin, and cadherins are representatives of molecules directly involved in ErbB-mediated cell migration.

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We found that no individual pathway signal is sufficient to predict drug efficacy on cell migration behavior from its target reduction—but we find that a partial least-squares regression model (Janes et al., 2005; Kemp et al., 2007; Miller-Jensen et al., 2007) that quantitatively integrates these three kinase pathway signals is successful in predicting the LY29004 and PD98059 effects despite their complex network modulations. We conclude that our multivariate computational modeling offers a novel and useful method for understanding how kinase inhibitors exhibiting direct and/or indirect effects on nontargeted signaling pathways influence downstream cell phenotypic behaviors.

Materials and Methods

Cell Culture and Stimulation. 184A1 human mammary epithelial parental line and HER2-overexpressing 24H subline cells were obtained and used as described previously, with defined serum-free DFCI-1 media (Kumar et al., 2006b; Wolf-Yadlin et al., 2006). Cells were stimulated with either 100 ng/ml EGF or 80 ng/ml HRG-β1 (Sigma, Inc., St. Louis, MO). For inhibitor experiments, cells were pretreated with either 20 μM LY294002 (Calbiochem, Inc., San Diego, CA) for 1 h, 25 μM PD98059 (Calbiochem, Inc.) for 1 h, 20 μM gefitinib (WuXi Pharmatech Co., Ltd., Shanghai, China) for 20 min, or 200 nM wortmannin (Calbiochem, Inc.) for 20 min.

Immunocytochemistry for Signal Profiling. Cells were seeded at ~50,000 cells/well in a 96-well plate (Nalge Nunc International, Rochester, NY) in full DFCI-1 media. After 4 to 6 h, full media were replaced with serum-free media. After 12 to 16 h of serum starvation, cells were preincubated with inhibitor or treated with EGF, HRG, or fresh serum-free media. A wound of width ~650 μm was then scraped in each well. Cells were stimulated for 0 (serum-free), 5, 15, 30, 60, and 90 min, with three or four wells used as biological replicates for each condition. After desired time had elapsed, cells were fixed, permeabilized, and stained as described in Supplemental Methods. The phospho-p44/42 MAPK (Erk, Thr202/Tyr204) and phospho-Akt (Ser473) rabbit monoclonal antibodies were purchased from Cell Signaling, Inc. (Danvers, MA) The phospho-p38 MAPK (pT180/pY182), phospho-EGFR (pY1068), and EGFR (pY1173) rabbit monoclonal antibodies were purchased from Epitomics, Inc (Burlingame, CA). Plates were imaged using an Odyssey instrument (Li-Cor, Lincoln, NE), and the integrated intensity was normalized to the loading control and then to parental serum-free intensity run on the same plate (see Supplemental Methods). All signaling data are plotted ± S.E.M., with $n = 3$ or 4.

Migration Assay. The migration assay was performed as described previously (Kumar et al., 2006b). In brief, cells labeled with 5-chloromethylfluorescein diacetate (Invitrogen, Carlsbad, CA) were diluted 1:20 with unlabeled cells and plated as described above, treated with ligand and inhibitor as described above, and wounded; cell movement was tracked for 12 h at 37°C and 5% CO_2 using an automated epifluorescent microscope. Individual cell trajectories in a monolayer were analyzed using Imaris (Bitplane, Inc., Zurich, Switzerland), and cell trajectories were then fit to the persistent random walk equation to derive cell persistence. All data were normalized by the persistence of 24H cells stimulated with 100 ng/ml EGF on the plate, to account for plate-to-plate variability. For each condition, $n > 300$ and all data are reported as ± S.E.M.

Multilinear Modeling Using Partial Least-Squares Regression (PLSR). We regressed the signaling matrix **X** onto the persistence matrix **P** using partial least-squares regression (PLSR) in SIMCA-P 11.0 (Umetrics) as described in Supplemental Methods and elsewhere (Kumar et al., 2006a; Wolf-Yadlin et al., 2006). **P** is a column vector (*Mx1*) of persistence values corresponding to *M* cellular conditions, and **X** is the signaling matrix (*MxN*) in which each column represents a signaling metric. Both the parental and 24H cell data were normalized to the parental serum-free condition data obtained from the same plate after averaging biological replicates. The quantitative metrics used in the model for each phosphorylation site corresponded to these normalized levels of phosphorylation at each time point as well as the integral of phosphorylation to characterize "net" phosphorylation over 90 min. Coefficients for each metric were extracted from SIMCA-P as previously (Kumar et al., 2006a). All data were mean-centered and scaled to unit variance before analysis.

Results

Inhibition with LY294002 Indicated a Significant Effect of Nontargeted Pathway Modulation. Akt and its upstream kinase PI3K have been identified as critical for directionally persistent cell locomotion (Merlot and Firtel, 2003; Sawyer et al., 2003). Thus, we set out to test whether this individual kinase signaling pathway might offer sufficient capacity for prediction of cell persistence. Our initial focus in this study was the quantitative relationship between Akt phosphorylation and HER2-mediated enhancement of mammary epithelial cell migration. As with our previous findings (Kumar et al., 2006b), HER2-overexpressing hMECs (24H cells) exhibited higher levels of directional persistence than those with low HER2 expression (parental cells) in the presence or absence of EGF or HRG (Figs. 2, A and B). Measurement of Akt phosphorylation under these same conditions revealed increased phosphorylation in response to HER2 overexpression, consistent with a putative role for Akt in persistent movement (Fig. 2C). Inhibition of Akt by treatment with LY294002 decreased persistence in 24H cells treated with HRG, further corroborating the role of the PI3K/Akt pathway in directional persistence (Figs. 2D,E). Inhibition with LY294002 in 24H cells treated with EGF, however, had no effect on directional persistence (Fig. 2F), despite the equivalent inhibition of Akt phosphorylation under both HRG and EGF treatments (Fig. 2D). Thus, the effect of LY294002 treatment on persistence could not be predicted solely by its effect on Akt phosphorylation, suggesting that assessing its effects on other pathways might be required to understand efficacy results.

