Temporal Profiling of Lapatinib-suppressed Phosphorylation Signals in EGFR/HER2 Pathways

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Lapatinib is a clinically potent kinase inhibitor for breast cancer patients because of its outstanding selectivity for epidermal growth factor receptor (EGFR) and EGFR2 (also known as HER2). However, there is only limited information about the in vivo effects of lapatinib on EGFR/HER2 and downstream signaling targets. Here, we profiled the lapatinib-induced time- and dose-dependent phosphorylation dynamics in SKBR3 breast cancer cells by means of quantitative phosphoproteomics. Among 4953 identified phosphopeptides from 1548 proteins, a small proportion (5–7%) was regulated at least twofold by 1–10 μM lapatinib. We obtained a comprehensive phosphorylation map of 21 sites on EGFR/HER2, including nine novel sites on HER2. Among them, serine/threonine phosphosites located in a small region of HER2 (amino acid residues 1049–1083) were up-regulated by the drug, whereas all other sites were down-regulated. We show that cAMP-dependent protein kinase is involved in phosphorylation of this particular region of HER2 and regulates HER2 tyrosine kinase activity. Computational analyses of quantitative phosphoproteome data indicated for the first time that protein-protein networks related to cytoskeletal organization and transcriptional/translational regulation, such as RNP complexes (i.e. hnRNP, snRNP, telomerase, ribosome), are linked to EGFR/HER2 signaling networks. To our knowledge, this is the first report to profile the temporal response of phosphorylation dynamics to a kinase inhibitor. The results provide new insights into EGFR/HER2 regulation through region-specific phosphorylation, as well as a global view of the cellular signaling networks associated with the anti-breast cancer action of lapatinib. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.019919, 1741–1757, 2012.

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Human epidermal growth factor receptor 2 (EGFR21 also known as HER2) is a key molecule in breast carcinoma cells. It is overexpressed and/or hyperactivated in ~25% of human breast cancer patients (1, 2), constitutively enhancing the downstream signaling pathways toward tumor growth and invasion by forming kinase-active dimers with EGFR family members (i.e. EGFR, HER2, HER3, and HER4) (3). A humanized monoclonal antibody-based drug, trastuzumab (Herceptin®) has been approved for the treatment of breast cancer patients with HER2 protein overexpression and HER2 gene amplification (4). It has been proposed that trastuzumab inhibits the downstream signaling pathways, including phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways (5, 6), and/or induces Fc receptor-mediated cytotoxicity through immune response (7). One critical issue is that most HER2-positive patients tend to be insensitive to trastuzumab treatment or acquire drug resistance (8, 9). On the other hand, small-molecular inhibitors such as gefitinib and imatinib have been launched as selective kinase inhibitors for several types of cancer (10). These inhibitors are generally designed to inhibit kinase activity by binding to the ATP-binding pocket of target kinase domains, thereby blocking the downstream signaling pathways that induce cell proliferation, growth, survival, and so on. However, most kinase inhibitors have multiple targets, including so-called “off targets” because of the similarity of ATP-binding pockets among more than 500 human protein kinases, and this can result in critical side effects such as cardiotoxicity (11). Although multitarget inhibitors (e.g. sunitinib and sorafenib) are effective in some cases (12), targeting specific protein kinase(s) would be a rational approach for the development of drugs without critical side effects. Among 12 kinase inhibitors so far launched on the US market, including imatinib, gefitinib, erlotinib, and dasatinib (11), lapatinib (Tykerb™, GSK572016) exhibits the highest selectivity and has a dual inhibitory effect on EGFR/HER2 (13). The dissociation

1 The abbreviations used are: EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; HPRD, Human Protein Reference Database; FPR, false-positive rate; SPL, shortest path length; PPI, protein-protein interaction; RNP, ribonucleoprotein.
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constant \( K_d \) values of EGFR (2.4 nM) and HER2 (7 nM) are 500–1800 times higher than those of off-target molecules not belonging to the EGFR family, such as serine/threonine-protein kinase 10 and receptor-interacting serine/threonine-protein kinase 2 (13). Further, because dissociation of lapatinib from EGFR/HER2 is much slower than that of other EGFR-selective inhibitors such as gefitinib and erlotinib, its inhibitory effects on EGFR/HER2 tyrosine phosphorylation are more prolonged (8). Lapatinib was reported to be effective against HER-2-overexpressing and trastuzumab-treated breast cancer cells (14). But, although lapatinib is currently one of the most potent clinical treatments for breast cancer patients (15), its global effect on EGFR/HER2 signaling networks in vivo is still poorly understood. Vazquez-Martín et al. performed a limited-scale phosphoproteome analysis of a lapatinib-resistant breast cancer cell line using a phospho-specific antibody array for 21 different oncogenic kinases (16). They found that the phosphorylation state of ribosomal protein S6 kinase beta-1 could be a potential marker for drug resistance. However, a proteome-wide picture of lapatinib’s inhibitory effects remains to be uncovered.

In recent years, mass spectrometry (MS)-based quantitative proteomics has been applied to large-scale drug profiling. Fabian et al. reported an in vitro competitive binding assay using recombinant kinase mixtures and defined interactions between 38 inhibitors and 317 kinases at various drug concentrations (13, 17). Bantscheff et al. developed a MS-based assay with a kinase-selective enrichment method (called “kinobeads,” on which nonselective kinase inhibitors are immobilized) to elucidate the mechanism of action of clinical ABL kinase inhibitors (18). Similarly, Sharma et al. introduced a multiplex SILAC (stable isotope labeling by amino acids in cell culture)-based proteomics approach combined with kinase inhibitor-containing beads to globally characterize drug-targeted kinases (19). More recently, KAYAK (kinase activity array for 21 different oncogenic kinases) was desalted using StageTips (32).

