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Multi-layered Proteomics Reveals Molecular Switches Dictating Biased Ligand-dependent EGFR Trafficking and Outcomes

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Running title: Molecular switches underlying EGF Receptor outputs.

SUMMARY

A fascinating conundrum in cell signaling is how stimulation of the same receptor tyrosine kinase with distinct ligands generates specific outcomes. To decipher the functional selectivity of EGF and TGF- α , inducing EGFR degradation and recycling, respectively, we devised an Integrated Multi-layered Proteomics Approach (IMPA). We analyzed dynamic changes in receptor interactome, ubiquitylome, phosphoproteome, and late proteome by quantitative mass spectrometry and identified 67 proteins regulated at multiple levels. Rab7 phosphorylation and RCP recruitment to EGFR were revealed as switches for EGF and TGF- α outputs, respectively, controlling receptor trafficking, signaling duration, proliferation and migration. By manipulating the level of RCP or phosphorylation of Rab7 in EGFR-positive cancer cells we demonstrated that the TGF- α -mediated response switched to an EGF-like response or vice versa as EGFR trafficking was rerouted. We propose IMPA as a general approach to uncover fine-tuned regulatory mechanisms in cell signaling.

INTRODUCTION

Living cells respond appropriately to their surroundings by activating plasma membrane spanning receptors such as Receptor Tyrosine Kinases (RTKs) and thereby adapting long-term outputs to various extracellular signals. How the core signaling cascades are orchestrated by different RTKs to elicit distinct cellular behaviors is still much debated. Signaling specificity can be modulated by rewiring of overlapping protein networks¹, protein-protein interactions², and differences in signal duration³. The spatiotemporal distribution of RTKs in subcellular compartments has also been associated with signaling specificity, often with contrasting results: for instance, a study showing that EGFR signaling occurs primarily at the plasma membrane⁴ challenged the finding that active receptors and signaling adaptors were functional in cytoplasmic endosomes following EGFR internalization⁵. The tight control of the endosomal distribution of activated EGFR⁶ or the dichotomy between receptor degradation in lysosomes and receptor recycling to the plasma membrane after internalization have been shown to profoundly affect signaling outputs⁷⁻¹⁰. Differential endocytic sorting of RTKs is now considered a fundamental process regulating signaling duration and thus long-term responses¹¹. Consequently, studying the molecular basis of RTK trafficking has tremendous implications for understanding diseases such as cancer that are caused by aberrant RTK signaling activation or derailed endocytosis¹².

The concept of biased ligand signaling, or functional selectivity, is well-established for G-protein coupled receptors (GPCRs), where ligands binding the same receptor activate full or only partial downstream signaling depending on cell-context¹³. However, it has only been scarcely explored in the context of RTKs, although a better knowledge of RTK functional selectivity could lay the foundation for revealing the molecular basis of signaling specificity and designing improved therapeutic drugs¹⁴. Using quantitative proteomics we have recently reported that FGFR2b trafficking, signaling duration, and responses are dictated by the engaged ligand⁹. It remains to be

determined whether this is specific to FGFR2b outputs or can be generalized to other ligand-RTK pairs. To systematically examine how biased ligands affect prototypical RTK signaling we focused on EGF Receptor (EGFR), the most studied RTK¹⁵. Among numerous EGFR ligands, we choose EGF and TGF- α , which induce differential sorting of internalized receptor¹⁶, yet their cellular effects have not been compared before to our knowledge.

As an alternative to multi-parametric image analysis or genetic screens combined with data-driven statistics^{17,18}, which are typically employed for this type of investigations, we performed a time-resolved analysis of EGFR signaling using mass spectrometry (MS)-based quantitative proteomics, a powerful technology for large-scale analysis of complex and dynamic signaling networks¹⁹⁻²². We developed an Integrated Multi-layered Proteomics Approach (IMPA) that combines five different layers of information on EGFR signaling (interactome, tyrosine and serine/threonine phosphoproteome, ubiquitylome, and late proteome) with functional assays to provide insights into the molecular mechanisms underlying EGFR responses. We analyzed the dynamic crosstalk between phosphorylation and ubiquitylation, the most prominent Post-Translational Modifications (PTMs) regulating RTK signaling, trafficking, and cellular responses²³. Our comprehensive resource of EGFR signaling events expanded previous proteomics studies focusing on single aspects of the EGFR signaling enigma²⁴⁻²⁷ and demonstrated the strength of a multidisciplinary approach for decoding functional selectivity and highlighting key regulatory protein hubs. Based on this we elucidated the role of Rab7 tyrosine phosphorylation and Rab-coupling protein (RCP) recruitment to EGFR as ligand-dependent molecular switches deciding EGFR trafficking and outputs in several cancer cell lines.

RESULTS

Integrated Multi-layered Proteomics Approach (IMPA) dissects dynamic EGFR signaling.

To study functional selectivity of RTKs, we focused on EGFR stimulated with EGF or TGF- α using the epithelial cervix carcinoma cell line HeLa as our model system, as this cell line endogenously express EGFR but not at overexpressing levels and is amenable for both proteomics, signaling and trafficking studies. We first analyzed EGFR trafficking by applying an immunofluorescence-based method that allows monitoring of receptor trafficking by confocal microscopy⁸. HA-tagged EGFR accumulated in the cytoplasm of cells stimulated for 8 or 40 min. with either ligand and recycled back to the cell surface after 90 min. (Fig. 1a-b). However, where TGF- α induced 91% EGFR recycling, EGF treatment only resulted in partial EGFR recycling (22%). This is consistent with previous reports that most of the EGF receptors are recycled after TGF- α stimulation, whereas EGF mainly causes receptor degradation^{16,28}. The ligand-dependent differential endocytic fate of EGFR was further confirmed by co-localization studies with GFP-tagged versions of Rab5, Rab7, and Rab11, known markers of early, degradation and recycling endosomes, respectively⁸. Endogenous EGFR co-localized with Rab5 and EEA1, another established marker of early endosomes, upon 8 min. stimulation with both ligands (Fig. 1c-d), confirming that receptor internalization did not depend on ligand type. At 40 min. however, EGFR displayed preferential accumulation in Rab7- or in Rab11-positive compartments by EGF or TGF- α , respectively (Fig. 1c-d), which indicated ligand-dependent differences in receptor degradation and recycling. We also confirmed that TGF- α was a stronger mitogen than EGF (Fig. 1e-f)²⁹. Together, these data demonstrate that EGF and TGF- α induce opposite EGFR endocytic fate and have different mitogenic potentials.

Based on our recent study of ligand-dependent FGFR2b signaling⁹, we reasoned that a global, multi-layered and time-resolved analysis of EGFR signaling in response to EGF and TGF- α would provide answers to the question of whether the molecular link between early events (e.g. receptor trafficking) and long-term outcomes is ligand/receptor pair-dependent. We used a quantitative proteomics approach (see Online Methods; Supplementary Fig.1a) to analyze cell lysates following EGF or TGF- α stimulation for 1, 8, 40 or 90 min. or 72h. These time points covered EGFR trafficking and early signaling events as well as long-term cellular outcomes. We collected information on five different layers of signaling events: EGFR interactome, tyrosine phosphoproteome, ubiquitylome, serine/threonine phosphoproteome and late proteome and defined this approach as Integrated Multi-layered Proteomics Approach (IMPA) (Fig. 2a; Supplementary Tables 1-4). The quality of our MS data was assessed by the high degree of reproducibility among two biological replicates for all five datasets, and the identification of peptides with high mass accuracy and in the high intensity range (Supplementary Fig. 1b-d). Samples from cells stimulated with EGF or TGF- α for 1-90 min. were analyzed for changes in phosphorylation and ubiquitin levels and we found 5541 phosphorylated and 1311 ubiquitylated sites mapping to 1949 and 782 proteins, respectively (class I sites; Fig. 2b, Supplementary Fig. 1e, and Supplementary Tables 1-2). Of the 5541 phosphorylated sites, 457 (8%) were Tyr-phosphorylated sites (Supplementary Fig. 1f), indicating a good enrichment of Tyr-phosphorylated proteins in our samples. Most of the PTM-modified peptides were singly phosphorylated or singly ubiquitylated (Supplementary Fig. 1g-h), in line with previous studies^{9,30}. We deemed sites to be regulated if their ratios were more than 2-fold in at least one experimental condition (see Online Methods). We found 1257 regulated phosphorylation sites (22.6%), 225 regulated ubiquitylated sites (17%) and more than half of all identified Tyr-phosphorylated sites (259 over 457), supporting the crucial involvement of Tyr phosphorylation in EGFR responses²⁶ (Fig. 2b). For the receptor interactome analysis we performed

