

1 **Elucidating synergistic dependencies in lung adenocarcinoma by**  
2 **proteome-wide signaling-network analysis**

3

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## 23 **Summary**

24 Signaling pathway models are largely based on the compilation of literature data from  
25 heterogeneous cellular contexts. Indeed, *de novo* reconstruction of signaling interactions from  
26 large-scale molecular profiling is still lagging, compared to similar efforts in transcriptional and  
27 protein-protein interaction networks. To address this challenge, we introduce a novel algorithm  
28 for the systematic inference of protein kinase pathways, and applied it to published mass  
29 spectrometry-based phosphotyrosine profile data from 250 lung adenocarcinoma (LUAD)  
30 samples. The resulting network includes 43 TKs and 415 inferred, LUAD-specific substrates,  
31 which were validated at >60% accuracy by SILAC assays, including “novel” substrates of the  
32 EGFR and c-MET TKs, which play a critical oncogenic role in lung cancer. This systematic, data-  
33 driven model supported drug response prediction on an individual sample basis, including  
34 accurate prediction and validation of synergistic EGFR and c-MET inhibitor activity in cells lacking  
35 mutations in either gene, thus contributing to current precision oncology efforts.

36

37 **Keywords:** signaling network, lung cancer, tyrosine kinase, combination therapy, proteomics,  
38 EGFR, c-MET

## 39 Introduction

40 Lung adenocarcinoma (LUAD) is a leading cause of cancer related deaths in United States,  
41 representing 40% of 225,500 new lung cancer cases every year, and has a 5-year survival rate  
42 of only 16 % (1). Excluding immunotherapeutic agents, which have recently shown significant  
43 success in a relatively small subset of patients (2), the most effective targeted therapies for this  
44 diseases were designed to inhibit tyrosine kinase proteins harboring genetic alterations that  
45 induce aberrant activation of downstream pathways (3-7). These the most frequent such  
46 actionable alterations include EGFR mutations and ALK-EML4 fusion events, in ~15% and ~3-  
47 7% of LUAD patients, respectively (8, 9). Yet, while targeted therapy is initially effective in a  
48 significant fraction of tumors harboring these genetic alterations, the vast majority of treated  
49 patients will either fail to respond or will develop resistance to mono-therapy (10, 11). In addition,  
50 most patient lack actionable alterations altogether. This suggests that novel approaches are  
51 critically needed.

52 A possible alternative to minimize emergence of resistance is combination therapy, a strategy  
53 that has been shown to be effective in many metastatic tumors, such as breast cancer and acute  
54 myeloid leukemia (12-14). However, systematic identification of effective drug combinations on a  
55 genetic alteration basis is difficult, because the number of patients presenting multiple actionable  
56 events is extremely low. As a result, combination therapy is generally hypothesized and tested on  
57 an empirical basis or based on elucidation of complex mechanisms of tumor cell adaptation. In  
58 addition, accurate prediction of response to available mono-therapy – including to EGFR inhibitors  
59 – in patients lacking any genetic alteration represents an equally relevant challenge, especially  
60 since a small fraction of EGFR<sup>WT</sup> patients have been shown to respond to Afatinib, even though  
61 a predictive biomarker is not available. To address these limitations, we and other have proposed  
62 that rational design of combination therapy and the identification of critical targetable

63 dependencies may require a more mechanistic and tumor-context-specific understanding of the  
64 molecular interactions that underlie their potential synergistic activity, starting with tyrosine  
65 kinases, which represent a critical class of pharmacological targets in cancer (15). Such an  
66 approach requires methodologies for the accurate and systematic elucidation of tumor-specific  
67 signaling transduction pathways.

68 Dissection of signal transduction networks represents a complex endeavor, requiring elucidation  
69 of hundreds of thousands of tissue-specific molecular interactions that mediate the post-  
70 translational modification of protein substrates. *In vitro* approaches generally fail to capture the  
71 tissue-specific nature of these interactions, thus providing “average” signal transduction pathways  
72 that are both incomplete and inaccurate. In addition, experimental approaches that have been  
73 successful in accelerating the analysis of molecular interactions in transcriptional regulation and  
74 protein-protein interaction in stable-complexes, such as those based on co-expression or yeast-  
75 2-hybrid assays, do not easily translate to elucidating signaling interactions. Similarly, approaches  
76 based on the use of phospho-specific antibodies, while elegant and effective, are limited to only  
77 a handful of proteins. Computationally, compared to the many algorithms that have been  
78 developed for the reverse engineering of transcriptional and protein-complex interactions (16, 17),  
79 only a handful of experimentally validated algorithms are available for the dissection of signaling  
80 networks, none of which works at the proteome-wide level or is tumor-context specific (16, 18,  
81 19).

82 Recent availability of proteome-wide molecular profile data, characterizing the abundance of  
83 phospho-tyrosine-enriched peptides by liquid chromatography coupled to tandem mass  
84 spectrometry (LC-MS/MS), suggests that additional methodologies may be developed to extend  
85 approaches that have been successfully applied to the dissection of transcriptional networks from  
86 gene expression profiling. In this manuscript, we propose extending the Algorithm for the  
87 Reconstruction of Accurate Cellular Networks (ARACNe) (20) for the reverse engineering of

88 signal transduction networks from large-scale phosphoproteomic profiles. The new method,  
89 *pARACNe*, addresses critical issues that prevented the direct application of the original ARACNe  
90 algorithm on phosphoproteomic profile data. Briefly, the new algorithm addresses critical  
91 computational challenges presented by LC-MS/MS and spectral counting data, while  
92 incorporating enzymatic signaling characteristics into the algorithm design. In particular,  
93 *pARACNE* is designed to handle three critical issues resulting from the use of LC-MS/MS assays,  
94 including the highly sparse nature of phosphopeptide abundance data, the large amount of noise  
95 and missing data, and the degenerate peptides-to-protein mapping.

96 We applied *pARACNE* to the analysis of previously published, genome-wide phosphoproteomic  
97 data from 245 lung adenocarcinoma (LUAD) samples, including 151 fresh-frozen biopsies, 46 cell  
98 lines, as well as 48 normal lung tissues. The resulting network comprised 46 tyrosine kinases  
99 (TK) densely connected with 415 candidate substrates (including 377 proteins lacking any TK  
100 activity), representing the first genome-wide, tumor-context-specific model for a TK signal  
101 transduction network, capturing both protein-specific and phospho-site specific events. We  
102 validated substrate predictions for two “hubs,” whose activity may play a key role in determining  
103 sensitivity to Erlotinib and Crizotinib, two FDA-approved drugs for LUAD, including the EGFR and  
104 c-MET tyrosine kinases by independent SILAC assays and database analysis, with >60%  
105 accuracy. Of particular note, the inferred TK-substrate network provided unique information about  
106 tyrosine kinase auto-phosphorylation events, either direct (*cis*) or via a second kinase (*trans*).

107 Analysis of the resulting TK-network – by extending the VIPER (Virtual Proteomics by Enriched  
108 Regulon analysis) algorithm (21), an established method for the inference of Master Regulator  
109 proteins – recapitulated established genetic determinants of LUAD and was effective in predicting  
110 sensitivity to Erlotinib and Crizotinib combination therapy. Predicted sensitivities were validated  
111 in an independent set of LUAD cell lines, the majority of which harbored no genetic alterations in  
112 the corresponding genes. Furthermore, predictions based on the analysis of the corresponding

113 patient cohort were strongly supported by genomic information, suggesting potential value in  
114 using these analyses for the identification of effective combination therapies in precision oncology.