EXPERIMENTAL PROCEDURES

Cell Culture and SILAC Labeling with Nondialyzed Serum—SKBR3 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Wako, Osaka, Japan) and culture media used in all experiments were supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA) and 100 \( \mu \)g/ml kanamycin. For dual labeling SILAC with nondialyzed serum (27), SKBR3 cells were grown in the presence of either \( ^{15} \text{N}_2 \)-Arg (+4) and \( ^{15} \text{N}_4 \)-Lys (+4) (“medium” labeling) or \( ^{13} \text{C}_6 ^{15} \text{N}_4 \)-Arg (+10) and \( ^{13} \text{C}_6 ^{15} \text{N}_4 \)-Lys (+8) (“heavy” labeling) under nondialyzed serum conditions. The amount of each labeled Arg and Lys used for supplementation followed the defined concentrations in DMEM. Quantitation error resulting from Arg-to-Pro conversion did not have to be considered because of the use of internal peak correction owing to the dual labeling with \( ^{15} \text{N}_2 \)-Arg and \( ^{13} \text{C}_6 ^{15} \text{N}_4 \)-Arg (28). All experiments using human cell lines were conducted according to the institutional regulation.

Drug Treatment—Lapatinib (Toronto Research Chemicals, Toronto, Canada) solution was prepared by mixing a stock solution of 1 mM lapatinib in dimethyl sulfoxide with culture medium. SKBR3 cells were treated with epidermal growth factor, EGF (Sigma-Aldrich) (100 ng/ml) in “medium” media as a control or EGF (100 ng/ml)-lapatinib (1 \( \mu \)M or 10 \( \mu \)M) in “heavy” media. Time points for the stimulation were set as 1, 5, 10, and 60 min. Two biological replicates for the series of time- and dose-dependent experiments were performed. H-89 was obtained from Calbiochem (San Diego, CA). For experiments involving H-89 treatment, 10 mM H-89 in dimethyl sulfoxide was diluted to a final concentration of 10 \( \mu \)M or 100 \( \mu \)M with culture medium. H-89 treatment was performed for 20 min before EGF (100 ng/ml)-lapatinib (10 \( \mu \)M) treatment for 60 min. After the treatments, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested on ice with ice-cold PBS containing protease inhibitors and protein phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich), and frozen at –80 °C until use.

Sample Preparation for Phosphoproteome Analyses—Pellets of medium- and heavy-labeled cells were combined in equal number, and cell lysis and subcellular fractionation were performed as described elsewhere (29). Briefly, the cells were lysed with 20 mM HEPES (pH 7.9), 0.25 mM sucrose, 1.5 mM MgCl\(_2\), 10 mM KCl, 0.5% Nonidet P-40, containing protease inhibitors and protein phosphatase inhibitor mixture 1 and 2 (Sigma-Aldrich), and left for 10 min on ice. Then, the lysates were centrifuged at 1000 \times g for 10 min at 4 °C. The supernatant (cytoplasmic extract) and the pellet (nuclear extract) were each dissolved in 0.1 M Tris-HCl (pH 8.0) and 8 \( \mu \)l urea, followed by ultrasonication with a Bioruptor (Cosmo Bio, Tokyo, Japan). After protein reduction with dithiothreitol, alkylation with iodoacetamide, and Lys-C/trypsin digestion were performed as described previously (30). Phosphopeptides were enriched from 200 \( \mu \)g of tryptic digests with a Phos-TIO Kit (GL Sciences, Tokyo, Japan), which is based on aliphatic hydroxy acid-modified metal oxide chromatography with lactic acid-modified titania beads (30, 31). The resultant peptides were desalted using StageTips (32).

Western Blotting—Cells were lysed with SDS sample buffer [125 mM Tris-HCl, 10% (v/v) sucrose, 10% (v/v) 2-mercaptoethanol, and 4% (w/v) SDS (pH 6.8)] and equal amounts of lysates were loaded on SDS gels. Polyvinylidene difluoride membranes (Amersham Biosciences) were soaked in methanol and placed in transfer buffer [25 mM Tris-HCl, 195 mM glycine, and 20% (v/v) ethanol]. Proteins were transferred on membranes and blocking was performed using blocking One-P (Nacalai Tesque, Kyoto, Japan). Proteins were probed against phospho-EGFR (Abcam, Cambridge, MA), phospho-HER2...
Three successive y- or b-ions with two or more y-, b-, and/or precursor-origin neutral loss ions should be observed based on the error-tolerant peptide sequence tag concept (36); this resulted in an estimated false-positive rate of 1%. Regarding counting the number of proteins identified, all matched proteins were separated first when any single peptide matched multiple proteins in the database search. Accordingly, peptides were separately counted as well when a single peptide matched multiple proteins. All MS/MS spectra are provided in supplemental Data and in our database “Pepbase” (http://pepbase.iba.keio.ac.jp/phospho/skbr/).

SIAL quantitation was performed by Mass Navigator (37). Mixing ratios of the medium- and heavy-labeled cells were corrected based on the mean values of SIAC ratios of nonphosphopeptide in each sample as described earlier (38). To obtain time-course phosphoproteome data, log2-transformed SIAC ratios (H/M; +EGF-lapatinib/+EGF) were plotted at each time point (Fig 1A). In this study, a twofold change (i.e., H/M ≥ 2 or H/M ≤ 0.5) cutoff was used for identifying phosphopeptides “regulated” by lapatinib treatment, which corresponds to p < 0.01 based on the distribution of SIAC ratios of nonphosphopeptides (39).

Phosphorylation Site Localization in Phosphopeptides—To re-evaluate phosphorylation sites determined by Mascot, we checked whether y- or b-ions between which the phosphorylated residue exists were observed or not in the peak lists of the fragment ions (30) (see “Manual confirmation” column in supplemental Table S1; all phosphopeptide list). This step was automatically performed using an in-house Perl script, by comparing the original peak lists with the theoretical m/z values of y- or b-ions from Mascot output files. For further assignment of the phosphorylation site localization, we calculated probability-based post-translational modification (PTM) scores (38) by using PhosCalc version 1.2 (40). Also, PTM localization probabilities were calculated and the three categories were defined as class I (localization probability, p > 0.75), class II (0.75 > p > 0.5), and class III (p < 0.5) (41) (see supplemental Table S2).

Computational Analyses—All computational analyses were performed for the proteins (n = 210) and the peptides (n = 367) that exhibited ≥ twofold phosphorylation changes in response to 10 μM lapatinib treatment unless otherwise described. Among the 210 regulated proteins, we assigned 21 as EGFR/HER2 pathway molecules for further analyses based on annotations from Human Protein Reference Database (HPRD (42), http://www.hprd.org/), Cell Signaling Technology (http://www.cellsignal.com/) and Reactome (http://www.reactome.org/).