immunoprecipitation of EGFR using lysates from the same cells described above and identified 855 proteins, of which 40 were dynamically recruited to EGFR at at least two time points (Fig. 2b, Supplementary Figure 1a, and Supplementary Table 3; see Online Methods for the definition of ‘EGFR interactor’). To analyze the late proteome changes we stimulated cells with the two ligands for 72h and identified 5926 proteins, of which 223 showed significant changes in abundance upon treatment (referred to as ‘regulated’) (Fig. 2b, Supplementary Table 4). By integrating the five data sets we covered 76.6% of the known proteome of HeLa cells³¹, of which 1036 proteins were regulated (Fig. 2b, Supplementary Table 5). Therefore, IMPA provides a good coverage of the EGFR-regulated proteome in HeLa cells.

We reasoned that central protein hubs in the EGF receptor network incorporate multi-level signals and would therefore be regulated on several levels. Consequently, to pinpoint potential candidates mediating ligand-dependent responses, we prioritized those that were regulated in more than one dataset. This quantitative filter short-listed 67 proteins, of which 30 were specifically regulated upon EGF and 15 upon TGF- α stimulation (Fig. 2c). Several proteins known to be involved in EGFR trafficking (Eps15, Cav1, Rab7)³² were regulated by both phosphorylation and ubiquitylation only in the EGF-treated cells (Fig. 2c). Another protein involved in endocytosis, RCP (also known as Rab11FIP1)³³, was not PTM-regulated, but TGF- α treatment changed both the abundance of this protein and its dynamic interaction with EGFR (Fig. 2c). These findings indicated that IMPA may represent a powerful means for studying signaling at multiple levels and suggest that the two ligands control EGFR trafficking, and possibly cellular outcome, through different mechanisms.

EGFR was the only protein regulated in all the datasets by both EGF and TGF- α (red in Fig. 2c). We confirmed the MS-based quantitation of the total EGFR levels by Western Blot (WB) analysis. EGF treatment resulted in EGFR degradation between 40 and 90 min., whereas TGF- α induced receptor stabilization upon 90 min. stimulation, in agreement with receptor recycling (Fig. 1, 2d-f).

We also found that TGF- α stimulation activated sustained Erk signaling, which is contrary to the transient Erk phosphorylation in response to EGF, as observed both in the phosphoproteome and by quantitation of WB analysis (Fig. 2 d-f). Erk phosphorylation was not detectable in cells treated with the specific EGFR inhibitor AG1478, confirming that the response to both ligands was mediated by EGFR (Fig. 2e-f). These data indicate that TGF- α -mediated stabilization of active EGFR influences signaling duration.

In conclusion, the IMPA analyses strongly indicated that each ligand/EGFR pair promotes specific mechanisms for the control of receptor endocytosis and signaling duration, resulting in distinct cellular outcomes.

Biased ligands specifically regulate PTMs.

To obtain insights into the molecular mechanisms underlying the opposite responses generated by EGF and TGF- α , we analyzed the distribution of regulated sites and EGFR interactors over time. The number of regulated Tyr phosphorylated sites was highest at 1 min., followed by regulated ubiquitylated sites at 8 min., and regulated Ser/Thr phosphorylation sites at 40 min.. Similarly to the regulation of phosphorylated Tyr, the highest number of proteins interacting with EGFR was found after one min. upon both EGF and TGF- α stimulation (Fig. 3a). Principal Component Analysis (PCA) showed a clear separation of early and late time points and pointed to 8 min. as the most divergent between EGF and TGF- α in all datasets (Fig. 3b), suggesting that signaling activated at 8 min. is the most stimulus-specific.

Gene Ontology (GO) enrichment analysis of the six temporal profiles (clusters) based on fuzzy c-means clustering of 330 regulated phosphorylated and 52 regulated ubiquitylated sites revealed that EGF and TGF- α shared the same enriched biological processes, like proliferation, migration, and endocytosis (Supplementary Fig. 2a-b). However, considering both the temporal pattern and the

total amplitude of the PTMs changes (defined by the similarity parameter S-score³⁴), EGF and TGF- α appeared to regulate phosphorylation in a similar way, but ubiquitylation differentially (Supplementary Fig. 2c). Together with a significant difference in the number of ubiquitylated sites in cluster 6 (recycling responders) in favor of TGF- α , this finding suggests that TGF- α enhances cellular ubiquitylation (Supplementary Fig. 2a-c). Enriched GO terms of ubiquitylated sites included signaling and ubiquitin-mediated activity but also long-term cellular events, like DNA repair and protein folding (Supplementary Fig. 2c). Of the 66 enriched proteins (79 sites) differentially regulated by the two ligands, 54 had more than one ubiquitylated sites and 27 (28 sites) were specifically regulated by TGF- α (Supplementary Fig. 2d). Interestingly, we found that the ubiquitylation sites of 20 TGF- α -regulated proteins belonging to sustained temporal profiles (3 out of 6 clusters) were static upon EGF stimulation (cluster 0) (Supplementary Fig. 2a and d, Supplementary Table 2), thus strengthening the idea that TGF- α induced sustained ubiquitylation compared to EGF in our experimental conditions (Fig. 3a). The chaperone protein Bag1 and the ubiquitin-conjugating enzyme UBE2T, involved in DNA repair^{35,36}, were also regulated by TGF- α in the proteome (Supplementary Fig. 2d, Fig. 2c), proposing a previously unknown link between TGF α -induced ubiquitylation, sustained signaling and long-term responses.

Next, we analyzed the dynamic regulation of phosphoproteome in more details. Firstly, we compared the dynamic EGFR Tyr phosphoproteome with the FGFR2b Tyr phosphoproteome previously measured in the same cell line⁹. PCA showed a clear separation between the two Tyr phosphoproteomes (Fig. 3c), suggesting that each ligand/RTK pair affects signaling duration and specificity in a unique way. Secondly, to identify protein kinases activated downstream of EGFR we looked for enriched amino acid residues adjacent to the regulated phosphorylation sites. This disclosed that TGF- α favored a proline in the position immediately C-terminal to the phosphorylation site (Fig. 3d), a feature of many Erk substrates. This finding was supported further

as we observed that TGF- α , but not EGF, mediated prolonged activation of Erk up to 72h stimulation (Fig. 3e-f).

Finally, we studied the crosstalk between phosphorylation and ubiquitylation upon EGFR activation by comparing the number of modified kinases, phosphatases, ubiquitin E3 ligases and deubiquitinating enzymes (DUBs), representing the writers and erasers of these two PTMs³⁷. Kinases (especially Tyr kinases) and phosphatases were more significantly phosphorylated than ligases and DUBs when compared to the expressed HeLa proteome³¹ (Fig. 4a, Supplementary Tables 1-2). Although writers and erasers were in general mainly modified by the PTM they regulate, there was an extensive crosstalk of the two PTM systems as well, with slightly higher spread of phosphorylation on ligases/DUBs compared to ubiquitylation on kinases/phosphatases (Fig. 4a). We found 260 proteins that were both phosphorylated and ubiquitylated and 25 of these were regulated at both PTM levels (Fig. 4b, Supplementary Tables 1-2). GO enrichment analysis of the regulated doubly versus singly modified proteins showed enrichment not only of signaling and endocytosis processes (e.g. EGFR, Eps15, Rab7, ERBB2), but also of long-term responses like metabolism and proliferation (e.g. HSPD1, HUWE1, TRIM28, EIF3B) (Fig. 4 c-e). We analyzed ligand-dependent regulation of these 25 doubly modified proteins, and found that EGF mainly induced phosphorylation at early time points (1-8 min.), whereas TGF α treatment resulted in sustained phosphorylation of the same proteins and in prolonged ubiquitylation of several of them (Supplementary Fig. 3). Overall, these data confirm that regulated phosphorylated and ubiquitylated proteins involved in signaling, trafficking and downstream responses have opposite temporal profiles in response to the two EGFR ligands.