115

## 116 **Results**

### 117 ***Overview of the pARACNe Algorithm***

118 Enzymatic activity of tyrosine kinase (TK) proteins – as assessed by the ability to phosphorylate  
119 their downstream substrates – is effectively determined by their phosphorylated isoform  
120 abundance (**Fig. 1A, B**). Therefore, we reasoned that computational inference of TK-substrate  
121 interactions (**TK→S**) could be effectively performed by measuring dependencies between their  
122 respective phospho-states by mutual information analysis (22) over a large sample compendium  
123 (**Fig. 1C**). Unfortunately, due to signal transduction cascade complexity and pathway cross-talk,  
124 such dependencies can manifest between protein pairs that are not involved in direct **TK→S**  
125 interactions. The ARACNe algorithm – previously designed for the reverse engineering of  
126 transcriptional networks – effectively addresses this problem by leveraging the Data Processing  
127 Inequality (23). This is a critical property of the mutual information that effectively allows  
128 disambiguating between direct and indirect interactions by assessing whether information transfer  
129 on any candidate direct interaction (e.g., **TK<sub>1</sub>→S**) is greater than transfer on every other indirect  
130 path (e.g., **TK<sub>1</sub>→TK<sub>2</sub>→S**). ARACNe has been highly successful in the experimentally validated  
131 dissection of transcriptional networks via analysis of large gene expression profile compendia.  
132 ARACNe-inferred targets of transcription factors were validated in multiple cellular contexts, with  
133 an accuracy of 70% to 80% (20, 24-27).

134 However, ARACNe relies on molecular profile data that is both continuous and non-sparse,  
135 properties that are not always provided by quantitative proteomic data sets, which can be

136 generated by a variety of methods. Those based on LC-MS/MS represent the most popular  
137 approaches (28), but different implementations have specific performance profiles in terms of  
138 analyte throughput, consistency of measurement of peptides across samples and linear dynamic  
139 range (29). Depending on the data acquisition method, one or both of these assumptions of  
140 ARACNe are violated in proteome-wide datasets generated by the most popular methods based  
141 on data-dependent acquisition. Particularly when employing quantification by spectral counting,  
142 as is typically conducted for global protein-protein interaction studies (30, 31), phosphoproteomic  
143 data is both discrete (i.e., generally represented by spectral counts) and very sparse, with a  
144 majority of peptides having zero spectral counts and presenting a significantly skewed distribution  
145 for low-abundance peptides.

146

147 To address these limitations, we developed a phospho-proteomic specific algorithm, pARACNe  
148 (phospho-ARACNe) (**Fig. 1C**), specifically designed to measure phospho-state dependencies  
149 between TKs and their candidate substrates from large-scale LC-MS/MS phosphoproteomic  
150 profiles. pARACNe thus extends the original ARACNe framework to allow systematic inference  
151 of **TK**→**S** interactions. Specifically, to handle the highly discrete nature of the data, we replaced  
152 the kernel-density and adaptive partitioning based mutual information estimators in the original  
153 algorithm with a bin-count based method (**Fig. 1C4**), using gold standard data to select the most  
154 effective number of bins [12] (see Methods). Furthermore, to deal with the skewed spectral count  
155 distribution, we introduce an iterative quantile discretization method, where samples are binned  
156 together, based on their spectral counts, to produce a distribution as close to uniform as possible  
157 (**Fig. 1C3**, Methods).

158

## 159 ***pARACNe-inferred LUAD-specific TK-phosphorylation Network***

160 We used pARACNe to reconstruct a LUAD-specific TK-signaling network, by analyzing  
161 phosphopeptide profiles obtained from 245 LUAD samples from Guo et al. (32). These data  
162 represent the abundance of peptides containing at least one phospho-tyrosine, as obtained by  
163 phosphoproteomic analysis of 46 LUAD cell lines, 151 LUAD tumors, and 48 adjacent normal  
164 samples. LC-MS/MS profiling produced spectral counts for 3,920 phospho-tyrosine containing  
165 peptides mapping to ~2,600 different proteins. Based on these data, pARACNE identified 2,611  
166 candidate phospho-peptide/phospho-peptide dependencies, which could be further mapped to  
167 2,064 unique **TK→S** interactions (**Table S1, S2**). These represent interactions between 46 unique  
168 TKs and their candidate substrates. These include 174 **TK<sub>1</sub>→TK<sub>2</sub>** interactions between two TKs  
169 (**Fig. 2A**), representing a statistically significant bias toward TK-TK interactions in the network  
170 ( $p = 10^{-62}$ ). This suggests that, within the complete TK signaling network, TKs themselves may  
171 form a more densely inter-connected subnetwork than previously assessed, providing potentially  
172 valuable novel information about adaptive response, pathway cross-talk, and auto-regulatory  
173 loops.

174 Such highly interconnected structure provides potential functional advantage compared to less  
175 interconnected (i.e., “flat”) architectures, including the ability to provide more fine-grain response  
176 to a highly heterogeneous variety of exogenous signals and conditions, the ability to provide rapid  
177 adaptive response to changing stimuli, and the ability to preserve cell state via autoregulatory  
178 feedback. Consistent with the underlying biology, and in contrast to transcriptional networks, the  
179 vast majority of pARACNe-inferred interactions have a positive Spearman correlation, with higher  
180 counts of TK-mapped phosphopeptides corresponding to higher counts of candidate substrate-  
181 mapped ones. This is consistent with the fact that TKs only phosphorylate their substrates, thus  
182 inducing positive phospho-state correlation. Only a negligible number of inferred interactions  
183 (0.5%) were associated with a negative Spearman correlation ( $N = 11, p \leq 0.05$ ). These may



184 represent either indirect interactions where the TK activates a substrate-specific phosphatase or  
185 direct interactions where phosphorylation of one phosphosite may prevent phosphorylation of  
186 another site on the same protein.

187

### 188 ***LUAD Network Accuracy and Sensitivity Analysis***

189 To estimate the accuracy of the inferred TK-signaling network, we investigated the substrates of  
190 two TK-proteins, EGFR and c-MET, representing high-affinity binding targets of existing FDA-  
191 approved TK inhibitors for LUAD. Specifically, we compared their pARACNe-inferred substrates  
192 to those reported in the phosphoDB database (33) and those supported by experimental  
193 evidence, based on previously published SILAC assays, following cell line treatment with  
194 associated, selective TK inhibitors. pARACNe inferred 123 EGFR substrates (**Fig. 2B**). Of these,  
195 5 (blue and cyan) were included as high-confidence EGFR substrates in phosphoDB, out of 13 in  
196 total (38%), including the established EGFR auto-phosphorylation site. Moreover, 50 additional  
197 proteins (45%, green) showed significant decrease (at least 2 fold) in the abundance of their  
198 phosphorylated isoforms in SILAC assays (32), following treatment of H3255 cells with the EGFR  
199 inhibitor Gefitinib. Similarly, pARACNe predicted 179 c-MET substrates (**Fig. 2C**). Notably, both  
200 of the established substrates reported in PhosphoDB were identified by pARACNE (100%, blue).  
201 Moreover, 126 additional proteins (71.5%, blue) showed significant decrease in the abundance  
202 of their phosphorylated isoforms in SILAC assays(32), following treatment of MKN45 cells with  
203 the first-generation c-MET-specific inhibitor Su11274.