We constructed two networks in this study: 1) the protein-protein interaction (PPI) network and 2) the phosphorylation dynamics-based network. First, human PPI network data was downloaded from a public PPI database, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) version 8.3 (http://string-db.org/) (43), and the high-confidence PPI network (score = 0.7) was constructed by importing the 210 proteins data set to the STRING database. Then, two sets of subrandom PPI networks were generated as negative controls by randomly extracting 210 proteins from a list of 1548 phosphoproteins identified in this study (subrandom PPI network 1) and from the UniProt/Swiss-Prot protein database 57.5 (subrandom PPI network 2). Note that because bias could be introduced by excluding EGFR/HER2 signaling proteins from random networks, despite the drug treatment of cells with the EGFR/HER2 specific inhibitor, the 21 EGFR/HER2 signaling proteins regulated by lapatinib were retained and were combined with 189 (= 210 – 21) randomly extracted proteins to create the subrandom networks, which gave us stricter negative controls compared with completely random selections from all of the protein lists. These random selections were repeated 1000 times. Second, a phosphorylation dynamics-based network was created by connecting the strongly correlated pairs of phosphorylation...
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dynamics-identified peptides (Pearson’s correlation coefficient value: \( R > 0.99 \)). Random PPI networks (\( n = 100 \)) were also created by random edge rewiring, as described before (44). We defined the closeness of two nodes in a network using the shortest path length (SPL), which represents the minimum number of steps (edges) between two nodes. The resulting networks were visualized with Cytoscape (45). We determined the statistical significance of differences between a single real value and the expected value from a group of repeatedly generated random values by calculating the proportion of random values that were greater than or equal to the real value (or less, depending on the case) (44).

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (46).

RESULTS AND DISCUSSION

Conditions for Time- and Dose-dependent Phosphorylation Profiling—The complex of EGFR and HER2 is the major heterodimer formed in response to stimulation with epidermal growth factor (EGF), and is of particular importance in breast carcinoma (47). In this study, therefore, we used an EGFR-expressing and HER2-overexpressing human breast cancer cell line, SKBR3 (48), and treated cells with lapatinib in the presence of EGF. Earlier studies performed lapatinib treatment for several hours (16, 49, 50), but here we chose 1–60 min treatment to monitor early phosphorylation events perturbed by lapatinib.

Recently we reported perturbation of the phosphoproteome expression profiles in breast cancer cell lines by the use of dialyzed serum during SILAC labeling compared with the usual cell culture condition with nondialyzed serum (27), and found that phosphorylation networks in SKBR3 cells were significantly suppressed by the depletion of hormones and/or growth factors in dialyzed serum, as described in other reports (51–53). Because we intended to examine the influence of lapatinib on as many phosphorylation networks as possible, the use of dialyzed serum was not preferable. Therefore, we employed dual-labeling SILAC with nondialyzed serum (27), which permits accurate quantitation even if nondialyzed serum is used during labeling and even if the labeling efficiency is limited. Also, the use of medium- and heavy-labeled Arg in the SILAC media allows accurate SILAC quantitation even when Arg-Pro conversion occurs (28). Note that the labeling efficiency in this study was very high even in the presence of unlabeled amino acids, as shown in Fig. 1B, and 99.0% of total identified phosphopeptides was labeled.

We next evaluated the effects of lapatinib concentration (0.1 \( \mu \)M, 1 \( \mu \)M, and 10 \( \mu \)M) on cell viability to examine suitable concentrations for this study. We found that 1 \( \mu \)M and 10 \( \mu \)M lapatinib dose-dependently inhibited the cell viability, whereas 0.1 \( \mu \)M lapatinib did not affect cell viability even after 48 h treatment (supplemental Fig. S1). Note that no significant change in the cell viability was observed within a few hours at any drug concentration. Thus, we decided to use 1 \( \mu \)M and 10 \( \mu \)M lapatinib treatments of SKBR cells for further phosphoproteome analyses. Indeed, we observed in our phosphoproteome analyses that 1 \( \mu \)M and 10 \( \mu \)M lapatinib induced inhibition of tyrosine phosphorylation at Y1172 on EGFR and at Y877 on HER2, both of which are known to be autophosphorylated (25, 54) (data obtained in the case of 1 \( \mu \)M lapatinib treatment for 60 min is shown in Fig. 1B as an example). Note that the well-studied autophosphorylation site of HER2 at Y1248 cannot be quantified by SILAC because of the lack of C-terminal Arg or Lys. Phosphorylation at Y1172 of EGFR, which regulates kinase activity, was markedly inhibited. Mild inhibition was observed for phosphorylation at Y877 of HER2, which is located in the activation loop of the kinase domain. This indicates that the inhibitory concentration for HER2 is higher than that for EGFR. This observation is consistent with the difference between the IC\(_{50}\) values of EGFR (3 \( \mu \)M) and HER2 (13 \( \mu \)M) (15). In contrast, 0.1 \( \mu \)M lapatinib did not inhibit phosphorylation at any of the sites of EGFR or HER2 (data not shown). In our phosphoproteome analysis, “medium”-labeled (Arg “4” and Lys “4”) cells were stimulated with EGF for 1, 5, 10, and 60 min as controls whereas “heavy”-labeled (Arg “10” and Lys “8”) cells were treated with EGF and either 1 \( \mu \)M or 10 \( \mu \)M lapatinib at the defined time points. Note that the results (SILAC ratios) reflect the effect of only lapatinib on phosphoproteome because EGF was added to both of the control cells and the drug-treated cells, and that the diffusion time of lapatinib to the target molecules are included in the time-course data. Then, the medium and heavy cell fractions at the same stimulation time were combined equally and analyzed by nanoLC-MS/MS in combination with lactic acid-modified metal oxide chromatography (30, 31). Finally, we obtained the time- and dose-dependent phosphoproteome dynamics, as illustrated in Fig. 1A.