EGFR degradation and outputs depends on Rab7 phosphorylation on Tyr183.

To assess whether IMPA is a powerful approach to uncover candidates mediating RTK functional selectivity, we focused on endocytic proteins specifically regulated on multiple levels upon either

EGF or TGF- α stimulation, as these two ligands induces opposite intracellular fate of EGFR (Fig. 1, 2c). One such candidate indicated by IMPA was Rab7, a known marker of late endosomes deregulated in several diseases^{38,39}. Rab7 was doubly modified by EGF at early time intervals, but its levels did not change upon 72h stimulation (Fig. 2c; see also Fig 7b). EGF promoted the phosphorylation of Rab7 on Tyr183 at 8 min. with a two-fold increase compared to TGF- α (observed in three independent biological replicates; Fig. 5a-b, Supplementary Fig. 4a-d, Supplementary Tables 1, 2, and 6). Upon transfection of cells with GFP-tagged wild type Rab7 (wt) or Rab7_Y183F mutant, in which Tyr 183 was changed to phenylalanine, we confirmed that EGF induced the phosphorylation of Rab7 on Y183 at 8 but not 40 min. twice stronger than TGF- α (Fig. 5c). We then studied EGFR trafficking in the presence of wt or Rab7_Y183F mutant. In EGF-stimulated cells for 40 min., EGFR mainly co-localized with Rab7 in wt cells, whereas it switched to co-localization with the recycling marker Transferrin receptor (Tf-R) in Rab7_Y183F expressing cells (Fig. 5d and 5e, middle panels). This finding suggests that the level of Y183 phosphorylation on Rab7 at 8 min. in the presence of EGF acts as a molecular switch determining EGFR fate at later time intervals (40 min.). This effect did not depend on a cellular de-localization of the Rab7 mutant compared to Rab7 wt (Fig. 5e, top panels) and was EGF-specific, as the majority of TGF- α -stimulated EGFR was still found in Tf-R-positive vesicles both in wt and mutant Rab7 cells (Fig. 5d and 5e, bottom panels). These data indicate that TGF- α -dependent receptor recycling was not affected by Rab7 Y183 phosphorylation. Furthermore, EGF induced receptor stabilization and sustained Erk activation in the presence of Rab7_Y183F but not in wt cells, whereas TGF- α -dependent prolonged response was not affected by the mutant Rab7 (Fig. 2e, 5f) Collectively, these results indicate a link between ligand-dependent receptor recycling and signaling duration^{8,9,28}. Finally, we verified the effect of Rab7 Y183 phosphorylation on cell proliferation in HeLa cells and other cancer cell lines of different origin. In the presence of wt Rab7 all the tested cell lines

proliferate less upon EGF than upon TGF- α stimulation (Fig. 1 e-f, 5g, Supplementary Fig. 4e). However, EGF and TGF- α stimulated proliferation to a similar extent in cells transfected with Rab7_Y183F (Fig. 5g), suggesting that EGF-mediated EGFR stabilization and sustained Erk activation due to increased receptor recycling in the absence of Rab7 phosphorylation was crucial for the magnitude of the downstream response.

Overall, these data point to the higher extent of EGF-mediated phosphorylation of Y183 on Rab7 compared to TGF- α as a molecular switch priming EGFR for degradation, ultimately affecting signaling duration and long-term responses. EGF stimulation resulted in EGFR ubiquitylation and in the recruitment of the known E3 ligase Cbl-b to the activated receptor (Supplementary Fig. 5 and Fig. 6a) that marks the receptor for degradation as previously established^{32,40,41}. Therefore, Rab7 phosphorylation is as significant as EGFR ubiquitylation in controlling receptor degradation.

RCP mediates TGF- α responses.

In our experimental conditions the ligand for EGFR recycling TGF- α induced sustained receptor ubiquitylation, prolonged phosphorylation of the Cbl family binding site Y1045 and of the Grb2 binding site Y1068 on EGFR⁴¹, and the recruitment of Cbl-b, Grb2 and Shc to the receptor with different kinetics (Supplementary Fig. 5 and 6a, Fig. 6a). We hypothesized that TGF- α -mediated EGFR recycling occurred because TGF- α , besides not regulating Rab7 phosphorylation to the same extent as EGF (Fig. 5), promoted the binding to the receptor of proteins belonging to the recycling pathway. Thus, we analyzed the interactome dataset for ligand-selective EGFR interactors (Supplementary Table 3; see also Online Methods). We found a strong network of proteins centered on EGFR, which could be divided into a group of 25 EGF-dependent interactors of EGFR including canonical RTK players (Cbl, Shc, Vav2, PI3K, SOS, PLC γ) and a group of 16 TGF- α enriched proteins comprising the cell cycle regulator Cdc23, the adaptor DVL3, and proteins involved in

vesicle transport (RCP/Rab11FIP1, Rab6a) (Fig. 6b). Immunoprecipitation followed by WB analysis demonstrated that TGF- α , but not EGF induced the persistent formation of the EGFR/Grb2/RCP or of the EGFR/Grb2/DVL3 complexes (Fig. 6c and Supplementary Fig 6). The sustained binding of Grb2 to the receptor is consistent with sustained phosphorylation of the Grb2 binding site Y1068 on EGFR upon TGF- α stimulation (Supplementary Fig. 5, Fig 6a). These findings validated the interactome data and supported the idea that TGF- α facilitates the recruitment of unique proteins to EGFR.

We focused on RCP because this Rab11 effector has previously been associated to integrin-mediated EGFR endocytosis³³. In addition, this protein was regulated in both the interactome and proteome datasets by TGF- α but not by EGF (Fig. 2c). TGF- α -mediated EGFR trafficking and signaling duration change in cells depleted of RCP by siRNA compared to cells transfected with control siRNA. The majority of EGFR co-localized with Rab11 and RCP in wt cells stimulated with TGF- α for 40 min., whereas the receptor was found in Rab7-positive compartments in the absence of RCP upon both EGF and TGF- α stimulation (Fig. 6d-e). Furthermore, in cells depleted for RCP and stimulated with TGF- α EGFR stability decreased resulting in transient Erk phosphorylation (Fig. 6f). These data suggest that RCP is a molecular switch for EGFR recycling and signaling in response to TGF- α . RCP bound to Rab11 and Rab5 upon TGF- α , but not EGF stimulation for 8 min. (Fig. 6g), indicating that the fast recruitment of RCP to Rab5-positive early endosomes primes EGFR for recycling in our experimental conditions. These data also support the idea that the early time point 8 min. is crucial for the regulation of trafficking and signaling upon each stimulus (Fig. 3b, 5, 6).

Next, we showed by WB that RCP protein levels were higher upon TGF- α than upon EGF treatment, whereas Rab7 levels were unaffected in lysates from HeLa cells and three other cancer

cell lines, confirming what we observed in the proteome dataset (Fig. 2c, 7a-b). As GO term analysis showed an enrichment of intracellular signaling and migration in TGF- α -stimulated cells for 72h (Fig. 7a), we wanted to address whether long-term Erk activation (Fig. 3e-f) and cell migration depended on RCP. In cancer cell lines depleted of RCP and stimulated for 24-72h with TGF- α , Erk phosphorylation was absent and cell proliferation was reduced compared to wt cells (Fig. 7b-c). Furthermore, we did not observe TGF- α -induced cell migration in the absence of RCP (Fig. 7d). Conversely, EGF still activated Erk at 24h but not 72h stimulation and induced cell proliferation and migration in both RCP-depleted and in wt cells (Fig. 7b-d). RCP is therefore required for TGF- α - but not EGF-dependent long-term responses in several cancer cells.