204 We used MKN45 to assess overall prediction accuracy, even though it represents a gastric cancer  
205 cell line, because signaling networks should be much more conserved across tissue contexts  
206 than transcriptional ones. Indeed, while lineage-specific chromatin state represents a major  
207 determinant of transcriptional regulation, it only affects signal transduction in terms of overall

208 protein availability. As a result, it is reasonable to expect that an even greater overlap of inferred  
209 vs. SILAC positive substrates may be achieved in native LUAD cells.

210

211 Taken together, these data suggest that pARACNe can identify a much larger subset of candidate  
212 substrates, while both identifying a significant proportion of established substrates (46% on  
213 average, based on phosphoDB) and maintaining high accuracy (~60% on average, by SILAC  
214 assays). This also suggest that, similar to transcription factor targets reported in the literature, TK  
215 substrates are still poorly characterized in existing repositories, even for highly relevant and  
216 exceedingly well-studied kinases such as EGFR and c-MET. As a result, pARACNe could provide  
217 significant novel hypotheses for TK→S interactions that can be validated as required. We should  
218 also note that the reported accuracy for pARACNe is estimated using SILAC data on a single cell  
219 line. SILAC assays have significant false negatives and it would be reasonable to expect that,  
220 once tested in additional cell lines, the accuracy of pARACNe could further increase. As a further  
221 performance benchmark, we used the same SILAC benchmark to test predictions by NetworkIN,  
222 a reverse engineering method based on protein sequence motif analysis and protein association  
223 networks (16). The analysis found almost no consensus with SILAC assays, with only one out of  
224 33 NetworkIN-predicted EGFR substrate identified as significantly dephosphorylated following  
225 treatment with TK-specific inhibitors.

226

### 227 ***Systematic, Network-based Inference of Pharmacological Dependencies***

228 Once an accurate model of signal transduction in LUAD cells was established by pARACNe  
229 analysis, we interrogated the corresponding TK→S network using phosphoproteomic signatures  
230 from 46 LUAD cell lines to identify key dependencies for experimental validation. For this purpose,  
231 we extended the VIPER algorithm (Virtual Proteomics by Enriched Regulon analysis)(21), which

232 was originally developed to identify the MR proteins that mechanistically regulate the  
233 transcriptional state of a tumor by assessing the enrichment of their transcriptional targets in  
234 differentially expressed genes in the tumor signature. VIPER and its predecessor MARINA  
235 (Master Regulator Inference algorithm) (34) have been instrumental in inferring MR proteins  
236 representing key functional determinants of tumor-related phenotypes in many cancer types, from  
237 glioblastoma (26, 27, 35), neuroblastoma (34), lymphoma (36, 37), and leukemia (38) to prostate  
238 (39-41) and breast adenocarcinoma(42-44), among others. We thus reasoned that VIPER could  
239 be modified to identify master regulator TKs, most likely to mechanistically regulate the differential  
240 phosphorylation pattern observed in a specific tumor sample (**Fig. 3A**). A specific additional value  
241 of the algorithm is that, as previously shown {Lefebvre, 2010 #1763}{Aytes, 2014 #2520}{Carro,  
242 2010 #4021}, it could not only identify MR TK proteins, representing individual, pharmacologically  
243 accessible dependencies of the tumor, but also TKs representing potential synergistic MR-pair as  
244 candidate dependencies for combination therapy.

245 VIPER can be easily modified to analyze phosphoproteomic signatures (pVIPER). Specifically,  
246 rather than assessing the enrichment of a protein's transcriptional targets (regulon) in differentially  
247 expressed genes, pVIPER is designed to measure the enrichment of a TK's substrates (signalon)  
248 in differentially phosphorylated proteins. Since inferred TK-substrate interactions are virtually all  
249 positive, this further increases the accuracy of the algorithm by supporting use of a single-tail  
250 enrichment analysis as opposed to the three-tail analysis of the original implementation. We first  
251 performed pVIPER analysis at the individual phosphopeptide level, rather than by averaging  
252 phosphopeptide state on a whole protein level. We then combined the result of the analysis across  
253 all phosphopeptides mapping to the same protein. Consistent with VIPER's experimentally  
254 validated ability to identify synergistic master regulators proteins by transcriptional network  
255 analysis, pVIPER inferred several candidate synergistic TK interactions based on the statistical  
256 significance of the signature-enrichment of substrates shared by both TKs compared to that of

257 substrates uniquely mapped to either one or the other TK (see Method section). Systematic  
258 VIPER analysis of phosphoproteomic profiles from 46 LUAD cell lines generated between 2 and  
259 13 master regulator TKs or synergistic TK-pairs, as candidate pharmacologically actionable  
260 dependencies, for each cell line, thus generating a plausible number of hypothesis for each cell  
261 line (**Fig. 3B** and **Fig. 3C**).

262

### 263 **pVIPER Identifies LUAD-specific Dependencies**

264 pVIPER analysis inferred several TK proteins as highly conserved individual dependencies across  
265 multiple cell lines, including the Ephrin type-A receptor 2 (*EPHA2*), epidermal growth factor  
266 receptor (*EGFR*), c-Met proto-oncogene (*MET*), and HER2 receptor tyrosine kinase 2 (*ERBB2*),  
267 suggesting a critical role of these proteins in the maintenance of LUAD cell line state. This is also  
268 in agreement with the functional role of these genes and the use of inhibitors of these kinases  
269 across a large panel of patients in multiple cancer types (45-49).

270 In contrast to these established LUAD cell line dependencies, we also identified several TKs as  
271 dependencies of specific cell lines. This can either be the result of associated genetic or  
272 epigenetic alterations in these cell lines or the result of field effects, where multiple genetic  
273 alterations or alterations in upstream pathway contribute to the cell line dependency on a specific  
274 TK activity. For instance, we identified ALK (Anaplastic Lymphoma Receptor Tyrosine Kinase) to  
275 be addiction point only in H2228 cell line. ALK is a conserved trans-membrane receptor tyrosine  
276 kinase (RTK) protein in the insulin-receptor super family. Chromosomal alterations involving *ALK*  
277 translocations and fusion events have been identified in several cancer types including LUAD (50,  
278 51), diffuse large B-cell lymphomas (52), neuroblastoma (53), and inflammatory myofibroblastic  
279 tumors (54), among others. Additionally, ALK fusion events with other genes, including EML4  
280 (Echinoderm Microtubule-associated protein Like 4) in LUAD lead to aberrant protein activity

281 eliciting “oncogene addiction” (51). Presence of ALK-EML4 fusion transcripts, in ~3–7% of LUAD  
282 patients (55-57), is a strong predictor of response to ALK inhibitors, such as Crizotinib, among  
283 others (58, 59). Interestingly, among all available LUAD cell lines for which a phosphoproteomic  
284 profile was available, H2228 was the only one with an established ALK-EML4 fusion event and  
285 with established sensitivity to ALK inhibitor (60, 61). This further reflects the specificity of our  
286 analysis as this was the only cell line predicted to depend on ALK activity. Interestingly, we  
287 identified 4 additional H2228 dependencies, namely EGFR, Epha2, c-MET, and PTK2. H2228  
288 sensitivity to EGFR inhibitors, in combination with ALK inhibitors, was already established (61).