EGF and HRG Stimulation Activated Multiple Kinase Pathways under Migration Assay Conditions. To assess cell signaling under the same conditions studied in the migration assay, we used a high-throughput immunocytochemistry assay to quantify phosphorylation in wounded monolayers of HMEC cells expressing either low (parental) or high (24H) levels of HER2 (Supplemental Fig. 1 and Materials and Methods). EGF treatment resulted in the broad activation of measured signaling kinases. Erk phosphorylation increased in response to EGF in both parental and 24H cells, with higher initial and sustained activation occurring in the 24H cells (Fig. 3A). EGF treatment also promoted Akt phosphorylation that increased with HER2 expression, although the dynamics of phosphorylation were similar (Fig. 3B). Phosphorylation at EGFR Tyr1068 and Tyr1173 also rose in response to EGF, as expected (Fig. 3, D and E). At the Tyr1068 site, we observed elevated levels of phosphorylation in 24H cells at the 0-min time point (serum-free condition), suggesting the existence of an autocrine loop. 24H cells exhibited increased absolute levels of Tyr1068 phosphorylation in response to EGF treatment compared with the parental cells. Both 24H and parental cells, however, had similar phosphorylated levels of Tyr1173.

In contrast to broad network activation by EGF, HRG

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stimulated the activation of only Akt, with 24H cells exhibiting a more sustained response than parental cells (Fig. 3B). Treatment with HRG did not stimulate substantial phosphorylation on Erk or EGFR (Fig. 3, A, D, and E), suggesting that the low levels of HER2-HER3 heterodimers formed in response to HRG can strongly activate the PI3K-Akt pathway relative to the MEK-Erk pathway.

Finally, p38 phosphorylation was higher in 24H cells but remained relatively constant in response to both HRG and EGF, although we observed a slight peak at 15 min under all conditions (only the 15-min HRG-stimulated peaks are significantly greater than the 0-min phosphorylation levels at $p \leq 0.05$, Fig. 3C).

Thus, the time-courses of measured phosphorylation levels indicate that upon ligand stimulation multiple signaling pathways in the network downstream of ErbB receptors respond to various levels and with diverse dynamics distinctly dependent on ligand and HER2 level. We hypothesize that migration persistence measured in Fig. 2, A and B, are integrated effects of these underlying signals.