In conclusion, based on IMPA, we uncovered two unique molecular switches underlying EGFR trafficking in response to the biased ligands EGF and TGF- α with different mitogenic potential. Our data demonstrate that TGF- α -dependent increase of RCP protein levels and of the RCP/EGFR/Grb2 interaction at early time points, which resulted in receptor accumulation into Rab5/Rab11/RCP-positive early endosomes followed by recycling, affected signaling duration and long-term responses. EGF instead induces a transient modification of the proteins necessary for EGFR degradation, including receptor ubiquitylation and Rab7 Y183 phosphorylation. The decrease of Y183 phosphorylation (e.g. in the presence of Rab7_Y183F mutant) switched EGF responses (receptor trafficking, signaling duration, long-term outputs) into TGF- α like responses (Fig. 7e).

DISCUSSION

This study provides a comprehensive resource quantifying temporal changes in EGFR responses upon stimulation with two ligands inducing opposite receptor trafficking. Sequential analysis of EGFR interactome, phosphoproteome, ubiquitylome and late proteome changes by MS-based

quantitative proteomics, an approach here defined as IMPA, revealed EGF- and TGF- α -specific mechanisms for the regulation of EGFR endocytic routes, signaling, and long-term outputs.

Although thousands of modified sites and proteins can be accurately and reproducibly identified and quantified in routine large-scale MS-based proteomic studies, it still remains challenging for the signaling community to pinpoint the key drivers in the process of interest²². IMPA offers an original solution to this current dilemma. The integration of five different PTM-proteome layers of information allowed us to restrict the number of candidates regulated in an EGFR ligand-specific manner from 7053 identified proteins to 30 EGF- and 15 TGF- α -specific proteins that were regulated at multiple levels (Fig. 2). Such a wide-ranging analysis comparing two ligands for the same RTK, stimulating cells for different time points, collecting five datasets might not reach the same depth in the number of identified proteins or modified sites as that obtained considering a single layer of information^{31,42,43}. However, we covered close to 80% of the known HeLa proteome without using any biochemical trick for boosting the number of identifications, like treatment with pervanadate or proteasome inhibitor^{42,43}. Furthermore, by manipulating Rab7 phosphorylation and RCP levels we demonstrated that we can convert one specific ligand-response into the other and vice versa, thus underscoring the power of our methodology. We envision that scientists who are looking for both an unbiased approach to address specific questions in cell signaling and a reliable method to prioritize candidates for further studies will adapt IMPA. In addition to the analysis of spatiotemporal regulation of signaling⁹ and of PTMs crosstalk³⁷, including a multi-layered proteomics workflow in routine experimental design will prove powerful to quantify differences in intracellular responses and solve cell signaling conundrums.

Recent mechanistic studies on EGFR ligand-induced dimerization⁴⁴ and our functional data on EGFR trafficking regulated by EGF and TGF- α challenged the thirty-year idea that EGFR is the prototype receptor for studying RTK biology¹⁵. Without disqualifying the literature on EGFR

signaling and the validity of EGFR as a model system, our findings support the hypothesis that each ligand-RTK pair (i.e. FGF10/FGFR2b, NCAM/FGFR1 or TGF- α /EGFR) has developed its own regulatory mechanism for the control of receptor trafficking and downstream events. Therefore, the findings on EGFR-dependent regulation of signaling cannot be generalized to all the other RTKs. For instance, FGF10 induces FGFR2b recycling by promoting the recruitment of SH3BP4 to the FGFR2b/PI3K complex⁹ and GGA3 binding to c-Met is necessary for receptor recycling¹⁰, whereas TGF- α -mediated EGFR recycling requires the binding of RCP to the receptor (Fig. 6). These data clearly indicate that RTK endocytic sorting for recycling depends on the recruitment of specific adaptors to the receptor, ultimately affecting biological outputs, i.e. cell proliferation and migration (this study, ⁷⁻¹⁰). Although the validity of this innovative concept needs to be confirmed in other RTKs as well, our findings suggest that recycling is not only an actively regulated process that affects long-term responses, but also that the manipulation of trafficking of certain RTKs might redirect signaling outputs. As perturbations of RTK trafficking machinery have been associated with cancer progression¹², building an atlas of endocytic proteins specifically regulated by each ligand-RTK pair (e.g. via multi-layered proteomics) may pave the way for targeted intervention in human cancer.

In the context of EGFR signaling, TGF- α deregulation has been associated to aggressive tumors, especially breast cancer, where its expression is controlled by estrogen²⁹. Given that the TGF- α -dependent EGFR/RCP/Grb2 complex is central for sustained signaling activation (e.g. Grb2-mediated activation of Erk) and cell migration (Figures 6-7), a possible therapeutic scenario in breast and perhaps other cancers might be to selectively target this complex or TGF- α responses. This idea is supported by the facts that 1) RCP is highly expressed in estrogen receptor-positive breast tumors and specifically controls breast cancer cells migration⁴⁵; 2) both TGF- α - and integrin, but not EGF-mediated migration depend on RCP and recycling in general (Fig. 7, ³³); 3) TGF-

α induces a unique signature of ubiquitylated and phosphorylated proteins regulating not only signaling and endocytosis but also transcription, DNA replication or damage, and proliferation, suggesting the possibility to fine-tune TGF- α signaling with surrogate ligands as successfully done for EpoR⁴⁶; 4) TGF- α signaling is sustained and signaling dynamics is now considered pharmacologically modifiable⁴⁷.

Thus, the multi-layered proteomics approach described here has the potential to improve our understanding of RTK functional selectivity⁴⁸, offering a defined wealth of novel candidates for hypothesis generation. In a broader perspective, it might guide individualized medicine when integrated with data from other omics studies⁴⁹.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession Codes The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium⁵⁰ via the PRIDE partner repository with the dataset identifier PXD001996. Project name: Multi-layered proteomics of EGFR signaling. Project accession: PXD001996. Reviewer account details: reviewer37607@ebi.ac.uk. Password: TVyVytn0.

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AUTHOR CONTRIBUTION

M.P. performed the experiments shown in Fig. 2e-f, 3e-f, 5f, S5b-c, 6a, 6g, S6. K.T.G.R. contributed to data analysis shown in Figures 2c, 3a-d, 4, 6b, 7a and S1, S2, S3, S5a. J.O.S. performed the proteome experiment described in Figures 2c, S1 and 7a. G.C. prepared the Rab7 mutants construct. B.B edited the manuscript and discussed the results. C.F. generated and analyzed the data shown in remaining figures. C.F. and J.V.O. designed the experiments, critically evaluated the results, supervised M.P. and J.O.S., and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents

The following commercial reagents were used: EGF and TGF- α (PreproTech, London, UK); DMSO and BrdU (Sigma-Aldrich, St. Louise, MO); the EGFR inhibitor AG1478 (Cell Signaling Technology, Danvers, MA); tetramethylrhodamine-conjugated transferrin (TRITC-Tf) (Invitrogen, Carlsbad, CA).

Antibodies: rabbit anti-EGFR (Upstate, Millipore, Billerica, MA, used in WB and immunoprecipitation analysis); mouse anti-EGFR (Calbiochem, Millipore, Billerica, MA, used in immunofluorescence and immunoprecipitation analysis); mouse anti-phosphotyrosine (4G10) (EMD, Millipore, Billerica, MA); mouse anti-vinculin (Sigma-Aldrich); rabbit anti-DVL3, rabbit anti-RAB7, rabbit anti-K48 and anti-K63, rabbit anti-PI3K, rabbit anti-phospho EGFR Y1045 and anti-phospho EGFR Y1068, mouse anti-phospho-ERK1/2 (E10) and rabbit anti-ERK1/2 (137F5), mouse anti-phospho-tyrosine (P-Y100), anti-SHC (Cell Signaling Technology); mouse anti-poly-ubiquitinated conjugates (clone FK1) (Enzo Life Sciences); mouse anti-CBLb (G-1), mouse anti-GFP (B-2), rabbit anti-RAB5A (S-19), mouse anti-ubiquitin (P4D1), goat anti-EEA1 (Santa-Cruz Biotechnologies, Santa Cruz, CA); mouse anti-GRB2 (BD Biosciences, San Jose, CA); rabbit anti-RAB11 (Invitrogen, Paisley, UK); rabbit anti-RAB11FIP (RCP) (Novus Biologicals, LLC, Littleton, CO); mouse anti-HA tag, used in immunofluorescence, (HA.11, Covance, Emeryville, CA)..