Lapatinib-induced Phosphoproteome Profiles—We identified 4953 unique phosphopeptides (1548 proteins) from biologically duplicate analyses. At least 5% (10%) and 7% (14%) of the identified phosphorylated peptides (proteins) were up- or down-regulated by at least twofold in response to 1 \( \mu \)M and 10 \( \mu \)M lapatinib, respectively (Table I). The 10 \( \mu \)M lapatinib-regulated phosphoproteins include almost all (99%) of the proteins regulated by 1 \( \mu \)M lapatinib. An overview of the lapatinib-induced temporal phosphoproteome dynamics is shown in Fig. 2. This represents a hierarchical clustering of the phosphopeptides showing \( \geq \)twofold phosphorylation changes in response to 10 \( \mu \)M lapatinib. Phosphorylation of key signaling molecules on the EGFR pathway, i.e. receptor EGFR, adaptor protein SHC1, MAPK ERK2, and transcription factor JUN/JUND, was time- and dose-dependently regulated and the dynamic profiles clearly reflected their locations in the signaling pathway (Fig. 2B). For example, EGFR (pY1197) and SHC1 (pY427) showed rapid inhibition, whereas downstream molecules, ERK2 (pT185/pY187) and JUN/JUND (pS73/pS100), had mild and slow inhibition profiles, respectively. We also validated phosphorylation dynamics of selected proteins, including EGFR, HER2, ERK1/2, and JUN/
JUND, using phospho-specific antibodies (Fig. 3). The temporal profiles obtained by the MS-based method (Fig. 2B and Fig. 4B) were consistent with those by the Western blot analysis.

In total, we identified 62 phosphorylated proteins out of more than 200 EGFR/HER2 pathway-related molecules derived from three major databases, HPRD (42), Reactome (http://www.reactome.org) and Cell Signaling Technology (http://www.cellsignal.com/) (Table I and Supplemental Fig. S2). Among these identified proteins, the phosphorylation states of 21 were affected by lapatinib, corresponding to 10% of the 210 regulated proteins. Although we identified other key signaling proteins in the EGFR/HER2 pathways, including protein kinases (e.g., HER3, BRAF1, GSK3, M3K1/2/4, p90S6K, RAF1, SRC/FYN/LCK/YES), transcriptional factors (e.g., MYC, STAT3), and other molecules (e.g., AB11, CRK, PTN12), no significant phosphorylation changes were observed in the identified peptides from these proteins, indicating that phosphorylation sites on these molecules may not directly contribute to the signal processing. We also identified 80 protein kinases, of which 20 exhibited changes in phosphorylation with lapatinib treatment. This result implies that...
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Table I
Summary of phosphoproteome data

| Summary                        | No.  |
|--------------------------------|------|
| Identified phosphopeptides     | 4953 |
| Regulated phosphopeptides      | 391  |
| (≥2-fold)                      |      |
| Identified phosphopeptides      | 1548 |
| Regulated phosphopeptides      | 222  |
| (≥2-fold)                      |      |
| Identified EGFR/HER2 signaling proteins | 62   |
| Regulated signaling proteins   | 21   |
| (≥2-fold)                      |      |
| Identified protein kinases      | 80   |
| Regulated protein kinases       | 20   |
| (≥2-fold)                      |      |

**Number of phosphopeptides regulated by either 1 μM or 10 μM lapatinib treatment.**

**EGFR/HER2 signaling proteins were collected from HPRD, Reactome and Cell Signaling Technology.**

protein kinases are significantly enriched among the regulated proteins, as demonstrated later in GO enrichment analyses, such as tyrosine/threonine phosphorylation by inactivation of the down-regulation profiles because of inhibition of phosphorylation by lapatinib (Fig. 2). In contrast, we also observed a group of events that showed higher phosphorylation states in lapatinib-treated cells than in nontreated cells, as reported elsewhere (22, 24, 25). This “up-regulated” group includes EGFR/HER2 pathway proteins, as well as a variety of functional proteins such as protein kinases (CDK12, CDK2, CDK2/3, CLK3, EGFR, HER2, M3K7, PKA, and PKC) and cytokines-related proteins (CLIP1/2, DESP, K1C18, MAP4, and PLEC1 shown in Fig. 2B). Some of them are thought to have functions associated with negative regulation of phosphorylation, such as CDC2 and CDK2/3 at T14, inducing enzymatic inactivation (55).

To globally characterize the functions and regulatory pathways of the lapatinib-regulated proteins, we used DAVID to extract the enriched GO terms and KEGG pathways. As expected, GO terms related to protein phosphorylation, protein kinase, and (EGFR) signal transduction were enriched in the data set (p < 0.01) (supplemental Figs. S3–S5). In addition, a number of keywords linked to cell cycle, cytokines organization, (messenger) RNA, and DNA were also observed (supplemental Figs. S3–S5). The KEGG pathway enrichment analysis successfully captured the ErbB (EGFR) signaling pathway (p = 5.8E-06) and MAPK signaling pathway (p = 7.9E-06), whereas other signaling pathways such as the GnRH (gonadotropin-releasing hormone) signaling pathway (p = 1.4E-06), focal adhesion (p = 2.3E-06), insulin signaling pathway (p = 2.6E-06), and vascular smooth muscle (p = 4.4E-06) were also highly ranked (supplemental Fig. S6). We will discuss later how such uncharacterized proteins may contribute to the known EGFR/HER2 signaling network, based on computational approaches.

**Impact of Lapatinib on EGFR/HER2 Phosphorylation**—In this phosphoproteomic study, we identified eight and 13 unambiguous (class I) phosphorylation sites on EGFR and HER2, respectively, including six novel serine and three novel threonine phosphorylation sites on HER2 (Figs. 4A, 4B and Table II). The identified tyrosine phosphorylation sites (i.e., pY1110, pY1172, pY1197 on EGFR and pY877 on HER2) exhibited rapid inhibition on lapatinib treatment (Fig. 4B upper), as would be expected from the fact that lapatinib is a dual tyrosine kinase inhibitor for EGFR/HER2. On the other hand, the serine and threonine phosphorylated peptides of EGFR, including pS1042 and pS1045, were more slowly down-regulated than the tyrosine phosphorylation sites (Fig. 4B middle left) This is consistent with the observation that EGFR tyrosine phosphorylation was immediately (1 min) induced by EGF-stimulation and the serine/threonine phosphorylation was subsequently activated (38). Considering that the inhibition states of tyrosine-phosphorylated peptides reached a plateau at 10 min for both EGFR and HER2, the diffusion equilibration of lapatinib inside cells and the competitive equilibration between lapatinib and intracellular ATP would presumably have been completed within that time after the drug treatment. In contrast, the inhibitory effects on serine/threonine phosphorylation were generally more prolonged. This observation is consistent with direct inhibition of tyrosine phosphorylation by lapatinib and indirect suppression of serine/threonine phosphorylation by inactivation of the downstream protein kinases.