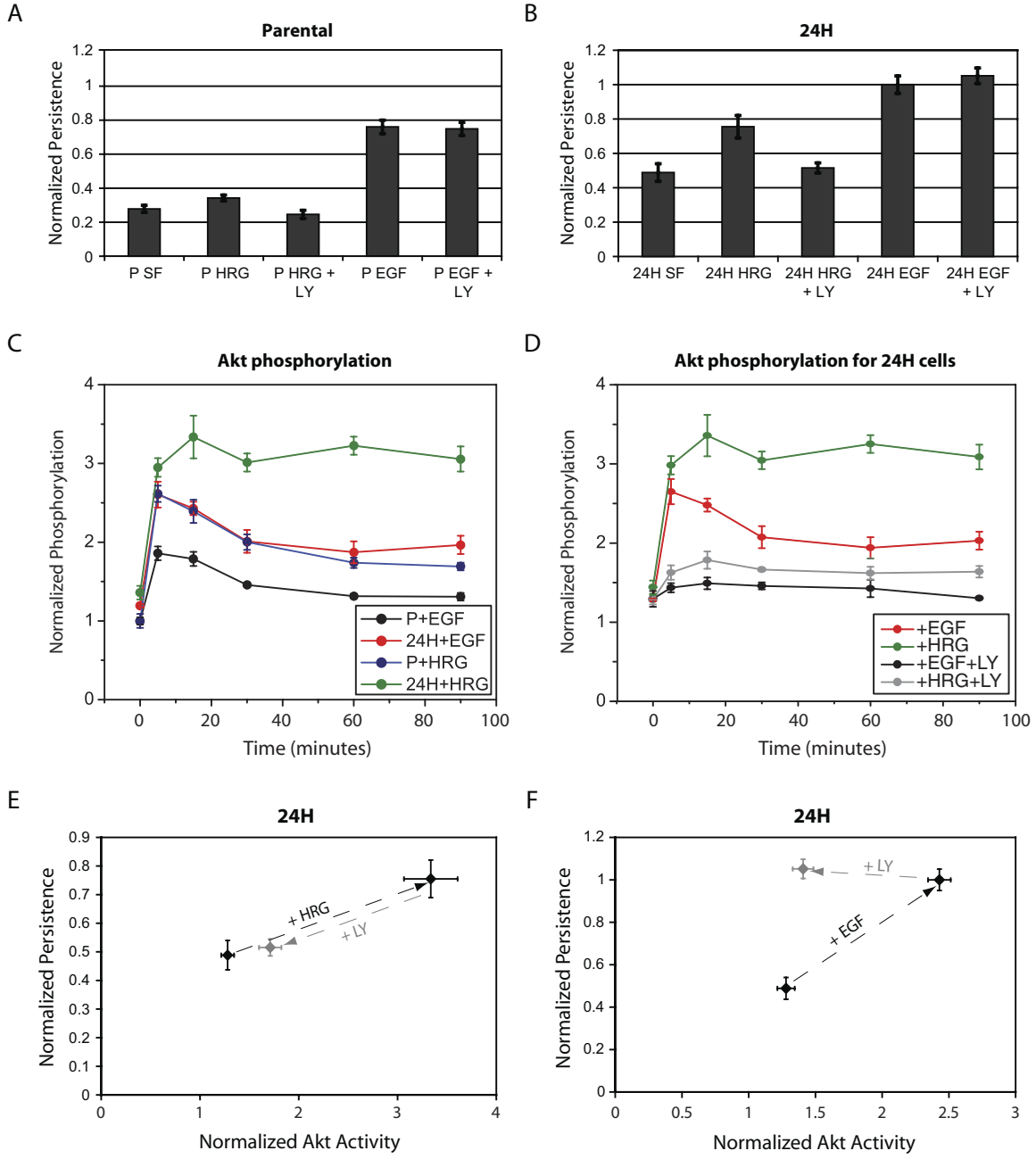

Fig. 2. Akt inhibition by LY294002 decreases persistent migration in HRG-stimulated 24H cells but not in EGF-stimulated 24H cells. A and B, directional persistence of migration of parental cells (A) and HER2 overexpressing 24H cells (B) over 12 h in serum-free conditions or under treatment with EGF (100 ng/ml) or HRG (80 ng/ml) in presence of absence of 1 h pretreatment with 20 μM LY294002. Persistence values are normalized to that of 24H + EGF. C, quantitative immunocytochemistry measurements of Akt (Ser473) phosphorylation in parental and 24H cells stimulated with EGF (100 ng/ml) or HRG (80 ng/ml). D, quantitative Western blotting measurements of Akt (Ser473) phosphorylation in 24H cells stimulated with EGF (100 ng/ml) or HRG (80 ng/ml) in the presence or absence of 1-h pretreatment with LY294002 (20 μM). Data are shown $\pm$ S.E.M. and normalized as indicated under *Materials and Methods*. E and F, replots of data from A, B, and D illustrating that Akt inhibition leads to reduced migration persistence in HRG-treated (E) but not in EGF-treated (F) 24H cells.

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Cell Migration Behavior Exhibited Ligand- and HER2-Dependent Inhibition Effects. To elucidate relationships between kinase phosphorylation and migration persistence, we measured cell movement in response to inhibition by the various compounds. As illustrated clearly in Fig. 2, E and F, treatment with LY294002 resulted in reduction of the migration of HER2-overexpressing 24H cells in the context of HRG stimulation but not EGF stimulation, even though Akt phosphorylation was diminished to comparable degrees for both ligand stimuli. This inhibitor similarly reduced migration of parental cells under HRG but not EGF induction (Fig. 4). Moreover, abrogation of Akt phosphorylation was found to be associated with diminished migration under serum-free conditions (Fig. 4), for which this functional behavior arises mainly from activation of EGFR by autocrine EGF family ligands (Wolf-Yadlin et al., 2006).

In analogous but not identical fashion, treatment with the MEK-Erk pathway inhibitor PD98059 reduced migration in a ligand- and HER2-dependent manner (Fig. 4). From our signaling data in Fig. 3, we hypothesized that PD98059 treatment would inhibit migration in response to EGF but not HRG. We were surprised to find that the inhibitor decreased levels of persistence in parental cells treated with EGF as well as HRG (Fig. 4). PD98059 reduced persistence in EGF-treated parental cells with greater efficacy than in 24H cells (~50% versus ~25% inhibition for parental and 24H cells, respectively), suggesting that HER2-overexpressing cells are less reliant on Erk for directional persistence. PD98059 treatment of HRG-stimulated 24H cells did not inhibit persistence (Fig. 4), further corroborating a diminished importance of Erk for 24H directional persistence. Thus, the varying efficacy of PD98059 treatment across different HER2 expression levels and ligand treatments indicates an important role for off-target and/or cross-talk effects in the governance of persistence.

Treatment with the EGFR kinase inhibitor gefitinib led to the greatest decrease in persistence across cell type and ligand treatment, indicating potential merit of further study into the potential use of gefitinib as an antimetastatic treatment in HER2-overexpressing tumors. Inhibition with gefitinib under EGF-stimulating conditions reduced persistence by ~70 to 90%. It is noteworthy that gefitinib decreased persistence significantly more in 24H cells compared with parental cells (Fig. 4). To summarize, increasing HER2 expression in the presence of EGF and gefitinib lowered persistence, and HRG-treated 24H cells treated with gefitinib had persistence values less than those observed in parental cells without inhibitor.

Quantification of Phosphorylation in Response to Treatment with LY294002, PD98059, and Gefitinib Reveals Pathway Cross-Talk Effects. To better understand the above patterns of inhibitor efficacy, we measured multipathway effects by quantifying the phosphorylation of the previously mentioned kinases under all inhibitor conditions. Treatment with LY294002 greatly reduced Akt phosphorylation, although small differences between 24H and parental levels remained (Fig. 5). It is noteworthy that this PI3K inhibitor also abrogated Erk phosphorylation in response to EGF treatment; both negative and positive cross-talk from the PI3K/Akt pathway to the Erk pathway have been reported in previous literature, depending on cell type and ligand conditions (see Fig. 1) (Zimmermann and Moelling, 1999; Chaudhary et al., 2000; Mograbi et al., 2001; Moelling et al., 2002; Hausenloy et al., 2004). Replication of this result using wortmannin further corroborated a PI3K-related inhibition of Erk (Supplemental Fig. 2). Treatment with LY294002 also increased EGF-stimulated phosphorylation at both EGFR Tyr1068 and Tyr1173 (Fig. 5). This effect was not due to decreased serum-free phosphorylation levels, because serum-free levels of phosphorylated EGFR were the same in the presence or absence of inhibitor (Supplemental Fig. 3). This observation could suggest potential regulation of EGFR by the PI3K pathway; this kind of relationship has been previously reported for wortmannin, with alterations of endocytic trafficking indicated as an underlying mechanism (Bolander, 1998), although off-target effects may be involved (Chen and Wang, 2001).