Expression Vectors

The pcDNA3.0 vector encoding N-terminally HA-tagged EGFR, in which the endogenous signal sequence of EGFR was replaced by an influenza virus hemagglutinin signal sequence followed by the HA epitope, was kindly provided by Y. Chen (Shanghai, China). The cDNA for Rab5-GFP was

from M. Zerial (Dresden, Germany). The cDNA for Rab11-GFP was from F. Senic-Matuglia and B. Goud (Institute Curie, Paris, France). The cDNA for Rab7-GFP used in the immunofluorescence analysis shown in Figure 1a was from B. van Deurs (University of Copenhagen, Denmark). The GFP-tagged human Rab7 (GFPRab7a) and its mutant containing phenylalanine (F) at position 183 (Rab7-Y183F) were synthesized by GeneArt (Invitrogen, Germany), cloned into the pCEP4 vector and used in all the other figures.

Cell Culture and SILAC labelling

Human epithelial cervix carcinoma HeLa cells, breast adenocarcinoma cells MDA-MB231, lung adenocarcinoma A549 cells were purchased from ATCC. Ovarian cancer cell line HeyA8 was kindly provided by Dr. U. Cavallaro (Milan, Italy). Cells were cultured in DMEM (Gibco, Invitrogen), supplemented with 10% fetal bovine serum, 100U/mL penicillin (Invitrogen), 100µg/mL streptomycin (Invitrogen), at 37°C, in a humidified incubator with 5% CO₂. All experiments were performed at 80% confluence.

For quantitative mass spectrometry, HeLa cells were labelled in SILAC DMEM (PAA Laboratories GmbH, Germany) lacking arginine and lysine. They were supplemented with 10% dialyzed fetal bovine serum (Sigma), 2 mM glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin for 15 days to ensure complete incorporation of amino acids (data not shown). Three cell populations were obtained: one labeled with natural variants of the amino acids (light label; Lys0, Arg0), the second one with medium variants of amino acids (L-[13C6] Arg (+6) and L-[2H4]Lys (+4)) (Lys4, Arg6) and the third one with heavy variants of the amino acids (L-[13C6,15N4]Arg (+10) and L- [13C6,15N2]Lys (+8)) (Lys8, Arg10). The light amino acids were from Sigma, while their medium and heavy variants were from Cambridge Isotope Labs (Massachusetts, US).

Transfection and RNA Interference

HeLa cells were transfected using Lipofectamine (Invitrogen), according to manufacturer's instructions, and all the assays were performed 36 hours after transfection, as described^{8,9}. Double-strand, validated Stealth siRNA oligonucleotides targeting human RAB11FIP (RCP) (sequences: 5'-GGUCCUCAAACAGAAGGAAACGAUA-3'; 5'-GAAGACUACAUUGACAACCUGCUUG-3' and 5'-UCCGCAUCCCGACUCAGGGUUGGCAA-3', called siRNA#1, siRNA#2 and siRNA#3, respectively) were purchased from Invitrogen. HeLa cells were transfected either with siRNA#1 and siRNA#2 (Figure 6f) to test off-target effects or with a mixture of all RCP-targeting siRNAs (in all the other experiments), as previously described⁹. A Negative Control Med GC siRNA duplex was used as a negative control (Invitrogen, Cat. Num. 12935300). Silencing of gene expression was monitored by immunofluorescence (Figure 6e) and immunoblotting of cell lysates with antibodies against RAB11FIP (RCP) (Figure 6f).

Cell Lysis, Immunoprecipitation, and Western blots

Cells were cultured in either Nunc® Petri dishes (100 or 150mm for WB and immunoprecipitation analysis) or 245mm dishes for SILAC-based experiments. Cells were cultured in complete medium, and then serum-starved overnight in serum-free medium. Cells were stimulated for the indicated time points with 100 ng/mL of EGF or TGF- α . Ligands have been replenished every 24 hours for long-term (24-72 h) stimulation. When needed, cells were pre-incubated for 2 hours with chemical inhibitors at the following concentrations: 500 nM AG1478. Control cells were pre-incubated with DMSO alone. After stimulation, cell extraction and immunoblotting were performed as described⁹. Each experiment was repeated at least three times.

Immunoprecipitation of EGFR, GFP-tagged Rab5 or Rab7 and its mutant or GRB2 from cell extracts was performed as described⁹, using a mixture of anti-EGFR antibodies, anti-GFP (B-2), and anti-GRB2 antibodies, respectively. Each experiment was repeated at least three times.

Quantification of immunoblotting was performed using the ImageJ software and images were processed with Photoshop and Illustrator software (CS5 version; Adobe).

Cell Proliferation Assays

HeLa cells transfected or not with Rab7-GFP and its mutant or depleted of RCP were seeded in quadruplicate on 24-well plates at 2×10^4 cells/well, serum-starved overnight and treated for three days with 100 ng/mL EGF or TGF- α , replenished every 24 hours. Viable cells were counted using the Trypan blue exclusion method and the ratio to unstimulated cells at time 0 was determined for each time point, as previously described⁹. Values represent the mean $\pm$ s.e.m. from at least four independent experiments. BrdU incorporation assay was performed as described⁹.

Cell Migration Assay

Cell migration was measured using a two-chamber Transwell system (5-mm pores; Boyden chamber; Costar, San Diego, CA). The lower side of the filter was coated with poly-lysine polymers, an inert attachment surface used to improve adherence of the cells after they migrate, as shown previously⁹. Forty-thousand serum-starved cells (HeLa cells depleted of RCP and negative control) were seeded in the upper well of Transwell in the presence of stimuli. Four-hundred microliters of complete medium was placed in the lower chamber as chemoattractant. Migration was allowed for 24 hours at 37°C. Cells remaining on the upper surface of filters were scraped off. Cells on the lower side were fixed and stained with DAPI. Cells were then counted in 10 random fields per filter, using AxioImager A2 (Carl Zeiss, Germany) with a Plan NEOFLUAR 20X/0.5 NA objective, equipped with a Monochrome AxioCam camera and the analysis software AxioVision. Results represent the mean $\pm$ s.e.m of at least three independent experiments.

Immunofluorescence

Immunofluorescence staining was performed as described⁹. Briefly, HeLa cells transfected with HA-EGFR (Figure 1a-b) were incubated with 10 μ g/ml of anti-HA antibody. After adding EGF or

TGF- α , cells were incubated at 37°C for different time points. At each time point, non-permeabilized cells were either fixed to visualize the receptor on the cell surface (plasma membrane) or acid-washed to remove surface-bound antibody. Acid-washed cells were then fixed and permeabilized to visualize the internalized receptor (cytoplasm). Finally, to detect HA-EGFR, cells were stained with AlexaFluor488-conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained with DAPI. Coverslips were then mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

Quantification in Figure 1a-b: for each time point and each treatment, the presence (total) and the localization (cell surface vs. internalized) of HA-EGFR were assessed in seven randomly chosen fields. Approximately 100 cells per condition (both acid-washed and not) were analysed from three independent experiments. The results are expressed as the percentage between the number of HA-EGFR-positive cells (green) and total cells (corresponding to DAPI-stained nuclei) and referred to the values obtained at time zero.