Importantly, several serine- and threonine-phosphorylated peptides of EGFR and HER2 were up-regulated following drug treatment (Figs. 4A, 4B and Table II), suggesting that some protein kinase(s) as well as phosphatase(s) activated by lapatinib-induced signaling increased the phosphorylation of these sites. Although these phosphorylation sites were located in the cytoplasmic tails of EGFR (amino acids 1162–1166) and HER2 (amino acids 1049–1083), these regions do not overlap after alignment (Fig. 4A and supplemental Fig. S7). Interestingly, these regions also do not have counterpart regions in the other molecule after alignment, though the overall degree of similarity between EGFR and HER2 is 50% (see supplemental Fig. S7).

**Protein Kinases Responsible for HER2 Serine/Threonine Phosphorylation**—The lapatinib-induced EGFR/HER2 phosphorylation raised a question as to which kinase(s) is responsible. It has been reported that several serine and threonine sites on EGFR are phosphorylated in vivo by cAMP-dependent protein kinase A (PKA) (56) and protein kinase C (PKC) (phosphorylates at T678 of EGFR) (57, 58), CDC2 protein kinase (at S1026) (59), calmodulin-dependent kinase 2 (CaMK2) (at S768, S1070, S1071, S1081, and S1166) (60, 61), and MAPKs (at T693) (62, 63). Threonine at 1172 of HER2 is also phosphorylated by CaMK2 (64). Although the up-regu-
lated sites at S1162 and/or S1166 on EGFR, found in this study, are presumably also phosphorylated by CaMK2 (61), little is known about the kinase(s) responsible for phosphorylation at HER2 serine/threonine sites other than pT1172.

Thus, to identify the upstream kinases for the HER2 phosphorylation sites, we first tried a motif-based analysis using Networkin (65), Phospho.ELM (66), and PhosphoMotif Finder (67). As a result, AKT, CaMK2, CK1, ERK, GSK3, MAPKAPK2, PKA, PKC, etc. were extracted as candidate kinases (supplemental Fig. S8A). To test whether these kinases phosphorylate the corresponding HER2 sites, we performed an in vitro kinase assay using recombinant protein kinases and HER2 synthetic peptides as substrates. We used four different HER2 synthetic peptides designed to cover the amino acid sequences containing the phosphorylation sites (amino acids 1041–1090 and amino acids 1240–1255) as illustrated in Fig. S2.
5A (upper). Also, we chose the following recombinant kinases; CDK2, CDK5, MAPKAPK2, ERK2, CK2α1, AKT1, PKCa, CaMK2α, and PKACA, based on the results from the motif-based analyses, as well as a HER2 recombinant protein to check the tyrosine phosphorylation at Y1248 through autocatalysis as a positive control. In this assay, we identified and quantified phosphorylated forms of the HER2 peptides by using LC-MS/MS. The results are summarized in Fig. 5 (bottom) and supplemental Fig. S9, in which red boxes indicate “phosphorylated” by a particular kinase(s). We confirmed that recombinant HER2 phosphorylated the peptide containing Y1248, and some of the candidate kinases phosphorylated particular serine/threonine sites of the peptides (Fig. 5A bottom). Collectively, the following kinases were validated by means of both approaches (i.e., the motif-based analyses and in vitro assay): CaMK2 and PKC (for pS1049 on HER2), PKA and PKC (for pS1050), AKT and PKA (for pS1051), and CDK5 and ERK (for pS1073).

We next focused on the protein kinases identified in our phosphoproteome data to further evaluate if the kinases used in the in vitro kinase assay phosphorylate HER2 in vivo (Table III). Among the candidate kinases, PKC, AKT, and ERK belong to the EGFR/HER2 signaling pathways, so it is unlikely that these kinases are activated by lapatinib. Indeed, ERK1 (pT202/pY204), ERK2 (pT185/pY187), and PKC (pS304) were down-regulated (Table III). Previous studies also suggested that AKT phosphorylation was inhibited by lapatinib treatment in breast cancer cell lines, including SKBR3 (14, 16). Further, we observed up-regulated phosphorylation at T14 for the CDK family (CDC2, CDK2, CDK3, and CDK5) in this study, in accordance with a report that the activity of this kinase family is down-regulated by phosphorylation (55). Consequently, CaMK2 and PKA are expected to be involved, and this is further supported by the fact that sequence motifs of CaMK2 (R-X-X-S/T) and PKA/PKC (R-X-S/T, R-R/K-X-S/T) were enriched from the up-regulated phosphopeptides data set by a motif-extraction analysis using motif-X (68) (Fig. 5B). Further, the PKA phosphorylation site at T198 (class II), which is required for the kinase activation (69), was increased within 1 min on lapatinib treatment (supplemental Fig. S8B and Table III), whereas the CaMK2 autophosphorylation site at T287 (70) was unchanged in this study. Therefore, PKA is one of the kinase candidates to phosphorylate the HER2 serine/threonine sites in the region of amino acids 1047–1072.

Another question is whether or not the up-regulated phosphorylation sites affect the HER2 kinase activity. The phosphorylation site at S1166 of EGFR, identified in this study, was previously shown to negatively regulate EGFR tyrosine kinase activity by 1.5- to 3.5-fold (61). Similarly, inactivation of tyrosine kinase activity was observed on PKA-induced phosphorylation of EGFR (56) and CaMK2-induced phosphorylation of HER2 at T1172 (64). Such a regulatory mechanism using negative feedback through EGFR serine/threonine phosphorylation could be important to modulate signal generation (56, 61). Considering these observations, the region-specific multiple phosphorylations on HER2 characterized in this study seems likely to regulate HER2 tyrosine kinase activity as well. To verify this hypothesis, we treated SKBR3 cells with H-89 (71), a selective inhibitor of PKA, for 20 min before lapatinib-EGF treatment and compared the resulting cells with the control cells treated with lapatinib-EGF in terms of phosphorylation changes of HER2, using a quantitative phosphoproteomic approach. We found that H-89 treatment inhibited the phosphorylation of the sites between amino acids 1047–1072 on HER2, which correspond to the sites phosphorylated by PKA in vitro, in a dose-dependent manner (Fig. 5C). Interestingly, the phosphorylation states at amino acids 1073–1096 were unchanged by H-89 treatment, suggesting that not only PKA, but also other kinases are involved in this phosphorylation event. It should be noted that tyrosine phosphorylation in HER2 at position Y877 was significantly down-regulated in a dose-dependent manner, showing that HER2 kinase activity was down-regulated by H-89 treatment via de-phosphorylation at amino acids 1047–1072 on HER2. Collectively, these results demonstrate that PKA phosphorylates the HER2 serine/threonine sites between amino acids 1047–1072 in response to lapatinib treatment and positively regulates the tyrosine kinase activity. It was shown that EGF stimulation did not change the PKA kinase activity via phosphorylation at T198 in HeLa cells (38), so lapatinib itself may induce the HER2 phosphorylation through PKA activation. Although the underlying mechanism of PKA activation by lapatinib remains unknown.
unclear, it should be accompanied with an increase of cAMP concentration. The next step would be to examine in detail the mechanism of PKA activation and the effect of PKA knock-down on HER2 serine/threonine phosphorylation as well as the clinical importance of these phosphorylations on HER2 in vivo.