Treatment with PD98059, like that with LY294002, resulted in substantial multipathway modulation effects. PD98059 dramatically decreased phosphorylation at EGFR Tyr1173 in response to EGF. PD98059 eliminated Erk phosphorylation in response to EGF treatment as expected, and Akt signaling remained relatively consistent with observed phosphorylation in the absence of inhibitor (Fig. 5). The difference between 24H and parental p38 phosphorylation levels decreased over the 90-min time course as a result of decreasing 24H phosphorylation concomitant with increasing parental phosphorylation (Fig. 5), suggesting the presence of another cross-talk effect. At 90 min, p38 phosphorylation in parental cells was higher than that in 24H cells. Phosphorylation at EGFR

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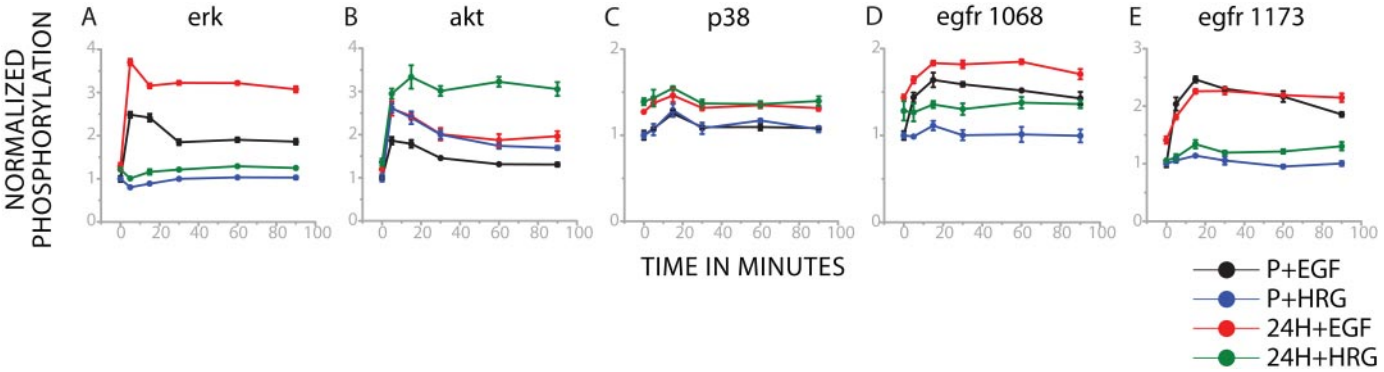

Fig. 3. Quantified phosphorylation for Erk, Akt, p38, and EGFR. Phosphorylation of Erk (A), Akt (B), p38 (C), EGFR Tyr1068 (D), and EGFR Tyr1173 (E) for parental and 24H cells stimulated with EGF (100 ng/ml) or HRG (80 ng/ml) measured via quantitative immunocytochemistry. All data are shown ± S.E.M.

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Tyr1068 was relatively similar to that observed in cells not treated with inhibitor, although phosphorylation levels in 24H cells stimulated with EGF were transiently activated, in contrast to the slight but sustained activity observed under normal circumstances (Fig. 5).

Gefitinib differentially reduced EGFR Tyr1068 phosphorylation depending on HER2 expression levels; EGF-stimulated 24H phosphorylation was similar in the presence or absence of gefitinib, whereas parental phosphorylation was significantly decreased in response to gefitinib treatment (Fig. 5). Tyr1173 phosphorylation was substantially reduced in both cell lines (Fig. 5). Erk and Akt phosphorylation were eliminated by gefitinib treatment, including HRG-stimulated Akt phosphorylation in both parental and 24H cells (Fig. 5). The initially surprising ablation of HRG-stimulated Akt phosphorylation by gefitinib is consistent with its documented ability to trap HER2 and HER3 receptors in an inactive form (Anido et al., 2003). p38 phosphorylation levels equalized between the two cell lines over 90 min under gefitinib treatment, as observed under PD98059 treatment as well, suggesting HER2-dependent differences in off-target and cross-talk effects for both inhibitors.

Taken together, these results show that kinase inhibitor treatment leads to modulation of multiple pathways and that the dependence of the downstream cell migration phenotypic response on inhibition of the targeted pathway is not straightforward. Our goal in this work is not to elucidate the probably numerous molecular mechanisms by which the pathway cross-talk occurs, but instead to provide a computational modeling approach by which the combined effects of multiple pathways on downstream phenotypic behavior can be comprehended.

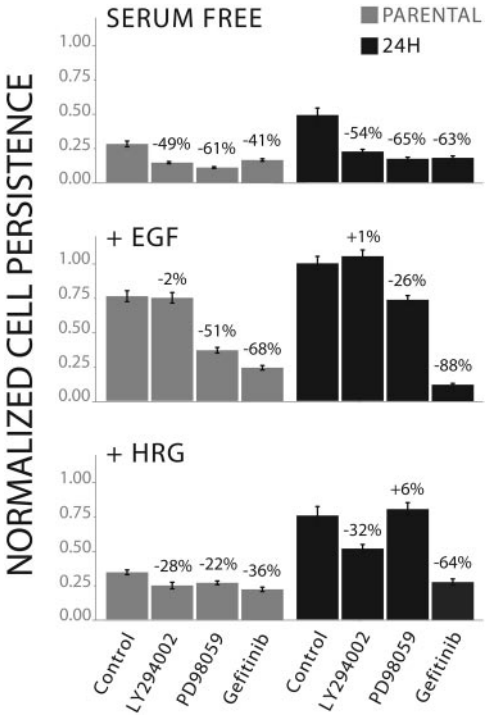

Fig. 4. Effects of LY294002, PD98059, and gefitinib on persistent migration. Directional persistence of parental and 24H cells in the presence of EGF (100 ng/ml), HRG (80 ng/ml), or serum-free media were quantified in the absence of or after pretreatment with one of three inhibitors: LY294002 (20 μM), PD98059 (25 μM), or gefitinib (20 μM). Migration persistence data were normalized as described under *Materials and Methods*; data are shown ± S.E.M. Numbers above bars indicate percentage change relative to no inhibitor control.