For co-localization experiments, cells were acid-washed, fixed, permeabilized with 0.02% saponin (Sigma), treated with a primary antibody against EGFR, EEA1 or RCP for 60 min. at 37°C, and stained with AlexaFluor488-conjugated donkey anti-mouse, CY3-conjugated goat anti-mouse or Alexa647-donkey anti-mouse, CY3-conjugated donkey anti-goat, Alexa647-donkey anti-mouse secondary antibodies, respectively. Samples treated with TRITC-Transferrin (to stain Transferrin Receptor, Tf-R), added to the medium at a final concentration of 50 μ g/ml, were kept in the dark. Nuclei were stained with DAPI. Coverslips were then mounted in mounting medium (Vectashield; Vector Laboratories). All the images were acquired at room temperature with a confocal microscope (LSM 710; Carl Zeiss) mounted on a confocal laser-scanning microscope (Axiovert 100M; Carl Zeiss) equipped with a Plan Apochromat 63 $\times$ /1.40 NA DIC M27 oil immersion objective using standard settings: DAPI and Alexa Fluor 488/GFP, Alexa Fluor 568, and Alexa

Fluor 647 dyes were excited with 405-, 488-, 546-, and 633-nm laser lines, and emitted light was collected through band pass 420-480-, 505-530- and 560-615-nm and long pass 615-nm filters, respectively. Pinhole size was set to 1 airy unit or opened slightly for all wavelengths acquired if the signal intensity was otherwise too low. Zoom was 2.5X. Image acquisition, analysis and quantification were performed with the ZEN2010 software (Carl Zeiss). Raw images were exported as TIF files, and if adjustments in image contrast and brightness were applied, identical settings were used on all images of a given experiment.

The quantitation of receptor subcellular localization shown in Figures 1c, 5d, and 6d was performed blindly on 10 images for each condition in triplicates by setting the same intensity threshold for all the images and using the Pearson's correlation coefficient, which provides information on the intensity distribution within the co-localization region (range -1/+1 where 0= pixels distributed in a cloud with no preferential direction), to relatively compare the co-localization of EGFR with the GFP-tagged Rab proteins or Tf-R in treated versus control cells.

Sample Preparation for Mass Spectrometry

Sequential enrichment of Post Translational Modifications (PTMs)

HeLa cells from the three SILAC conditions were lysed at each time points in modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA, 5 mM β -glycerolphosphate, 5 mM sodium fluoride, 1 mM sodium orthovanadate) supplemented with 1 complete® protease inhibitor cocktail tablet (Roche, Basel, Switzerland) and N-ethylmaleimide to block cysteine-based proteases such as deubiquitylases (Sigma). Lysates were incubated for 15 min on ice and cleared by centrifugation at 16,000g. Proteins were precipitated in four-fold excess volumes of ice-cold acetone overnight at 20 °C and subsequently re-dissolved in denaturation buffer (6 M urea, 2 M thiourea in 10 mM HEPES pH 8). The SILAC-labelled lysates were mixed 1:1:1 based on protein concentration (Bradford). The untreated controls were used as

the reference point to combine the four triple SILAC experiments, each obtained in biological duplicates (Supplementary Fig. 1a). Cysteines were reduced with 1 mM dithiothreitol (DTT) and alkylated with 5.5 mM chloroacetamide (CAA). About 20 mg of proteins were digested with endoproteinase Lys-C (Wako, Osaka, Japan) and sequencing grade modified trypsin (modified sequencing grade, Sigma) after four-fold dilution in deionized water. Protease activity was quenched by addition of trifluoroacetic acid (TFA) to a final concentration of 1%. Precipitates were removed by centrifugation for 10 min at 3,000 g. Peptides were purified using reversed-phase Sep-Pak C18 cartridges (Waters, Milford, MA) and eluted with 50% acetonitrile. Peptides were lyophilized and re-dissolved in MOPS immunoprecipitation buffer (10 mM sodium phosphate, 50 mM sodium chloride (NaCl) in 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) pH 7.2) overnight. Ubiquitylated peptides were enriched as described previously⁵¹ with some modifications. The peptide mixture were transferred in a clean tube containing immobilized di-Gly-lysine-specific beads (Cell Signaling Technology) for 4 h at 4 °C on a rotation wheel. The immunoprecipitates were washed three times in the MOPS immunoprecipitation buffer followed by three washes in deionized water, and immunoprecipitated peptides were eluted using 0.1% TFA in milliQ water. Ubiquitylated peptides were fractionated using microcolumn-based strong cation exchange (SCX) chromatography as described previously⁵² and eluted at increasing pH.

The supernatant from ubiquitylated-enriched immunoprecipitates was kept on ice for subsequent phosphorylated tyrosine peptide enrichment, performed as described⁵³. Briefly, peptides were transferred to a new tube containing immobilized phospho-tyrosine antibody beads (pY100-AC, pY1000-AC, Cell Signaling Technologies) and incubated for two hours at 4°C. After five washes with the MOPS immunoprecipitation buffer followed by two washes with 50 mM NaCl, the enriched peptides were eluted from the beads three times with 50 µl of 0.1% TFA. The experiment

shown in Supplementary Fig. 4 has been performed by incubating the digested proteins from each condition with the containing immobilized phospho-tyrosine antibody beads.

The supernatant was mixed with all the washes from phosphorylated tyrosine-enriched peptides immunoprecipitates and kept on ice before further enrichment of phosphorylated serine and threonine-containing peptides. After further concentration on a C18-SepPak cartridge and elution with 50% acetonitrile, 0.1% TFA, phosphopeptides were enriched using titansphere chromatography as described previously⁵³. Briefly, titanium dioxide (TiO₂) beads (10 μ m, Titansphere, GL Sciences, Japan) were incubated with a solution of 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich) in 80% acetonitrile, 0.1% TFA for 30 min. Approximately 1 mg of TiO₂-DHB beads was added at room temperature. All the samples were incubated twice with TiO₂-DHB beads for better coverage. The beads were washed and transferred in 80% acetonitrile, 0.5% acetic acid on top of a C8 STAGE-tip. The bound phosphopeptides were eluted directly into a 96-well plate by 5% NH₄OH followed by 10% NH₄OH, 25% acetonitrile. The eluate was immediately concentrated in a speed vacuum at 60°C and acidified with 5% acetonitrile, 1% TFA.

Pull-downs

After stimulation and lysis as described before, SILAC labelled HeLa cell lysates (10 mg for each SILAC condition) were kept separated, pre-cleared with anti- rabbit and mouse IgG (Sigma-Aldrich), supplemented with Protein G Sepharose beads (Invitrogen), and left on inversion rotation for at least 4 h at 4°C. After centrifugation at 1000g for 5 minutes, cleared samples were incubated with anti-EGFR antibodies overnight at 4°C. Protein G Sepharose beads were added for 1 h at 4°C and the immunoprecipitated proteins were washed 5 times with ice-cold lysis buffer. Eluates were then combined and resolved on SDS-PAGE. After staining with the Colloidal Blue Kit (Invitrogen) according to the manufacturer's protocol, each gel lane was divided into seven different slices and

each gel slice was cut into 1 mm³ cubes. Gel slices were destained with 50% ethanol in 25 mM ammonium bicarbonate solution and dehydrated with absolute ethanol. Proteins were digested with trypsin (modified sequencing grade, Sigma) overnight. Trypsin activity was quenched by acidification with TFA and peptides were extracted from the gel pieces with increasing concentrations of acetonitrile. Organic solvent was evaporated in a speed vacuum centrifuge, as described⁵⁴.

Proteome analysis

Cells from light, medium and heavy SILAC conditions treated for 72 h were lysed separately at 4°C in modified RIPA buffer. 50 µg protein amounts from each SILAC condition were mixed prior to SDS-PAGE and digestion as described above. Gel-lanes were divided into ten different slices and each gel slice was cut into 1 mm³ cubes.