289

### 290 ***EGFR and c-MET are Predicted Dependencies in Multiple LUAD Cell Lines***

291 As discussed, pVIPER analysis revealed several TK-pairs as candidate synergistic dependencies  
292 across several cell lines, such as Epha2/c-MET, EGFR/PTK2, EGFR/Epha2, Epha2/c-MET, and  
293 EGFR/c-MET. Among these the EGFR/c-MET pair emerged as the most conserved synergistic  
294 TK-pair across the available cell lines. In addition, EGFR and c-MET were also identified as  
295 candidate TK MRs in several of these cell lines, suggesting either a complementary or synergistic  
296 role for these proteins and a potential therapeutic opportunity for combination therapy in LUAD  
297 (62, 63).

298

299 To validate pVIPER-predicted, cell line specific EGFR/c-MET synthetic lethality, we selected a  
300 panel of 14 cell lines, 11 of which were predicted to be synergistically dependent on EGFR/c-MET  
301 (H226, H2122, H1666, H2172, Cal-12T, H2023, H1568, Calu-3, H1650, HCC78, and A549), as  
302 well as 3 negative controls with no predicted synergistic or individual dependencies on the two  
303 TKs (H2170, H460, and H520). To measure sensitivity to these agents, we used two different and  
304 complementary assays, including: (a) colony formation assay to assess long term sensitivity (**Fig.**  
305 **4A and Methods**) and (b) 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTS)  
306 assay for short term sensitivity analysis (**Fig. 5A, Table S3 and Methods**). For colony formation

307 assays, cells were treated with either an EGFR inhibitor (Erlotinib, 1uM) or a c-MET/ALK inhibitor  
308 (Crizotinib, 0.1uM), either individually or in combination (see Methods). To evaluate synergistic  
309 dependency on EGFR/c-MET we used Excess Over Bliss (64), which measures the difference  
310 between the observed effect on colony formation and the effect expected from a purely additive  
311 model. For MTT assay, first, cells were treated with EGFR inhibitor (Erlotinib) or MET inhibitor  
312 (Crizotinib) individually at various concentrations to identify IC50 (concentration resulting in 50%  
313 cell death). Next, cells were treated with 1 uM of Erlotinib and varying concentrations of Crizotinib  
314 to identify combinations resulting in IC50 and used the combination index (CI) statistic (65) to  
315 measure interaction between the two drugs.

316  
317 Across all 11 cell lines tested in colony formation assay, 8 showed significant sensitivity to either  
318 individual inhibitors (H226<sub>E,C</sub>, H2122<sub>E</sub>, H1666<sub>E,C</sub>, Cal-12T<sub>E</sub>, Calu-3<sub>E,C</sub>, H1650<sub>E</sub>) or synergistic  
319 sensitivity to the combination (HCC78<sub>E+C</sub> and H2023<sub>E+C</sub>) (**Fig. 4B-C**). Surprisingly, all of these cell  
320 lines were EGFR<sup>WT</sup>, ALK<sup>WT</sup>, and c-MET<sup>WT</sup>, except for H1650, which was EGFR<sup>Mut</sup>. Thus, based  
321 on standard of care criteria, 7 out of 8 cell lines would not have been considered as sensitive to  
322 either EGFR or ALK/c-MET inhibitors. Several cell lines presented striking sensitivity to either one  
323 (H2122<sub>E</sub>, Cal-12T<sub>E</sub>, H1650<sub>E</sub>) or both inhibitors (H226<sub>E,C</sub>, H1666<sub>E,C</sub>, Calu-3<sub>E,C</sub>) in isolation, thus  
324 making the assessment of synergistic drug sensitivity difficult. In addition, three EGFR<sup>WT</sup> cell lines  
325 harboring BRAF (Cal-12T and H1666) or KRAS (H2122) mutations were also highly sensitive to  
326 Erlotinib as a single agent, as predicted by pVIPER, despite the fact that KRAS pathway mutations  
327 are mutually exclusive with EGFR mutations and predictive of Erlotinib resistance (**Fig. 4C**).  
328 Finally, none of these cell lines was predicted to be sensitive to ALK inhibitors, suggesting that  
329 Crizotinib sensitivity is mediated by c-MET specific dependencies. Of the negative controls, only  
330 one (H2170) showed high sensitivity to Erlotinib. Taken together, 8/11 cell lines (73%) predicted  
331 as sensitive to the inhibitors were validated long term colony formation assays, while only 1/3  
332 negative controls showed sensitivity to these agents (33%).

333

334 To evaluate the short-term interaction between EGFR and c-MET, we performed MTT assay  
335 across 11 cell lines (HCC78, H2023, H1650, Calu-3, H2172, H2122, H1568, A549, H1666, H520  
336 and H2170) including 2 negative control cell lines (H520 and H2170). Similar to colony formation  
337 assay, we found synergistic sensitivity to EGFR and c-MET inhibitors in 6/9 cell lines (67%), with  
338 5 cell lines showing strong synergy ( $CI \leq 0.8$ ) (**Fig. 5B-C**) and 1 borderline synergy ( $CI = 0.82$ ),  
339 showing the consistency between two assays. However, for two cell lines, H1666 and H2170 (a  
340 negative control), results were inconsistent between long term colony formation and MTT assays.  
341 For both H1666 and H2170 cell lines, colony formation and MTT assay to showed sensitivity to  
342 Erlotinib alone, where colony formation assay has complete abrogation of colonies at 1  $\mu\text{M}$  of  
343 Erlotinib, and later had  $IC_{50} = 1.25 \mu\text{M}$  and  $3.7 \mu\text{M}$  for H1666 and H2170 respectively. However,  
344 in combination therapy, MTT assay showed antagonism ( $CI > 1$ ), despite the fact that colony  
345 formation assay still showed complete abrogation which could be associated to the accumulation  
346 of new mutations in these cell lines. However, this is just hypotheses and needs to be verified by  
347 further experiments such as sequencing of these cell lines pre-and post-treatment.

348

#### 349 ***Phosphosite-specific Phosphorylation Predicts EGFR/c-MET Inhibitor Synergy.***

350 In previous section, we assessed the pVIPER predictions after consolidating the result at protein  
351 level. Following the results from MTT and colony formation assays, we reanalyzed the pVIPER  
352 predictions at the phosphopeptide level. Interestingly, this revealed that whenever synergistic  
353 EGFR/c-MET dependencies were predicted from phosphosite EGFR<sub>1197</sub> and phosphosites other  
354 than c-MET<sub>1003</sub> (H1666, Cal-12T, H1650), cell lines responded to Erlotinib in isolation, while when  
355 predictions were based on phosphosites EGFR<sub>1197</sub> and c-MET<sub>1003</sub> (HCC78, H2023, and Calu-3),  
356 cells exhibited bona fide synergistic sensitivity to the two inhibitors, with the only possible  
357 exception of Calu-3, which showed synergistic sensitivity in MTT assays and additive sensitivity  
358 to both inhibitors in colony formation assays. Conversely, when predictions were not based on



359 either phosphotyrosine, cells exhibited no sensitivity to the individual inhibitors or the combination  
360 (H2172, H226, A549, H460, H520, H1568). Thus, predictions based on these two phosphosites  
361 produced no false positives (6 out of 6 predicted and validated as non-sensitive) and only 2 false  
362 negatives (H2170 and H2122), resulting in an error rate of only 2 out of 14 cell lines (14%,  $p =$   
363 0.0093 using fisher exact test).