Multivariate Computational Model Enables Prediction of How Multiple Pathways Are Integrated to Influence Cell Migration. To quantify the contribution of each kinase to directional persistence, we constructed a PLSR model that linearly related our signal dataset to our migration dataset, following established methods (see Janes and Yaffe, 2006). In essence, weighted linear combinations of the most informative subsets of signal measurements are determined from a matrix algebraic equation relating the signal data across the full landscape of treatment conditions to the corresponding migration data across the same conditions; thus, this technique belongs to a class termed "data-driven modeling" approaches (Janes and Lauffenburger, 2006). We found that the resulting multivariate model (the "Full Model"; see *Materials and Methods* for computational details) accurately recapitulated measured migration persistence values (R^2 = 0.89, r = 0.94, Fig. 6A). The high goodness-of-fit and correlation values helped validate the application of the multilinear modeling approach, consistent with previous successes in other cell systems (Janes et al., 2005; Kemp et al., 2007; Miller-Jensen et al., 2007).

Next, we tested whether information about an individual kinase could lead to accurate prediction of persistence under treatment with its corresponding inhibitor. As is clear from Fig. 2, we knew that a model based on Akt alone could not predict persistence after treatment with LY294002. More generally, PLSR models based on any given individual kinase could not accurately predict a priori changes arising from treatment with the associated inhibitor (average r = 0.02 with a high of 0.34; data not shown). We then tested our hypothesis that incorporation of pathway cross-talk effects, through the measurement of multiple kinase pathways, could lead to accurate predictions of inhibitor efficacy.

A PLSR model constructed only from data obtained in the absence of inhibitor predicted a priori values of persistence in the presence of LY294002 and PD98059 that positively correlated with measured values of persistence (reduced model, r = 0.93; Fig. 6B). The reduced model accurately predicted the previously confounding effects of LY294002 on persistence under all ligand treatment conditions (Fig. 6D). Increased EGFR signaling in the case of EGF treatment under LY294002 compensated for the decrease in Akt signaling, explaining the multivariate model's accurate prediction of inhibitor nonefficacy and underscoring the role of indirect pathway cross-talk. In contrast, the model failed to accurately predict persistence in the presence of gefitinib (r = −0.36, Fig. 6C). Plausible explanations for this contrary result might lie in the fact that this inhibitor operates centrally on a kinase at the receptor level instead of downstream at the highly integrative "bow tie" of the signaling network (Oda et al., 2005) (see Fig. 1) and/or in substantial off-target effects on pathways besides those we measure here (Brehmer et al., 2005).

Inhibitor efficacy in 24H cells was accurately captured by the full multilinear model under both EGF and HRG stimulation (Figs. 6, E and F). In the case of HRG stimulation, the model accurately captured the ineffectiveness of treatment with PD98059, and the increased efficacy of gefitinib over LY294002 (Fig. 6E). It is noteworthy that the model not only captured trends but also accurately recapitulated absolute levels of persistence. In the case of EGF stimulation, the model captures the

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trend of increasing efficacy from LY294002 to PD98059 to gefitinib (Fig. 6F). In the case of gefitinib, the model failed to capture the absolute magnitude of migration persistence, presumably for the same reasons noted above. Nevertheless, the model, by accurately recapitulating measured persistence levels, defines a set of signal-integration rules by means of which the multipathway data effectively predict modulation of migration behavior.

The regression coefficients associated with each phosphorylation metric reveal the metric's contribution to persistence. Coefficient values, plotted in Fig. 6G, indicated that Akt most positively correlated with persistence. However, EGFR