**FIG. 4.** Impact of lapatinib on EGFR/HER2 phosphorylation. A, Landscape of identified and quantified EGFR/HER2 phosphorylation sites. Each identified site was assigned as up-regulated (red) or down-regulated (green) based on the quantitative results of the corresponding phosphopeptides (see also Table II). The reported phosphorylation sites were taken from the databases UniProtKB, PhosphoSitePlus, Phospho.ELM, and Networkin. Parenthesized sites indicate class II sites. B, Examples of time- and dose-dependent phosphorylation dynamics of EGFR/HER2 phosphopeptides. Boldface and underline show Class I phosphorylation sites (localization probability, \( p > 0.75 \)), whereas parenthesized sites are ambiguous phosphorylation sites. C, MS/MS spectra of HER2 phosphopeptides containing novel phosphorylation sites.
Temporal Profiling of Suppressive EGFR/HER2 Pathways

Table II

List of phosphorylated peptides from EGFR/HER2

*Novel phospho sites which had not been reported in UniProt-KB/Swiss-Prot 57.7, PhosphoSite (CST), PhosphoELM and PHOSIDA.

**EGFR**

| Peptide sequence* | No. of phosphosites | Identified sites (Class I: p > 0.75)* | Ambiguous sites (0 < p ≤ 0.75)* | Mascot score | PTM score | Regulation* |
|-------------------|---------------------|---------------------------------------|---------------------------------|-------------|-----------|-------------|
| TPLLSSLATSNNSTVACIDR | 1 | S1042 | – | 43 | 65 | n.a.* |
| TPLLSSLATSNNSTVACIDR | 2 | S1042, S1045 | – | 44 | 43 | down |
| TPLLSSLATSNNSTVACIDR | 3 | S1039, S1042, S1045 | – | 34 | 44 | down |
| NGLOSCQIKEFSLFQR | 1 | S1064 | – | 31 | 53 | down |
| RPAGSVQNYTHNQLPNAPSR | 1 | Y1110 | – | 40 | 72 | down |
| G1@H@Q@L@DNP@D@Q@D@F@P@K | 1 | – | S1162/S1166 | 36 | 88 | up |
| GSHQ@S@L@D@N@P@D@Q@D@F@P@K | 1 | Y1172 | – | 55 | 167 | down |
| GSHQ@S@L@D@N@P@D@Q@D@F@P@K | 2 | S1166, Y1172 | – | 50 | 92 | down |
| GSTAÑÀEYLR | 1 | Y1197 | – | 49 | 92 | down |

**HER2**

| Peptide sequence* | No. of phosphosites | Identified sites (Class I: p > 0.75)* | Ambiguous sites (0 < p ≤ 0.75)* | Mascot score | PTM score | Regulation* |
|-------------------|---------------------|---------------------------------------|---------------------------------|-------------|-----------|-------------|
| LLQET́LEVEPL[T]PSGAMPNQAQM | 1 | – | T701/T708 | 53 | 79 | down |
| LLIDIDETEYADGGK | 1 | Y877 | – | 47 | 102 | down |
| FVQVQEDLPAPLDSTFYR | 1 | S998 | – | 70 | 120 | down |
| S@S@S@R@GG@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 2 | – | S1049*|S1050*/S1051*/T1052* | 55 | 107 | up |
| S@S@S@R@GG@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 2 | S1049* | – | 32 | 32 | up |
| HR@S@S@R@GG@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 3 | S1050*, S1051*, T1052* | S1050*/S1054/T1060* | 40 | 96 | up |
| HR@S@S@R@GG@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 3 | S1051* | S1050*/S1054/T1060* | 43 | 109 | up |
| HR@S@S@R@GG@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 4 | S1051*, T1052*, S1054 | S1049*/S1050* | 47 | 122 | up |
| SS@G@D@L@T@L@G@E@P@S@E@E@A@P@R | 1 | S1054 | – | 99 | 135 | up |
| SPLAPSE@G@S@V@F@D@G@D@L@G@M@G@A@A@K | 1 | S1083* | – | 85 | 177 | up |
| SPLAPSE@G@S@V@F@D@G@D@L@G@M@G@A@A@K | 2 | S1078*, S1083* | – | 64 | 163 | up |
| GLQLP@T@H@D@P@S@P@L@Q@R | 1 | S1100* | – | 47 | 126 | down |
| GLQLP@T@H@D@P@S@P@L@Q@R | 1 | T1103* | – | 49 | 90 | down |
| GLQLP@T@H@D@P@S@P@L@Q@R | 1 | S1107 | – | 48 | 105 | down |