364  
365 This finding is in agreement with the established role of EGFR<sub>1197</sub> as a predictor of EGFR inhibitor  
366 sensitivity (66). Intriguingly, when sensitivity was predicted using phosphosites other than  
367 EGFR<sub>1197</sub> and c-MET<sub>1003</sub>, cell lines did not respond to the inhibitors, either individually or in  
368 combination. For these two peptides, we found their common substrates to be hyper  
369 phosphorylated in the sensitive cell lines (**Fig. 6A**) compared to the specific substrates of each of  
370 them, whereas cell line responding only to EGFR inhibitors showed more hyper phosphorylation  
371 of EGFR only substrates (**Fig. 6B**). Cell lines resistant to both EGFR and c-MET inhibitors either  
372 showed no change in the phosphorylation status or hypo-phosphorylation compared to the normal  
373 samples (**Fig. 6C**). Hence, either the common substrates of EGFR and c-MET, or the  
374 phosphorylation status of EGFR<sub>1197</sub> and c-MET<sub>1003</sub> could potentially be used as biomarkers for  
375 predicting therapy with the dual inhibitors. However, this conclusion is based on a very limited  
376 number of observations and lacks the statistical power. This finding needs a re-  
377 evaluation/validation using larger cohort of samples to establish an appropriate biomarker for  
378 combination therapy.

379

### 380 ***Systematic Inference of Patient-specific Dependencies***

381 Similar to cell lines, when applied on patient data (32), pVIPER identified EGFR as one of the  
382 most common addiction points (**Fig. 6**). We inferred EGFR dependency in 12 patients. Of these,  
383 5 harbored EGFR mutations, while the remaining 7 patients had not been tested for this mutation,  
384 showing a high consistency between our predictions and the genetic predisposition for sensitivity



385 to EGFR inhibitors. In the entire cohort, there were only 3 patients with EGFR mutations that were  
386 not identified as EGFR dependent by pVIPER, resulting in an overall sensitivity of 62.5% (5/8).  
387 However, it is well known that >50% of patients harboring EGFR mutations do not respond to  
388 Erlotinib, suggesting that these may not be false negatives but rather patients with low activation  
389 of downstream EGFR pathways, despite their EGFR<sup>Mut</sup> state. Similarly, our analysis identified  
390 candidate ALK dependencies in 4 patients. Of these one had an established TFG-ALK fusion,  
391 whereas the others had not been tested for ALK fusion events.

392 Across all patient samples, we observed Discoidin Domain Receptor-1 (DDR1) to be the most  
393 frequent addiction point, which was not predicted for any of the 46 cell lines. One reason for the  
394 difference is that DDR1 is collagen dependent and there may be differences in the 3D structure  
395 of the tumor and the cell lines growing on the plate. An independent study (67) in a cohort of 83  
396 lung cancer specimens found that silencing of DDR1 in these samples leads to the hampering of  
397 cell survival, reduced invasiveness in collagen matrices, increased apoptosis in basal condition  
398 and decreased metastatic activity in model of tumor metastasis to bone, signifying it as a potential  
399 novel therapeutic target.

## 400 **Discussion**

401 In this paper, we developed pPARACNe to infer Tyrosine Kinase (TK) signaling network using  
402 published genome-wide phosphoproteomic data from lung cancer. The network prediction was  
403 validated using SILAC experiments, with high accuracy. Interrogation of the predicted TK-  
404 substrate network generated biologically meaningful hypotheses, followed by experimental  
405 validations illustrating the effectiveness of predicted kinase inhibitor combination, EGFR and c-  
406 MET combination inhibitors, in treating lung cancer cell lines. Furthermore, Master Regulator  
407 Analysis using patient proteomics data provides implications for using targeted agent  
408 combinations to treat patients based on their proteomic profile data.

409

410 Notably, pARACNe is significant and powerful as of its genome-wide scale and context-specificity  
411 in discovering global signaling cascading relationships, which were missing by previous methods.  
412 For example, methods proposed by Linding et al. (16) combine motif-based phospho-site  
413 predictions with information of physical association, co-occurrence, and co-expression to identify  
414 substrates with high specificity and accuracy, but with low coverage and lack of contextual  
415 specificity. Bender et al (17) used reverse phase protein assay data after various stimulations to  
416 cells and inferred signaling network using hidden Markov models and genetic algorithms. Even  
417 though the resulting networks are context specific, they lack genomic-scale coverage. There have  
418 been methods which used existing large-scale protein networks and prune them using  
419 transcriptomic information to identify signaling pathways (68-70). In addition, attempts have been  
420 made to reconstruct signaling network using gene expression data (71, 72). However, as signaling  
421 complexity lies mostly in upper level of cellular processes, inferring the cascades from  
422 downstream gene expression fails to capture all the dynamics. Also, PrePPI proposed by Zhang  
423 et al. (73) used protein structure-based methods to infer global protein-protein interaction, but this  
424 approach fails to address phosphorylation context specificity. Innovative uses of multiplex and  
425 microarray-based approaches, where multiple antibodies can be used to probe an ensemble of  
426 phosphoproteins, are finally becoming sufficiently mature to allow characterization of small  
427 pathways. Yet, these methods are still far from providing an unbiased, genome-wide view of  
428 signal-transduction processes and continue to be completely dependent on antibody specificity  
429 and availability. Similarly, assays developed specifically to monitor phosphorylation pathways,  
430 such as Stable Isotope Labeling with Amino acids in Cell culture (SILAC), provides a simple and  
431 straightforward approach to detect differential protein abundance. Coupled with phosphorylation  
432 enriched assays, it can provide high quality quantification for post-translation phosphorylation  
433 changes in cell lines. However, these methods are 1) laborious and costly; 2) can only be

434 performed to dissect the substrates of a single enzyme at a time and 3) do not differentiate  
435 between direct and indirect targets.

436

437 To be noted, as the LC-MS/MS experiments used here was generated based on Tyrosine-kinase  
438 enrichment, which is only about ~2% of whole phosphoproteome. pARACNe is shown only on  
439 TK-substrates network. The current methodology could be extended to whole phosphoproteomic  
440 data based signaling network reconstruction where the data is available. In addition to label free  
441 based LC-MS/MS proteomics data used in this work, label based approaches, such as ITRAQ or  
442 TMT, could generate higher throughput whole proteomic profiles which might require future  
443 redesign of ARACNe to incorporate both kinases and phosphatases in regulating their  
444 downstream substrates. It is reasonable to expect that a version of ARACNe developed  
445 specifically to dissect signaling networks should work at least as well as its transcriptional  
446 counterpart. Since the relationship between the mRNA abundance of a gene encoding a  
447 transcription factor (TF) and the activity of the corresponding protein is much looser than that  
448 between the abundance of a phospho-isoform of a kinase and its enzymatic activity.