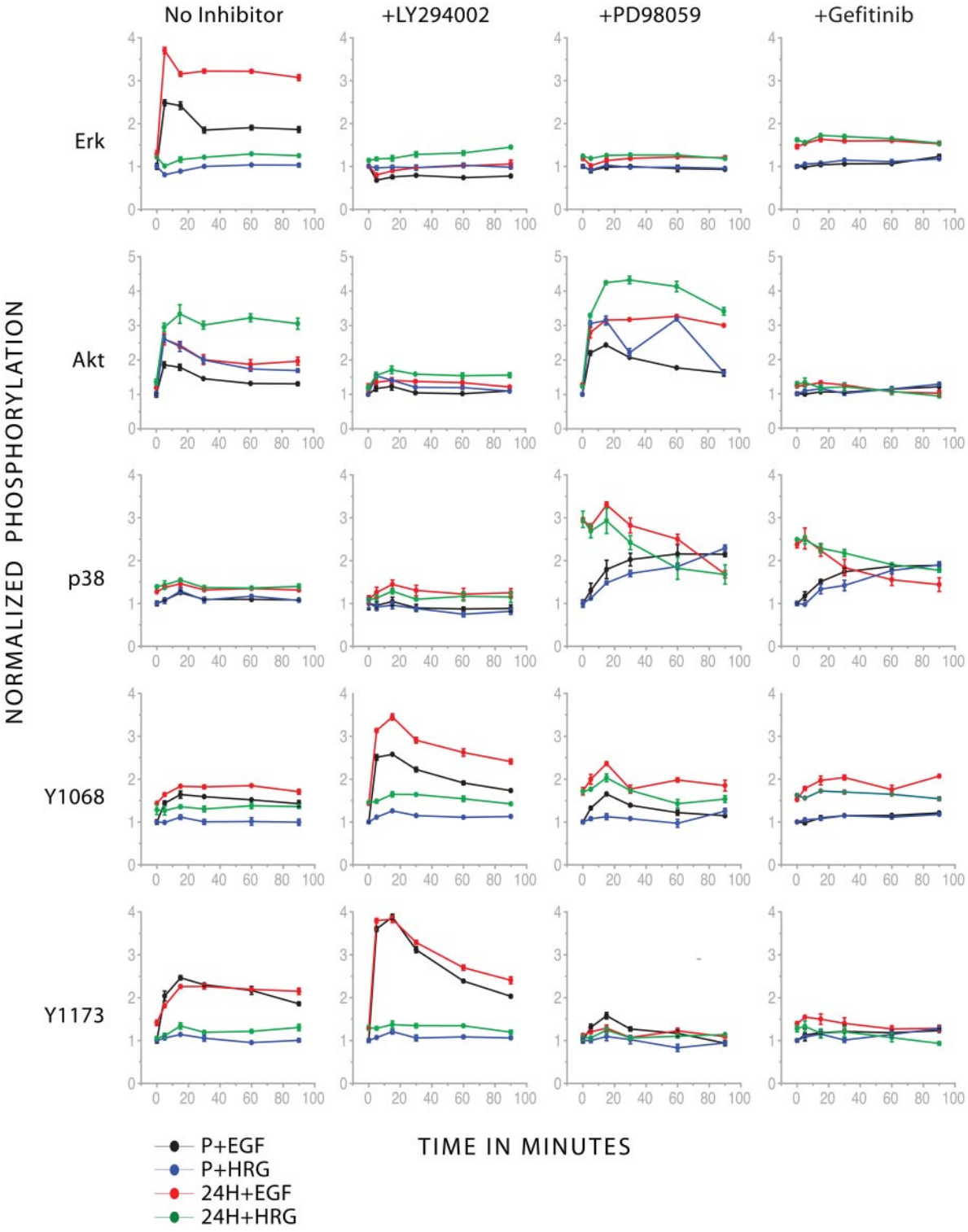

Fig. 5. Effects of LY294002, PD98059, and gefitinib on the phosphorylation of Erk, Akt, p38, and EGFR. Erk, Akt, p38, EGFR Tyr1068, and EGFR Tyr1173 phosphorylation for parental and 24H cells stimulated with EGF (100 ng/ml) or HRG (80 ng/ml) in the absence of inhibitor, pretreated with LY294002 (20 μM), pretreated with PD98059 (25 μM), or pretreated with gefitinib (20 μM) as measured by quantitative immunocytochemistry. Data are shown ± S.E.M.

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Tyr1173 and early-phase Tyr1068 data also strongly correlate with persistence, indicating that an increase in phosphorylation at those sites helps to keep persistence high even in the absence of significant Akt phosphorylation. Erk plays a positive but limited role in stimulating persistence, consistent with the current view that Akt is more important for directional migration. p38 negatively correlates with migration in our context, which contrasts to results obtained in MCF10A breast epithelial cells (Shin et al., 2005). We do not observe acute p38 stimulation under any conditions, however, so our conclusions may not extend accurately to systems where p38 is transiently activated to a more dramatic degree.

Overall, we have found that a partial least-squares regression modeling framework based on multipathway integration yields a substantial advance in successfully accounting for kinase inhibitor effects on downstream cell phenotypic response behavior.

Discussion

Our objective in this study was to ascertain whether we might be able to predictively understand how kinase inhibitor treatments influence cell phenotypic behavior when those inhibitors exhibit significant unintended effects on other pathways. These multipathway effects can arise from direct, "off-target" interactions with additional proteins beyond the one intended or from indirect "pathway cross-talk" arising