Liquid chromatography tandem Mass Spectrometry (LC-MS/MS)

Peptides from all samples were concentrated using a sample concentrator, acidified with 0.1% TFA before desalting on reverse phase C18 StageTips as described previously⁵⁵, and eluted using 40% ACN, 0.5% acetic acid prior to online nanoflow LC-MS/MS analysis. Peptide mixtures were analyzed using an EASY-nLC system (Proxeon, Odense, Denmark) connected to a Q Exactive or a LTQ-Orbitrap Velos (for samples shown in Supplementary Fig. 4) mass spectrometers (both from Thermo Fisher Scientific, Bremen, Germany) through a nanoelectrospray ion source. Peptides were separated in a 15 cm analytical column (75 µm inner-diameter) in-house packed with 1.9 µm reversed-phase C18 beads (Reprosil-Pur AQ, Dr. Maisch, Ammerbuch, Germany) with a 80 min. (for ubiquitylated peptides, EGFR interactome, proteome analysis) or 135 min. (for phosphorylated peptides analysis) gradient from 3% to 60% acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min. Standard mass spectrometric parameters were as follows: spray voltage, 2.1 kV; no sheath and auxiliary gas flow, heated capillary temperature, 275°C; S-lens RF level of 50%. Both the

instrument were operated in data-dependent acquisition mode, as described⁵⁶. Full-scan MS spectra (m/z 250-1,250 for ubiquitylated peptides analysis and m/z 300-1,750 for all the other experiments, resolution 70,000 at m/z 200) were detected in the Orbitrap analyzer after accumulation of ions at $1e6$ target value based on predictive AGC from the previous scan. For every full-scan the 10 most intense ions (12 in case of EGFR interactome analysis) were isolated and fragmented (collision energy: 25%) by higher-energy collisional dissociation (HCD) with a fixed injection/fill time of 250 ms and 70,000 resolution for ubiquitylated and phosphorylated peptides analysis; 100 ms and 35,000 resolution for EGFR interactome analysis; 120 ms and 35,000 for the proteome analysis.. These parameters were optimized for each dataset. Finally, the dynamic exclusion was set to 30 seconds.

Raw MS Data Analysis

Raw data were analyzed by the MaxQuant software suite⁵⁷, either version 1.0.14.7 using the MASCOT search engine (v. 2.3.02) (EGFR interactors analysis) or version 1.1.2.7 (analysis shown in Supplementary Fig. 4), 1.3.10.9 (ubiquitylated and phosphorylated peptides) and 1.4.1.4 (proteome analysis) with the integrated Andromeda search engine⁵⁸. Proteins were identified by searching the HCD-MS/MS peak lists against a target/decoy version of the human International Protein Index (IPI) database (v. 3.37) (EGFR interactors analysis) or the complete human Uniprot database supplemented with commonly observed contaminants such as porcine trypsin and bovine serum proteins. Tandem mass spectra were initially matched with a mass tolerance of 7 ppm on precursor masses and 0.02 Da or 20 ppm for fragment ions. Cysteine carbamidomethylation was searched as a fixed modification. Protein N-acetylation, N-pyro-glutamine, oxidized methionine, di-Gly-lysine and phosphorylation of serine, threonine, and tyrosine were searched as variable modifications for the experiment of the ubiquitylated and phosphorylated peptides enrichment. Protein N-acetylation, oxidized methionine and deamidation of asparagine and glutamine were

searched as variable modifications for the pull down and the proteome experiments. Labeled lysine and arginine were specified as fixed variable modification, depending on prior knowledge about the parent ion (MaxQuant SILAC triplet identification). False discovery rate was set to 0.01 for peptides, proteins and modification sites. Minimal peptide length was six amino acids. Site localization probabilities were calculated by MaxQuant using the PTM scoring algorithm²⁶. The dataset were filtered by posterior error probability to achieve a false discovery rate below 1% for peptides, proteins and modification sites. Only peptides with Andromeda score >40 were included.

Data Analysis

For the analysis of ubiquitylated and phosphorylated peptides, only peptides with site localization probability of at least 0.75 (class I, shown in Supplementary Tables 1, 2 and 6)²⁶ were included in the bioinformatics analyses. The ratios of identified and quantified modified sites were normalized to the ratio of EGFR at 1 min. upon stimulation with EGF or TGF- α using the unmodified peptides to account for uneven efficiency during individual experiments performed in parallel. To identify significantly regulated modification sites we compared the distributions of the ratio of all quantified modified peptides with all unmodified peptides that specify our technical variance and sites with a SILAC ratio higher than 2-fold or lower than 0.5-fold were considered regulated. Each time point was considered an independent experiment and we defined a site regulated if its SILAC ratio was higher than 2-fold or lower than 0.5-fold in at least one experimental condition (upon EGF or TGF- α stimulation for one of the time points in one of the replicates). We considered proteins to be TGF- α -dependent EGFR interactors if their TGF- α /EGF SILAC ratio at each time point was higher than 2. We considered proteins to be EGF-dependent EGFR interactors if their TGF- α /EGF SILAC ratio at 1-40 min. (when EGFR was still present and not degraded) point was lower than 0.5. Therefore, our definition of regulated site (either up- or down-regulated) is quite broad This allowed the best comparison of the two dynamic responses described here. The definition of ligand-specific EGFR

interactor takes into account the total level of EGFR at each time point. Counting of different protein categories was based on this definition (Figures 2b-c, S1e-h, 3a, 4a-b, S3, 6b). To visualize the temporal regulation of modified sites (Figures S3 and S5a), heat maps of the log2 of the EGF or TGF- α ratios were created using the software R.

For unsupervised cluster analysis of the data the log2-ratios of all phosphorylation and ubiquitylation sites that showed a ratio at all the time points were z-scored (by subtracting the mean and dividing for the standard deviation) and subjected to clustering by the fuzzy c-means algorithm in GProX 1.1.12., by requesting six clusters with a fuzzification parameter of 2 and 100 algorithm iterations⁵⁹. Subsequently, all Gene Ontology (GO) biological process terms that occurred at least five times in a cluster were tested for enrichment in the cluster compared to the group of non-regulated sites (cluster 0, static sites) by Fisher's exact test. GO terms that obtained a p-value below 0.05 after correction for multiple testing by the the Benjamini and Hochberg method was regarded as significantly enriched.

The S-score was calculated essentially as described³⁴. Briefly, for each phosphorylation or ubiquitylation site the p-value for the correlation between the log2-ratios observed after EGF or TGF- α stimulation at each time point was calculated. This provided a measure of the degree of similarity in terms of the overall pattern of regulation observed after the two treatments (the "P-score"). To account also for potentially unequal magnitude of the regulation the difference in the area under the curve for the ratios observed after the two treatments was also calculated (the "M-score"). Finally, to obtain one value for the similarity in regulation the P-score and the M-score were multiplied and the resulting value was log10 transformed and multiplied by -10. To identify the biological functions associated with different ranges of S-score the modified sites were divided into quintiles based on their attained S-score. Subsequently, Fisher's exact test was used to extract for each quintile the GO biological process terms enriched compared to the sites in the remaining

four quintiles. Only GO terms occurring at least three times in a quintile were included in the analysis and a p-value threshold of 0.05 after correction for multiple testing by the Benjamini and Hochberg method was used (Supplementary Fig. 2c).

The overview of proteins shown in Supplementary Fig. 2d, and in Figure 4e was manually curated based on either STRING databas⁶⁰ and visualized in Cytoscape (version 3.1). Clusters represented in Figure 4e were obtained using the Cytoscape plug-in ClusterONE. PCA was performed using the Perseus software (Max-Planck Institute of Biochemistry, Department of Proteomics and Signal Transduction, Munich, Germany). For identification of potential kinase motifs the sequence window of the regulated phosphorylation sites were compared to the non-regulated sites using the IceLogo resource⁶¹ with default parameters.

For interactome and proteome data, a minimum of three to seven peptide identifications with at least two being uniquely assigned to the particular protein were required. Sequence coverage of the identified proteins was at least 5%. In the EGFR dataset ratios of identified and quantified dynamic interactors were normalized to the ratio of EGFR upon 1 min. stimulation with each ligand to account for uneven efficiency during individual pull downs performed in parallel. From the 855 identified proteins all contaminants, ribosomal and proteasomal proteins have been filtered, resulting in 278 proteins. Proteins with a SIALC ratio quantified in at least two time points are shown in Supplementary Table 3. The overview of the EGFR interactome shown in Fig. 6b was based on the STRING database and colour-code based on the difference in the regulation between the two stimuli.