449

450

451 Even though research has attempted to identify addiction points based on gene expression data  
452 (74), predictions based on phosphoproteomic data appear superior in a way that they can directly  
453 reflect contextual specific signaling activity and are able to be directly targeted by kinase  
454 inhibitors. It is important to note that clinically, only patients with base-pair deletion at exon 19  
455 (del746\_A750) or a point mutation at exon 21 mutation (L858R) in EGFR shows sensitivity to  
456 EGFR inhibitor such as Cedirinib or Erlotinib (75).

457

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464

## 465 **Author Contributions**

466 M.B. and M.K. developed the algorithm. M.B. and J.H. performed the analysis. M.P. performed  
467 the experimental validation. A.I., M.B., M. W., J.M and A.C. guided the experiments. M.B. J.H.,  
468 A.I. and A.C wrote the manuscript. M.B. and A.C. provided supervision and guidance. A.C.  
469 conceived, funded and administrated the project.

## 470 **Declaration of Interests**

471 A.C.is a founder and shareholder of DarwinHealth Inc. and a member of the Tempus Inc. SAB  
472 and shareholder. Columbia University is a shareholder of DarwinHealth Inc.

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664

## 665 **Figure Legends**

### 666 **Figure 1. Framework for the reverse engineering of TK signaling networks from** 667 **phosphoproteomic profiles.**

668 **(A)** Schematic diagram of a **TK**→**S** interaction. The non-phosphorylated kinase is inactive in terms  
669 of phosphorylating a substrate, while the active isoform successfully phosphorylates the  
670 substrate. **(B)** Schematic diagram showing the correlation between TK phosphorylation and that  
671 of its potential substrates. The first two rows in the heatmap show proteins representing candidate  
672 TK substrates **(C)** Illustration of the pARACNe framework including 6 steps. Step-1 depicts  
673 peptides collection from primary lung cancer tissue and cell lines for whole phosphotyrosine  
674 proteomics quantification. Step-2 depicts inferences of TK→S interactions using Mutual  
675 Information by Step-3 Naïve-Bayes estimator and Step-4 of the iterative quantile discretization  
676 methods. Step-5 and 6 depict network pruning and bootstrapping to construct final network. **(C).**  
677 Workflow of pARACNe from LC-MS/MS data normalization, IQD process, MI calculation, DPI  
678 process, bootstrapping to network consolidation.

### 679 **Figure 2. Predicted TK-TK network and validation of EGFR and c-MET prediction**

680 **(A)** pARACNe-inferred densely inter-connected TK-TK network, with red nodes representing  
681 candidate TKs involved in auto-phosphorylation, where the phospho-state of a tyrosine is  
682 correlated with the phospho-state of a different tyrosine on the same TK protein. **(B)** pARACNe-  
683 inferred EGFR and **(C)** c-MET substrate overlap with SILAC-based and Database reported  
684 substrates, respectively.

685

### 686 **Figure 3. Inference of Master Regulator and combination**

687 **(A).** Schema of Master Regulator analysis in lung cancer using pVIPER. Prioritized Master  
688 Regulators **(B)** and Prioritized Master Regulator Pairs **(C)** as significantly activated (red circle) or

689 de-activated (blue) in different lung cancer cell lines (column). Red color represents an enrichment  
690 of substrates hyper-phosphorylation by a Master Regulator or Master Regulator Pairs. Blue color  
691 represents that of hypo-phosphorylation.

692 **Figure 4. Experimental validation of EGFR and c-MET combination by colony**  
693 **formation assay**

694 **(A)** Colony formation assay schema. **(B)** shows the image of long-term EGFR and c-MET double  
695 inhibition effects in HCC78 cell line with different treatments. **(C)** shows long-term colony formation  
696 data for 14 cell lines with different EGFR, BRAF and KRAS genomic mutation status.

697 **Figure 5. MTT Assay validation of EGFR and c-MET combination**

698 **(A)**. MTT assay experimental schema. **(B)** MTT assay of HCC78 cell line shows synergistic effects  
699 of Crizotinib and Erlotinib treatment. **(C)** shows short-term effects of EGFR and c-MET inhibitors'  
700 combination index in 11 cell lines include 2 control cell lines (red).

701 **Figure 6. Master Regulating peptides in primary lung cancer samples**

702 EGFR and c-MET co-regulate in three scenarios **(A)** when their common substrates are  
703 hyperphosphorylated, the patient responds to combination treatment well; **(B)** when most EGFR  
704 substrates are hyper-phosphorylated, the patient responds to EGFR inhibitor; **(C)** when  
705 substrates of both EGFR and c-MET are mostly hypophosphorylated, the patient does not  
706 respond. **(D)** show the Master Regulator and Master Regulator Pairs regulating hyper/hypo-  
707 phosphorylation of their network substrates in each primary samples.

708

## 709 **Methods and Data**

### 710 ***Phosphoproteomic Data***

711 The previously published phosphoproteomic data used to reconstruct signaling network was  
712 download from (50). This dataset, representing the abundance of phospho-tyrosine containing  
713 peptides, was obtained by tandem mass spectrometry analysis of 46 non-small cell lung cancer  
714 (NSCLC) cell lines, 151 NSCLC tumors, and 48 normal lung tissue samples.  
715 Immunohistochemistry and a phospho-tyrosine specific antibody were used to screen 96 paraffin-  
716 embedded, formalin fixed tissue samples from NSCLC patients as described by Rikova et al.(50).  
717 About 30% of tumors showed high-levels of phospho-tyrosine expression. Immunoblotting of 46  
718 NSCLC cell lines with a phospho-tyrosine specific antibody also showed heterogeneous reactivity  
719 especially in the molecular weight range characteristic of receptor tyrosine kinases.

720 Since phospho-tyrosine represents less than 1% of the cellular phosphoproteome, as determined  
721 by tandem mass spectrometry (MS/MS), and is difficult to analyze by conventional methods,  
722 immuno-affinity purification was performed with a phospho-tyrosine antibody to enrich for  
723 phospho-tyrosine containing peptides prior to tandem mass spectrometry. All tumors were  
724 identified as NSCLC based on standard pathology. Only those tumors with greater than 50% of  
725 cancer cells were considered for further analysis. NSCLC cell lines were grown overnight in low  
726 serum to reduce background phosphorylation from culture conditions.

727 Tandem MS profiling identified 3920 tyrosine phosphorylation sites on approximately 2600  
728 different proteins. 85% of these sites appeared to be novel when compared against PhosphoSite  
729 (<http://www.phosphosite.org>), a comprehensive resource of known phosphorylation sites.