Impact of Lapatinib on Signaling and Protein-protein Interaction Networks—To obtain a global view of lapatinib-regulated protein networks, we mapped all of the 210 regulated phosphoproteins onto a curated PPI network derived from the STRING database (43), yielding 221 connections (edges) among 101 proteins (nodes) based on high confidence scoring (score = 0.7) (Fig. 6A). The numbers of both nodes and edges in the actual phospho-PPI network were significantly higher than those in the two sets of random networks (43), yielding 221 connections (edges) among 101 proteins (nodes) based on high confidence scoring (score = 0.7) (Fig. 6A). The numbers of both nodes and edges in the actual phospho-PPI network were significantly higher than those in the two sets of random networks (n = 1,000) generated from either the whole protein data set identified in this study or the UniProt/Swiss-Prot protein database (p < 0.05) (see Experimental Procedure) (Fig. 6B left and middle). In addition, in the actual networks, the number of interactions between molecules previously undefined as EGFR/HER2 pathway proteins and the known EGFR/HER2 pathway proteins is significantly higher than for other combinations of randomly extracted proteins (p < 0.05) (Fig. 6B right). These results suggested that the network formed in the obtained phospho-PPI networks was much denser than that in the random networks, and the uncharacterized proteins are regulated together with the EGFR/HER2 pathways at the network level. As expected, EGFR/HER2 pathway proteins (shown in pink in Fig. 6A) were highly enriched. Further, this analysis identified protein networks involved in transcription/translation regulation (purple in Fig. 6A) and cytoskeletal organization (yellow in Fig. 6A), which have not previously been linked to EGFR/HER2 pathways. This result is consistent with the results of GO enrichment analyses in which the relevant terms (e.g., microtubule, cytoskeleton, RNA-binding, nucleotide-binding) were enriched (p < 0.01) (supplemental Fig. S3–S5).

Several phosphoproteomic studies have used public PPI databases to profile protein networks modulated by perturbations (24, 26, 72–74). However, there has been no previous quantitative evaluation of the significance of applying phosphoproteome data to PPI network analysis. We addressed this issue by using computational approaches and investigated whether the phospho-PPI networks reflect the biological properties of signaling networks. To do this, we hypothesized that adjacent signaling proteins in the actual phospho-PPI net-
works tend to have similar phosphorylation dynamics profiles, as the phosphorylation cascade is spatiotemporally extended between proteins via protein kinases from receptors to transcriptional factors. In other words, there should be a linear correlation between the path lengths of nodes (proteins) in the actual network and similarity of phosphorylation dynamics profiles. To test this hypothesis, we performed data mining using the phospho-PPI network and phosphorylation dynam-

**Fig. 5.** Screening for putative protein kinases that mediate HER2 phosphorylation. A, Four HER2 synthetic peptides were used as substrates for in vitro kinase assay (upper). Three peptides that cover the up-regulated phosphosites on HER2 and one peptide containing Y1248 were used. Bold red indicates phosphorylation sites identified in this study. The results of in vitro kinase assay are summarized at the bottom. Peak areas of phosphorylated forms of the four peptides were quantified by LC-MS after in vitro reaction with a particular kinase. Red color represents peptides “phosphorylated” by particular kinases. AKT, RAC-alpha serine/threonine-protein kinase; CaMK2a, calcium/calmodulin-dependent protein kinase type II subunit alpha; CDK2/5, cyclin-dependent kinase 2/5; CK1a, casein kinase I isoform alpha, CK2a1, casein kinase II subunit alpha; CLK3, dual specificity protein kinase CLK3; DNA-PK, DNA-dependent protein kinase; ERK2, mitogen-activated protein kinase 1; MAPKAPK1/2, MAP kinase-activated protein kinase 1/2, GPRK7, G protein-coupled receptor (GPCR) kinase 7; GSK3a, glycogen synthase kinase-3 alpha; PKAα, cAMP-dependent protein kinase catalytic subunit alpha; PKCa, protein kinase C alpha type; PKD1, 3-phosphoinositide-dependent protein kinase 1; STK39, STE20/SPS1-related proline-alanine-rich protein kinase. B, A result from Motif-X analysis of the phosphopeptides showing up-regulation profiles. C, Effects of H89 treatment on HER2 phosphorylation. The white, gray, and black boxes indicate the phosphorylation changes in the control and after 1 µM H89 and 10 µM H89 treatments, respectively.
ics-based network (44) in which pairs of peptides with strongly correlated phosphorylation dynamics ($R > 0.99$) were connected (Fig. 6C upper). This phosphorylation dynamics-based network included known interacting partners, such as EGFR-SHC1 and NCBP-NUP98 (Fig. 6C upper right). We calculated all possible short path lengths (SPLs) for any two proteins in the PPI network as well as for any two peptides in the phosphorylation dynamics-based network. Note that SPLs are defined as the minimum number of steps (edges) between two nodes. As expected, we observed a linear correlation between SPLs of the actual network and those of the dynamics-based network (Fig. 6C bottom left), whereas no correlation was observed for the negative control using random PPI networks ($n = 1000$) (Fig. 6C bottom right). This result suggests that quantitative phosphoproteome analysis combined with PPI-based analysis can successfully capture signaling networks in a biological system.

It is noteworthy that this PPI-networks analysis implies that lapatinib impacts on nuclear protein networks such as transcriptional factors, spliceosome, and ribonucleoprotein (RNP) complexes (i.e. heterogeneous nuclear RNP, small nuclear RNP, telomerase, and ribosome) (see purple proteins in Fig. 6A right). Enrichment of the RNP complexes (HNRPC, PCBP1, HNRNPH1, HNRNPH2, NCBP1, SMG6, and ASCC3L1) suggests that lapatinib targets the pre-mRNA splicing process as a result of inhibition of EGFR/HER2 pathways. We observed that telomerase reverse transcriptase (TERT)-associated proteins (SMG6, PTGES3, and HNRPC) showed repression of phosphorylation. It has been reported that more than 85% of human tumors have high telomerase

### Table III

**List of protein kinases regulated by lapatinib**

Number of phosphosite(s) is indicated following the sequence.