For the analysis of late proteome, proteins showing significant changes in abundance ($P < 0.05$, significance B test) were referred to as ‘regulated’ (Supplementary Table 4). The column “Significance B” and “Significance B - BH adjusted p-values” report values calculated by the Perseus software⁵⁷ before and after correction for multiple testing by the Benjamini and Hochberg

method, respectively. GO analysis shown in Figure 7a was performed using DAVID⁶². Significantly over-represented GO terms within the proteome data were represented in bar plots. For the Venn diagram shown in Figure 2c, we collected in Supplementary Table 5 the 7053 non-redundant accession keys found in the "Uniprot protein ID" columns in Supplementary Tables 1-4. All counts of regulated proteins were based on this gene-centric table.

Statistical Analysis

The SILAC experiments have been performed in duplicates. All the other experiments have been performed in at least triplicates. Results shown are mean $\pm$ SD or $\pm$ s.e.m. and P value was calculated by Student's two tailed t-test or Fisher's exact test, as indicated. In Figure 2f P-values were calculated using a two-sample Student's t-test on slopes calculated by the linear least squares regression model. Bonferroni correction was used to correct for multiple t-test comparisons. A statistically significant difference was concluded when $P < 0.05$ or $P < 0.01$ as indicated by * in the figures and as reported in the figure legends.

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FIGURE LEGENDS

Figure 1. EGFR trafficking and responses depend on biased ligands.

(a) Quantification (see Online Methods) of total EGFR level (left panel), cell surface EGFR (middle panel) or internalized EGFR (right panel) upon EGF- or TGF- α -stimulation for different time intervals. Values in the graph represent the means $\pm$ SD. of three experiments. (b) Representative images from (a), showing the internalization (cytoplasm) and recycling (plasma membrane) of EGF- or TGF- α -stimulated HA-EGFR (green) at different time intervals. Arrows indicate internalized receptor. Asterisks indicate cells with the receptor recycled to the cell surface. Bar, 5 μ m. (c) Quantification (see Online Methods) of the co-localization of EGFR with endocytic markers upon EGF- or TGF- α -stimulation. Values in the graph represent the means $\pm$ SD. of three experiments. A.U., arbitrary units. *, p value < 0.01 compared to the other stimulus. (d) Representative images from (c), showing the co-localization of EGFR (green or red) with

intracellular markers in stimulated cells. Bar, 5 μ m. Cell proliferation (**e**) and BrdU incorporation (**f**) of stimulated cells. Data represent the mean $\pm$ s.e.m. of three experiments. *, p value<0.05 compared to EGF. Black line represents control cells.

Figure 2. Multi-layered proteomics of EGFR signaling shows ligand-specific EGFR regulation.

(**a**) Overview of the time scale for proteomics of EGFR signaling. (**b**) Number and percentage of identified and regulated sites and proteins. (**c**) Venn diagram indicating the number of proteins specifically regulated at different levels upon EGF (blue) or TGF- α (green) stimulation. *na*, none of the proteins were specifically regulated by either ligand. EGFR is highlighted in red. (**d**) Quantitation by MS of Erk1 and Erk2 Y202/T204-containing doubly phosphorylated peptide (right) and EGFR protein (left) upon EGF (blue) or TGF- α (green) stimulation. Values are the median $\pm$ SD of two replicates. (**e**) The immunoblotting for EGFR, ERK and vinculin is quantified in (**f**). *, p value<0.05 (two-sample Student's test on slopes). AG1478, EGFR inhibitor.

Figure 3. Analysis of dynamic EGFR signaling indicates ligand-dependent regulation of phosphorylation and ubiquitylation.

(**a**) Number of sites and EGFR interactors regulated at each time point. (**b**) Principal Component Analysis (PCA) of the log₂-transformed ratios from the indicated datasets. Magenta line indicates a clear separation of EGF and TGF- α responses at 8 min.. (**c**) PCA of Tyr phosphoproteome from the indicated studies. (**d**) Sequence motif analysis of the $\pm$ 6 amino acid residues flanking the regulated phosphorylation site. (**e**) Immunoblotting for ERK and vinculin upon prolonged stimulation quantified in (**f**). *, p value<0.05 (two-sample Student's test on slopes).

Figure 4. Biased EGFR ligands differentially promote the crosstalk between phosphorylation and ubiquitylation.

(a) Number of modified kinases, phosphatases, E3 ligases and deubiquitinating enzymes identified in our dataset (left) and the same expressed as percentage of the total number of these enzyme categories identified in total in HeLa cells (right). *, p value<0.05 (Fisher's exact test). (b) Number of total (left) and regulated (right) modified proteins. (c) GO term enrichment analysis of the 25 doubly modified proteins against proteins modified by one of the PTMs. (d) List of the 25 doubly modified proteins. Regulated over total number of identified sites is shown. (e) Functional network analysis of the 25 doubly modified proteins based on the STRING database and visualized using Cytoscape (ClusterONE plugin). Cluster 1, p value=7*10⁻⁶. Cluster 2, p value=0.049. EGFR and Rab7A are highlighted in orange and blue, respectively.

Figure 5. Rab7 phosphorylation on Y183 is important for EGF-dependent EGFR degradation and outcomes.

(a) Representative MS/MS spectrum of the phosphorylated Y183 peptide of Rab7. (b) SILAC ratio of the phosphorylated Y183 peptide of Rab7 upon stimulation for 1 or 8 min.. (c) Lysates from stimulated cells transfected with Rab7-GFP or its mutant were immunoprecipitated and immunoblotted as indicated. (d) Quantification (see Online Methods) of the co-localization of EGFR with Rab7-GFP or Transferrin Receptor (Tf-R) upon EGF- or TGF- α -stimulation. Values in the graph represent the means $\pm$ SD. of three experiments. A.U., arbitrary units. *, p value<0.05 compared to the other stimulus. (e) Representative images from (d), showing the co-localization of EGFR (blue) with GFP-Rab7 or its mutant (green) or Tf-R (red) in 40 min.-stimulated cells. Bar, 5 μ m. (f) Lysates from stimulated cells transfected with Rab7-GFP or its mutant were immunoblotted as indicated. (g) Cancer cell proliferation assay in stimulated cells upon transfection with Rab7 or its mutant. Data represent the mean $\pm$ s.e.m. of three experiments. *, p value<0.05 compared to the indicated stimulus. Black line represents control cells.

Figure 6. RCP promotes TGF- α -dependent EGFR recycling.

(a, c) Lysates from stimulated cells were immunoprecipitated and immunoblotted as indicated. The inputs are shown in Supplementary Fig. 6. (b) Overview of the EGFR interactome based on the STRING database and colour-code based on the difference between EGF over TGF- α regulation. Each square indicates a time point. (d) Quantification (see Online Methods) of the co-localization of EGFR with Rab7 or Rab11 upon EGF- or TGF- α -stimulation in cells depleted or not for RCP. Values in the graph represent the means $\pm$ SD. of three experiments. A.U., arbitrary units. *, p value<0.05 compared to the other stimulus. (e) Representative images from (d), showing the co-localization of EGFR (red) with Rab7 or Rab11 in 40 min.-stimulated cells depleted or not of RCP. Bar, 5 μ m. (f) Lysates from stimulated cells where RCP expression was depleted by two different siRNA sequences or not were immunoblotted as indicated. (g) Lysates from stimulated cells transfected with GFP-Rab5 were immunoprecipitated and immunoblotted as indicated.

Figure 7. RCP mediates TGF- α -dependent cellular responses.

(a) Ratio vs. Intensity plot of the EGF (blue)- and TGF- α (green)- induced differentially expressed proteins upon 72h stimulation. Insert, GO terms enrichment analysis. (b) Lysates from RCP-depleted and stimulated cells (HeLa or other cancer cells) were immunoblotted as indicated. (c, d) Cancer cell proliferation (c) or migration (d) assays in stimulated cells upon RCP depletion. Data represent the mean $\pm$ s.e.m. of three experiments. *, p value<0.05 compared to the indicated stimulus. Black line represents control cells. (e) Model of EGFR trafficking, signaling and responses based on this study. The orange arrow indicates the EGFR-mediated phosphorylation of Rab7.

Fig. 1

a

b

e

f

c

d

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

a

b

c

d

e