730 ***pARACNe Algorithm***

731 ARACNe is originally designed for gene expression data, where expression of genes is usually  
732 continuous and non-sparse. Quantitative data obtained from label-free LC-MS/MS by data-  
733 dependent acquisition via spectral counting is discrete and very sparse, with many  
734 phosphopeptides counts not observed for multiple peptides in each sample causing the current  
735 version of ARACNe to be not suitable for this data, which thus required major modifications to  
736 handle discrete data. To handle discrete abundances, we modified the mutual information  
737 computation approach from a kernel density estimation based method to a Naïve based  
738 estimation of mutual information, which is a histogram based technique(76). Briefly, consider a  
739 collection of  $N$  simultaneous measurements of two genes  $X$  and  $Y$ . Data is partitioned into  $M$   
740 discrete bins  $a_i$ , and  $k_i$  denotes the number of measurements that lie within the bin  $a_i$ . The  
741 probabilities  $p(a_i)$  are then approximated by the corresponding relative frequencies of  
742 occurrence  $p(a_i) \rightarrow \frac{k_i}{N}$  and the mutual information  $I(X,Y)$  between datasets  $X$  and  $Y$  is  
743 expressed as

744 
$$I(X,Y) = \log N + \frac{1}{N} \sum_{ij} k_{ij} \log \frac{k_{ij}}{k_i k_j}$$

745 Here  $k_{ij}$  denotes the number of measurements where  $X$  lies in  $a_i$  and  $Y$  in  $a_j$  and  $N$  total number  
746 of samples.

747 Accuracy of mutual information is dependent on correct numbers of bins,  $M$ . To find the optimal  
748 number of bins we applied ARACNe on the whole dataset by varying  $M$  from 1 to 20 and testing  
749 the connections in predicted sub-network against the set of known connections (gold standard)  
750 from databases (phosphoDB) (33).

751 In case of continuous data, partitioning can be achieved by dividing the range of data into M  
752 equally spaced distance bins. Our data being discrete, equally spaced partitioning was not  
753 possible. So, to overcome this problem, we used an iterative approach of partitioning (**Fig. 1C**,  
754 **Fig. S1A**). The basic idea is to divide the number of N data points into M with each bin containing  
755 equal number of data point. If the data point(s) with the same value falls into consecutive bin(s),  
756 we put those data point(s) into current bin and repartition the remaining points into remaining  
757 number of bins. We keep iterating this till we finish either the bins or there are no more data points  
758 to bin. For example, in **Fig. S1A**, we initially partition N points into 4 bins. The data points with 0  
759 value does not fit into first bin and falls into subsequent bins, so we assign all data points with 0  
760 value into first bin and repartition the remaining points into 3 bins. We keep on doing this process  
761 till we achieve 4 bins.

762 To evaluate initial performance and decide number of bins, we computed the network among all  
763 tyrosine kinases and substrates, parsed the sub-network between 49 tyrosine kinases and 114  
764 substrates which were present in PhosphoSite database and compared the results with the  
765 connections present in database. From our analysis, we found that M=10 to be an optimal number  
766 (**Fig. S1B**) which gave us precision of 14% and sensitivity of 24%. This precision is an  
767 underestimate of real precision as in the gold standard many interactions are not present.

### 768 ***Master Regulator Analysis***

769 To discover the master regulator in various cell lines, we interrogated the network obtained from  
770 pARACNe using a novel algorithm, VIPER (Virtual Proteomics by Enriched Regulon analysis)  
771 (21), designed to infer kinases that are key players in a particular cell line. Protein activity is a  
772 good indicator of key kinases in a particular phenotype but often phosphorylated state of a protein  
773 is not sufficient to determine its activity both due to measurement noise in phosphorylated state  
774 as well as LC-MS/MS technique noise. To overcome this problem, VIPER infers kinase activity

775 from the global kinase substrate relationship and its biological relevance by overlapping this  
776 information in a particular phenotype-specific program.

777 VIPER requires a network model and signature of the phenotype transition (i.e., all genes ranked  
778 by their differential phosphorylation in two phenotypes). Here, the signature,  $S_{kin}$ , was obtained  
779 by t-test analysis by comparing each cell line against all normal samples. First, we associate each  
780 kinase with positive and negative activity targets, by computing the correlation between each  
781 kinase and its predicted substrates and selecting only those substrates which had a significant  
782 correlation ( $p\text{-value} \leq 0.05$ , Bonferroni corrected). Second, for each kinase we computed an  
783 *activity* by measuring the enrichment of the  $S_{kin}$  signature in predicted substrates list, separately  
784 for both positive and negative correlated, ( $S_{kin}\text{-enrichment}$ ). Enrichment was computed by Gene  
785 Set Enrichment Analysis (GSEA). Since very small percentage of kinases are found to have  
786 negative correlation, we did not use those interactions to evaluate enrichment.

### 787 **Cell Culture**

788 All cell lines were grown in RPMI-1640 with 5% fetal bovine serum and incubated at 37°C in a  
789 humidified atmosphere containing 5% CO<sub>2</sub>. Cell lines were fingerprinted using the Perplex 1.2  
790 system (Promega, Madison, WI). Fingerprints were compared to those generated at ATCC and/or  
791 our internal database.

### 792 **MTS Assays**

793 Short term MTS assays (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega,  
794 Madison WI) were performed as previously described in (77). Specifically, each drug  
795 concentration is octuplicated and the mean with standard deviation of all replicates were used to  
796 generate a curve to allow calculation of the drug IC<sub>50</sub> (Inhibitory Concentration of 50%) value.  
797 The assays were repeated at least 3 times and the IC<sub>50</sub>s are the average of all replicates.

798

## 799 **Colony-formation Assays**

800 Long term colony formation assays were performed in triplicate in 6-well plates. Cells were added  
801 to media containing drug and incubated for 1-2 weeks such that control wells (no drug) contained  
802 colonies of 50-70 cells each. At such time media was removed and all wells stained with a solution  
803 containing 0.5% crystal violet and 6% glutaraldehyde for 1 hour. The plates were then rinsed,  
804 dried, and colonies were manually counted.

## 805 **SILAC Experiments**

806 EGFR SILAC experiment was performed in H3255 cell line by treating samples with Gefitinib. c-  
807 MET SILAC experiment was performed in c-MET-driven gastric cancer cell line, MKN45, by using  
808 c-MET inhibitor Su11274. For both genes, cells were treated with inhibitors for 3 and 24hr. For  
809 control, cells were grown in same conditions but were not treated with the drug. For our  
810 comparison we combined the peptides, which were differentially obtained between treated and  
811 untreated samples, for 3 and 24 hr. More details about the experiment can be obtained from Guo  
812 et al (32).

813 **Gold standard:** In PhosphoSite database, there were 282 connections between 49 tyrosine  
814 kinases and 114 substrates.

815

## 816 **Supplemental Information**

### 817 ***Fig. S1. Performance of the pARACNe Algorithm.***

818 **(A).** To select optimal bin number in pARACNe algorithm, precision and recall curves for various  
819 number of bins were computed. Black curve is when no binning of data is done. When using 10  
820 bins, the algorithm achieved the best performance.