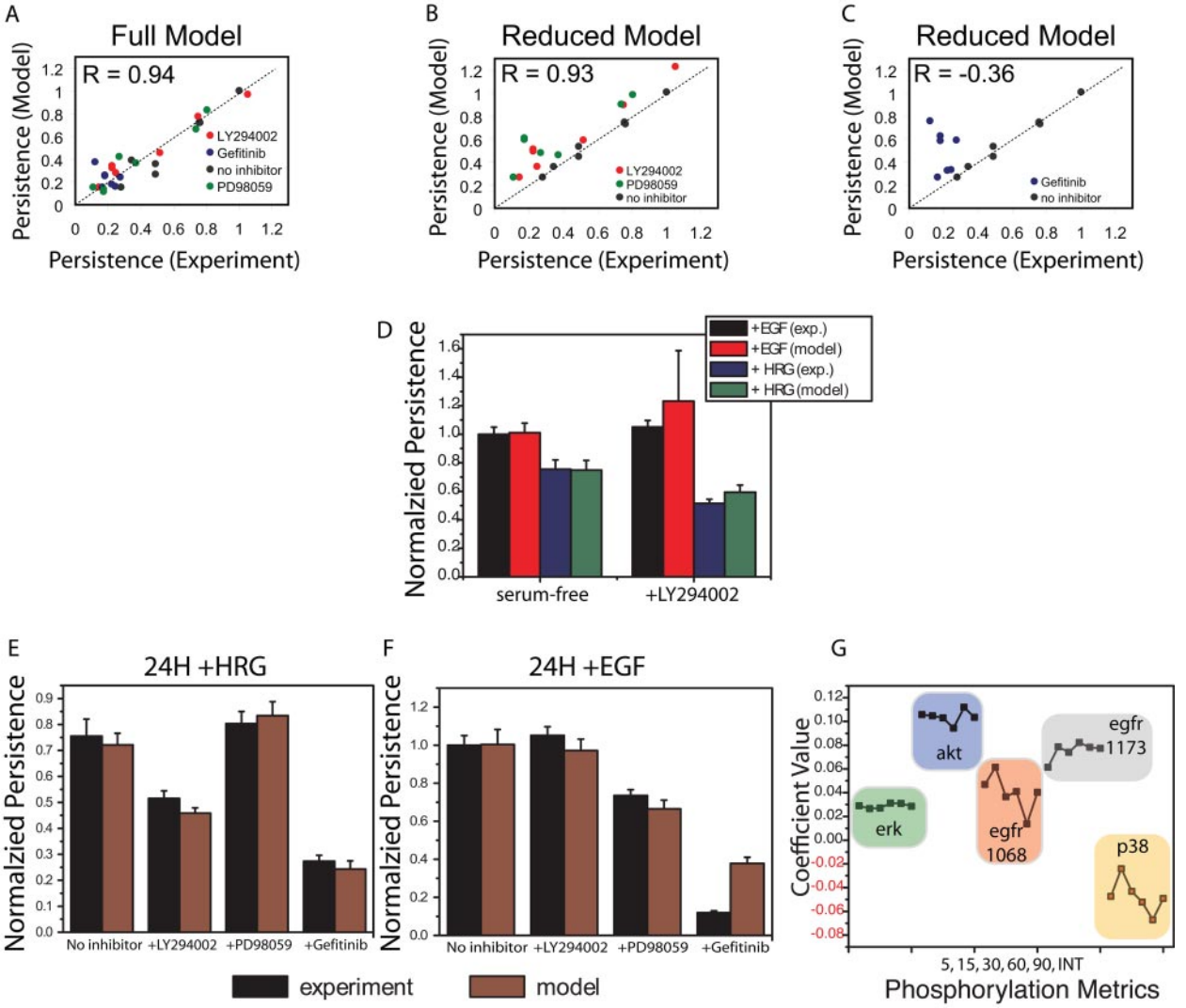

Fig. 6. A multivariate partial least-squares regression model captures changes in persistence and identifies quantitative roles for phosphorylation in control of persistence. A, experimentally measured values of persistence are correlated with predictions from a multilinear model constructed from the complete set of available experimental data presented in Figs. 1–4 (see Supplemental Materials and Methods for full description). B, a priori predictions from a multivariate model constructed only from signaling measurements and migration persistence data in absence of inhibitor are compared with experimentally measured values of migration in the presence and absence of pretreatment with LY294002 (20 μM) and PD98059 (25 μM). C, a priori predictions from a multivariate model constructed only from signaling measurement to migration persistence data in the absence of inhibitor are compared with experimentally measured values of persistence in the presence and absence of pretreatment with gefitinib. For A, B, and C, the broken line represents perfect correlation between model and experiment. D, reduced model a priori predictions compared with experimental data of persistence after pretreatment with LY294002 (20 μM) in the presence of HRG (80 ng/ml) or EGF (100 ng/ml). Predictions of the full model generated from all available data, compared with experimental measurements for 24H cells stimulated with HRG (80 ng/ml) (E) or EGF (100 ng/ml) (F). G, PLSR model weight coefficient values for all phosphorylation metrics included in the full model. For each kinase, phosphorylation at 5, 15, 30, 60, 90, and the integral of phosphorylation are represented. Data are shown ± S.E.M.

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from connection of the targeted pathway with others involved in regulating the downstream cell behavior. With most current kinase inhibitors, both of these effects are likely to participate in confounding a straightforward understanding of how phenotypic responses are altered by treatment. Whatever the underlying mechanisms, advances in comprehending multipathway network modulation will be required to improve rational analysis of prospective pharmaceutical efficacy of small molecule kinase inhibitor drugs. Because we have previously found a quantitative multivariate computational modeling approach to be useful in elucidating relationships by which activities across multiple signaling pathways are integrated to yield an ultimate cell behavior response (Janes et al., 2005; Kemp et al., 2007; Miller-Jensen et al., 2007), we reasoned that a similar approach could be usefully applied to this kinase inhibitor efficacy problem.

As a particular manifestation of this general problem, in this new study, we tested whether we could predict efficacy of two fairly nonspecific kinase inhibitors, one directed against PI3K (LY294002) and the other directed against MEK (PD98059), in decreasing persistent migration of normal and HER2-overexpressing hMECs across a landscape of serum-free, EGF, or HRG stimulatory treatments. Our computational model was based on measurements of phosphoprotein levels in three key migration-related pathways downstream of EGF receptor family activation—Akt (serine 473), Erk (threonine 202/tyrosine 204), and p38 (threonine 180/tyrosine 182)—along with phosphorylation of EGF receptor (tyrosine 1173 and tyrosine 1068). For both of these inhibitors, we demonstrated that the decreases in migration they produced could not be predicted simply from their corresponding reduction of their targeted kinase, but that a PLSR model constructed on the five phosphopeptide levels could successfully predict the migration responses in a priori manner (see Fig. 6). We conclude that our multivariate computational modeling provides a framework for comprehending how downstream cell phenotypic responses are integratively influenced by the modulation of the signaling network caused by the combination of direct and/or indirect effects of kinase inhibitors on nontargeted pathways.