| Gene name | Down-regulation profile | Up-regulation profile |
|-----------|-------------------------|-----------------------|
| CDC42BPB | Serine/threonine-protein kinase MRCK beta | Serine/threonine-protein kinase MARK2 |
| EEF2K | Elongation factor 2 kinase | CDK2 |
| EGFR | Epidermal growth factor receptor | Cyclin-dependent kinase 2 |
| ERBB2 (HER2) | Receptor tyrosine-protein kinase erbB-2 | Cyclin-dependent kinase 3 |
| PAK4 | Serine/threonine-protein kinase PAK 4 | Cell division control protein 2 homolog |
| PDK1\(^c\) | 3-phosphoinositide-dependent protein kinase 1 | Cell division cycle 2-related protein kinase 7 |
| PDPK2\(^c\) | 3-phosphoinositide-dependent protein kinase 2 | Dual specificity protein kinase CLK3 |
| PRKCD | Protein kinase C delta type | Cyclin-dependent kinase 2 |
| MAPK3 | Mitogen-activated protein kinase 1 | Cyclin-dependent kinase 3 |
| MAPK3 | Mitogen-activated protein kinase 3 | Cell division cycle 2-related protein kinase 7 |
| MARK2 | Serine/threonine-protein kinase MARK2 | Dual specificity protein kinase CLK3 |
| PRKACA\(^e\) | cAMP-dependent protein kinase catalytic subunit alpha | Cyclin-dependent kinase 2 |
| PRKACB\(^e\) | cAMP-dependent protein kinase catalytic subunit beta | Cyclin-dependent kinase 3 |
| PRKACG\(^e\) | cAMP-dependent protein kinase catalytic subunit gamma | Cell division cycle 2-related protein kinase 7 |
| PRKCD | Protein kinase C delta type | Dual specificity protein kinase CLK3 |
| MAP3K7 (TAK1) | Mitogen-activated protein kinase kinase 7 | Cyclin-dependent kinase 2 |

\(^a\) Underline indicates unambiguous (Class I) sites while parentheses indicate ambiguous (Class II, III) sites.

\(^b\) Kinases sharing the same peptide sequence were separately counted.
FIG. 6. Phospho-PPI network of 10 μM lapatinib-regulated phosphoproteins. The phospho-PPI network was constructed from 210 phosphoproteins regulated by 10 μM lapatinib using the STRING database. A, Overview of the extracted protein networks (left). Pink boxes were predominantly assigned as EGFR/HER2 signaling proteins. Yellow, purple, and gray boxes indicate proteins involved in transcriptional/translational regulation, cytoskeletal organization, and other functions, respectively, based on UniProtKB annotation. (right) Note that EGFR/HER2 signaling proteins involved in transcriptional/translational regulation and cytoskeletal organization were also included in the PPI networks shown in purple and yellow. B, Comparison of numbers of nodes, edges and interactors between the actual and random networks (see experimental procedure). C, Phosphorylation dynamics-based network analysis along with the STRING PPI-network data. SPL, shortest path length; NCBP1, nuclear cap-binding protein subunit 1; NUP98, nuclear pore complex protein Nup98-Nup96; CLIP1, CAP-Gly domain-containing linker protein 1; AHNK, neuroblast differentiation-associated protein AHNK.
activity owing to transcriptional up-regulation of TERT. Thus, telomerase inhibition is regarded as an effective anti-cancer therapy for breast and colorectal cancer (75). Further, cap-dependent translation seems to be affected by lapatinib through inhibition of phosphorylation of eIF4F (eIF4G1), ribosomal proteins (RPS6, RPLP1, and RPLP2), and a nuclear cap-binding protein (NCBP1), leading to an anti-cancer effect by preventing assembly of the eIF4F complex (76). We also identified transcriptional factor networks including PPARBP, GATAD2B, JUNB, ARID1A, ATF2, TRAP3, FOSL2, NCOA2, and TRIM28, which are mainly involved in hormone receptor signaling (77–80). Because growth and progression of breast cancer have been shown to be highly correlated with expression levels of hormone receptors, regulating such transcriptional factors through phosphorylation may be important to suppress breast cancer progression. In addition to the protein networks mentioned above, a microtubule-associated protein network (CLIP1/2, CLASP2, MAP4, STMN1, MARK2, and PRKACB) was identified (see yellow proteins in Fig. 6A). STMN1 acts as a regulatory relay integrating diverse intracellular signaling pathways (81) and its phosphorylation site at S25 (known to be targeted by MAPK (82)) was found to be down-regulated in this study, so STMN1 could be one of the key proteins in this network.

To uncover what kinds of proteins (networks) are modulated as the lapatinib concentration is increased, we analyzed the 71 unique proteins regulated only by 10 μM lapatinib, using STRING. Interestingly, we captured a core protein network involved in translation, as shown in Fig. 6A (supplemental Fig. S10), indicating that the higher drug concentration affects translation-associated proteins such as eIF4F, ribosomal proteins, and nuclear cap-binding protein, as mentioned above. Recently, two research groups have reported a new mode of action of anti-cancer drugs through spliceosome inhibition, followed by alteration of the pattern of gene expression in human cancer cells (83, 84). Thus, the phosphoprotein network involved in translation (e.g. spliceosome and RNP complexes) that we have identified may have a potential role in tumor progression. This information could be useful for exploring novel drug targets other than well-studied protein kinases.

CONCLUSIONS

Lapatinib, a dual tyrosine kinase inhibitor for EGFR/HER2, is of importance in the clinical treatment of breast cancer, and has the highest selectivity for EGFR/HER2 among current kinase inhibitors. To our knowledge, the present study is the first large-scale profiling of the perturbation of temporal phosphoproteome dynamics by a kinase inhibitor.

Our quantitative phosphoproteomic profiling study revealed that lapatinib treatment induces phosphorylation in specific regions of EGFR/HER2, i.e. its regulation of EGFR/HER2 phosphorylation is region-specific. The integrative approaches used here (i.e. literature, informatics analyses of phosphorylation motif, in vitro and in vivo kinase profiling and H89 treatment) identified PKA as the putative kinase mediating HER2 serine/threonine phosphorylation, and we also demonstrated that the induced HER2 phosphorylation contributes to the regulation of HER2 tyrosine kinase activity.

With respect to the impact of lapatinib on downstream signaling targets, as expected from the specificity of lapatinib, only a few percent of the 4953 identified phosphopeptides were regulated. The STRING PPI network analysis allowed us to identify lapatinib-regulated protein networks related to cytoskeletal organization, transcription, and translation, which have not previously been implicated in EGFR/HER2 signaling pathways. Although several proteome and phosphoproteome studies have used PPI- and pathway-based analyses for profiling the global impacts of a particular perturbation on entire protein networks, there has been no further in-depth data analysis of the obtained protein networks. We employed novel data-mining approaches with PPI network data and quantitatively showed that the uncharacterized PPI networks obtained by means of STRING form a part of the EGFR/HER2 signaling networks.

Taken together, our results provide a novel map of the EGFR/HER2 signaling network, including new molecules, based on the integration of quantitative phosphoproteomics using a EGFR/HER2-specific inhibitor with a variety of computational approaches.

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