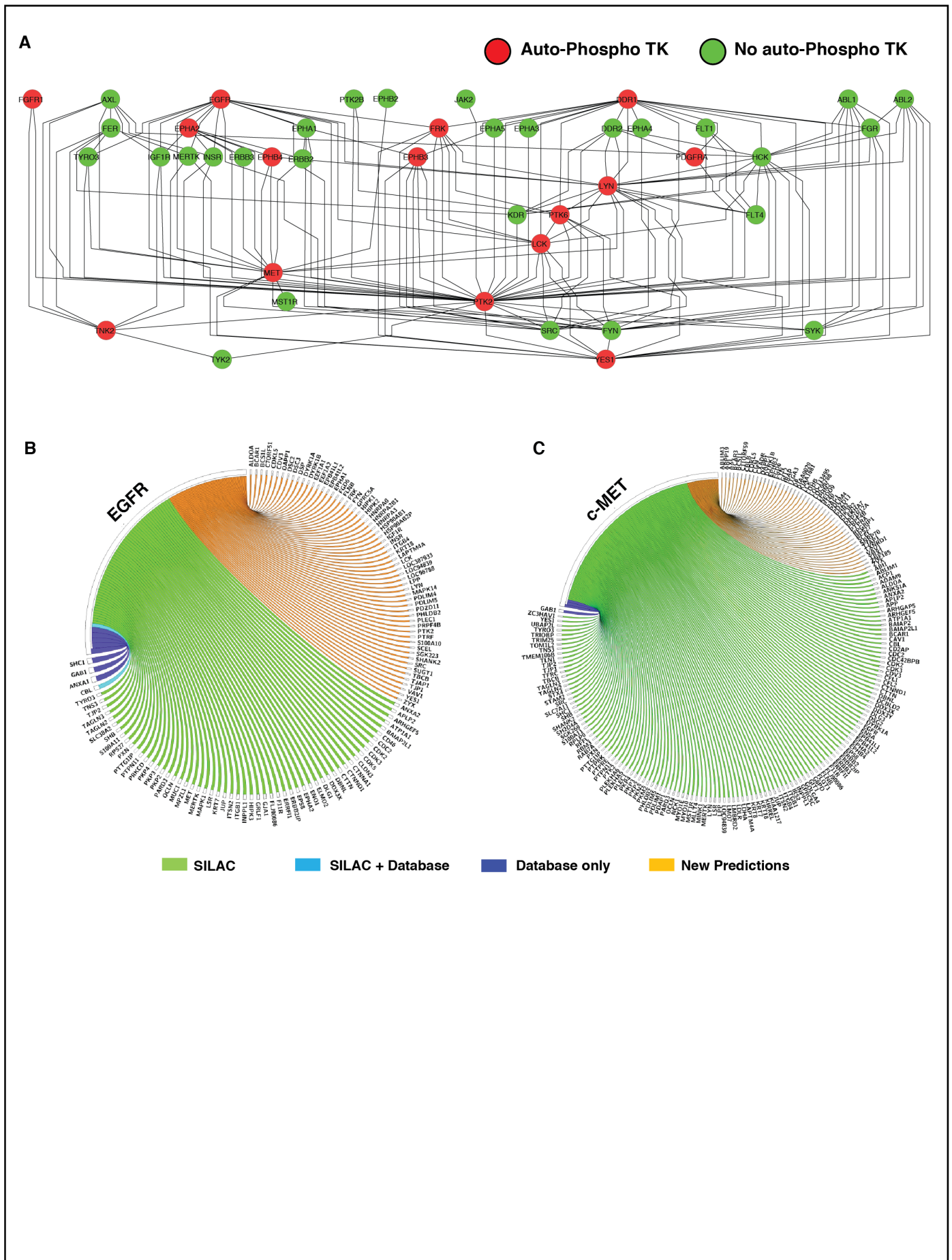
821 ***Table S1. pARACNe-inferred TK-peptides/substrate-peptides Interaction Network.***

822 ***Table S2. pARACNe-inferred TK-Protein/Substrate Interaction Network.***

823 ***Table S3. Colony Formation Assay and MTS Assay Results.***

824





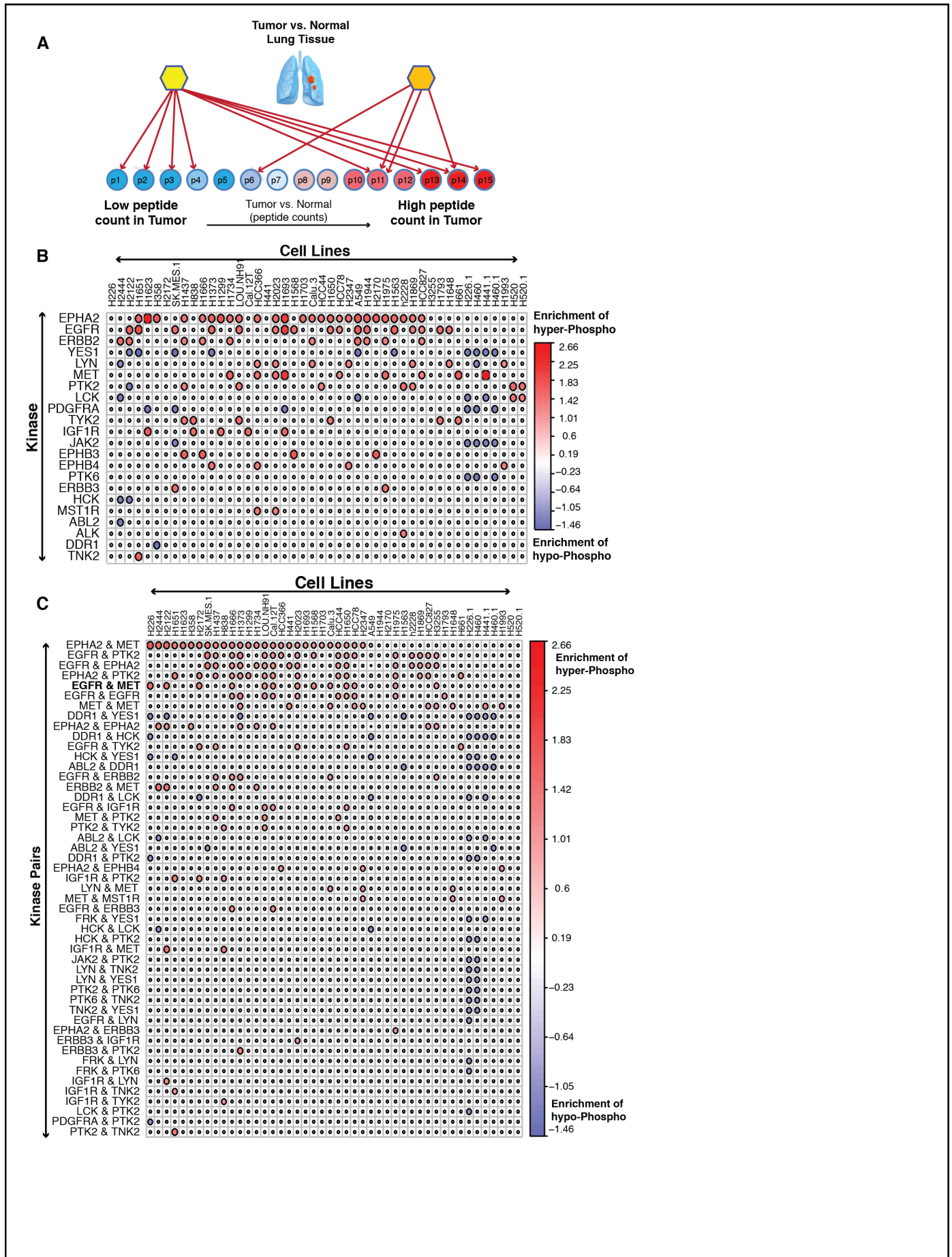


Figure 4