It is noteworthy that the PLSR model predictions for the EGFR kinase inhibitor gefitinib substantially under-predicted the diminution of cell migration by this compound (Fig. 6C); i.e., gefitinib treatment produced a much stronger reduction of migration persistence than was expected from the associated changes measured in the Akt, Erk, and p38 kinase signaling pathways. Our best explanation for this disparity relative to the highly successful results for the Akt and Erk pathway inhibitor model predictions are that inhibiting the upstream receptor kinase activity leads to significant modulation of additional pathways outside the realm of what is reflected in the Akt, Erk, and p38 signals. We have argued elsewhere (Kumar et al., 2006a; Miller-Jensen et al., 2007) that kinase pathway signals in the "bow-tie" of intracellular regulatory networks possess what can be viewed as "surrogate information," because they can integratively represent activities distributed more broadly across other pathways as a result of the the substantial degree of interpathway cross-talk (Oda et al., 2005). In contrast, more distal upstream measurements, such as at a receptor level, are much less likely to incorporate this kind of "surrogate information" arising from multipathway cross-talk integration.

It might be surprising that effects of kinase inhibitory compounds on downstream cell phenotypic functions can be effectively predicted using measurements from only 3 to 5 phosphoprotein signals [Akt, Erk, p38, EGFR (2 sites)] as we have found here. An immediate basis may reside in appreciating that our predictions were tested across a fairly limited experimental landscape: two related cell types (normal and HER2-overexpressing hMECs) and three environmental cue conditions [serum-free media (in which ErbB system activation arises mainly from autocrine ligands), EGF treatment (binding to EGFR, leading to EGFR-EGFR homodimers, EGFR-HER2 heterodimers, and possibly EGFR-HER3 heterodimers), and HRG treatment (binding to HER3, leading to HER3-HER3 homodimers, HER3-HER2 heterodimers, and perhaps HER3-EGFR heterodimers)]. Nevertheless, this landscape represents a more stringent test than is generally the case, for signal inhibition studies most typically consider only one environmental cue condition (whether in high-throughput assay format or not). Contemplating this situation more broadly, it must be recalled that the standard approach for attempting to predict drug efficacy relies on single biomarkers, which for the most part are inadequate (e.g., HER2 expression levels for trastuzumab (Herceptin; Kroese et al., 2007). Therefore, our PLSR model that quantitatively combines five measurements (in what might be considered a "model-based biomarker set") can be seen to offer increased power by taking advantage of an increased number of variables. Moreover, we have previously demonstrated that a relatively small fraction of available network variables can adequately represent the information content of the network more broadly, as a result of the surrogate information about other pathway activities that is reflected in the measured set because of the high degree of pathway cross-talk (Kumar et al., 2006a). Finally, we note that although genome-wide transcriptomics permits inclusion of hundreds to thousands of prospective variables for analogous models, recent study has found that a two-gene model is successfully diagnostic for distinctive diagnosis between two challengingly similar mesenchymal tumors (Price et al., 2007). Hence, a phosphoprotein-based model incorporating three to five signal measurements should not be considered an unreasonable success.

The signaling changes resulting from inhibitor treatments revealed modulations in the pathway activities beyond the targeted kinase pathway, consistent with either direct off-target or indirect cross-talk effects. For instance, treatment with LY294002 and Wortmannin diminished phosphorylation of Erk (Fig. 5, Supplemental Fig. 2). Previous studies documenting inhibition of Raf by Akt have proffered the hypothesis that PI3K inhibition should increase Erk phosphorylation (Wennström and Downward, 1999; Zimmermann and Moelling, 1999). Our data here show that PI3K inhibition can result in strong ablation of Erk phosphorylation, possibly through a MEK-independent pathway, because treatment with LY294002 or PD98059 reduces Erk phosphorylation in a similar manner. As demonstrated in a recent study of signaling network responses across different cell types, it is quite likely that multipathway cross-talk is cell-type dependent and accordingly difficult to anticipate in absence of dedicated experimental measurements (Miller-Jensen et al., 2007).

The full PLSR model suggested that Akt phosphorylation

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correlates positively with persistent migration (Fig. 6G). An explicit role for Akt, other than as a surrogate for upstream PI3K activity in breast cancer cell migration is not clearly established. Recent reports indicate that Akt may act as either an inhibitor or promoter of metastasis (Arboleda et al., 2003; Yoeli-Lerner et al., 2005), but our results indicate that Akt inhibition via LY294002 treatment can lead to an effective decrease of HER2-mediated cell migration in the absence of off-target effects. Because both HRG and EGF-family ligands have been found to circulate in breast cancer tumors (Normanno et al., 2006), we speculate that inhibition of Akt with a PI3K inhibitor similar to LY294002 might be effective against cell migration in tumors with high levels of HRG but not necessarily in those with high EGF-family ligand expression, as a result of the LY294002 treatment consequence of increasing EGFR phosphorylation. Our data also contrast with an earlier report suggesting that PI3K does not play a role in directed migration in the absence of chemical attractant gradients (Pankov et al., 2005) but cell-type or substratum differences could underlie this disparity.

The likelihood of indirect, network cross-talk-mediated off-target effects raises the consideration that this kind of off-target effect cannot be engineered away through the creation of more target-specific drugs, and thus must motivate broader strategies including computational modeling approaches such as those we have described here. In fact, prospects are now being raised regarding the potential advantage for promiscuous, multitarget compounds in explicitly acting across multiple pathways intentionally (Zhang and Meier, 2006; Cascone et al., 2007). If this promising avenue is followed, our quantitative, multipathway integration framework will be absolutely required to effectively predict resulting effects on cell phenotypic behaviors.

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