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The Proximal Signaling Network of the BCR-ABL1 Oncogene Shows a Modular Organization

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Abstract

BCR-ABL1 is a fusion tyrosine-kinase, which causes multiple types of leukemia. We used an integrated proteomic approach that includes label-free quantitative protein complex and phosphorylation profiling by mass spectrometry to systematically characterize the proximal signaling network of this oncogenic kinase. The proximal BCR-ABL1 signaling network shows a modular and layered organization with an inner core of three leukemia transformation-relevant adaptor protein complexes (Grb2/Gab2/Shc1 complex, CrkI complex, and Dok1/Dok2 complex). We introduced an “interaction directionality” analysis, which annotates static protein networks with information on the directionality of phosphorylation-dependent interactions. In this analysis, the observed network structure was consistent with a step-wise phosphorylation-dependent assembly of the Grb2/Gab2/Shc1 and the Dok1/Dok2 complexes on the BCR-ABL1 core. The CrkI complex demonstrated a different directionality, which supports a candidate assembly on the

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Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at Oncogene’s website

Supplementary Information

Supplementary Text: with an extended methods and discussion section

Supplementary Figures (combined with Supp. Text in Titz_et_al_SupplementaryMaterial.pdf)

Supplementary Figure 1: *Mass spectrometry-based protein interaction quantification*

Supplementary Figure 2: *Evaluation of protein expression and protein purification*

Supplementary Figure 3: *Identification of protein clusters*

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Supplementary Tables (combined in a single .xls file)

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Nedd9 (Hef1, CasL) scaffold. Since adaptor protein family members can compensate for each other in leukemic transformation, we compared members of the Dok and Crk protein families and found both overlapping and differential binding patterns. We identified an additional level of regulation for the CrkII protein via binding to 14-3-3 proteins, which was independent from its inhibitory phosphorylation. We also identified novel components of the inner core complexes, including the kinases Pragmin (Sgk223) and Lrrk1 (Lrrk2 paralog). Pragmin was found as a component of the CrkI complex and is a potential link between BCR-ABL1/CrkI and RhoA signaling. Lrrk1 is an unusual kinase with a GTPase domain. We detected Lrrk1 as a component of the Grb2/Gab2/Shc1 complex and found that it functionally interacts with the regulator of small GTPases Arap1 (Centd2) and possibly participates in the MAP-kinase response to cellular stresses. This modular and phosphorylation-driven interaction network provides a framework for the integration of pleiotropic signaling effects of BCR-ABL1 towards leukemic transformation.

Keywords

adaptor protein; BCR-ABL1; phospho-complex; quantitative mass-spectrometry; signaling network; systems biology

Introduction

BCR-ABL1 is a constitutively active oncogenic fusion kinase that arises through a chromosomal translocation and causes multiple types of leukemia. It is found in many cases (~25%) of adult acute lymphoblastic leukemia (ALL) and in the overwhelming majority (>90%) of patients suffering from chronic myeloid leukemia (CML). BCR-ABL1 kinase inhibitors have been developed (e.g., imatinib and dasatinib), which show outstanding clinical responses, especially in early phase CML (Druker et al., 2001). However, drug resistance can develop and these kinase inhibitors are much less effective for the treatment of ALL and late (blast) phase CML (Pui & Jeha, 2007; Kantarjian et al., 2007).

BCR-ABL1 induces cellular responses and causes leukemic transformation through a complex interconnected signaling network (Wong & Witte, 2004). BCR-ABL1 is coupled to several downstream signaling modules, which show synergistic and compensatory interactions (Goga et al., 1995; Sonoyama et al., 2002). Understanding BCR-ABL1 signaling from a network perspective can also support the development of leukemia treatment options. For example, network-level signaling differences have been observed between drug resistant BCR-ABL1 mutants (Graeber et al., 2010; Skaggs et al., 2006) and several studies point to an intricate interplay between BCR-ABL1 signaling and Src-family kinase (SFK) signaling, especially in the development of ALL (Hu et al., 2004, 2006).

Adaptor proteins are important components of signaling networks, whose function does not depend on their enzymatic activity, but rather on their capability to orchestrate the formation of signaling complexes. Adaptor proteins are not simply static coupling devices; instead, they play important dynamic roles in many signal transduction processes, where they are regulated by mechanisms such as phosphorylation and act as signal sensors, signal integration devices, and context-dependent output devices (Pawson, 2007). Adaptor proteins also play important roles in mediating the effects of BCR-ABL1 on cellular phenotype

(Brehme et al., 2009; Ren, 2005). The binding of the adaptor protein Grb2 to Tyr177 of BCR-ABL1 is crucial for the leukemic transformation of cells by BCR-ABL1 (Pendergast et al., 1993), Dok-1 is a negative regulator of BCR-ABL1-mediated proliferation (Di Cristofano et al., 2001), and the adaptor protein Gab2 plays a critical role for BCR-ABL1 induced leukemic transformation of bone-marrow cells (Sattler et al., 2002).

Whereas most studies focus on single adaptor proteins or on small subsets of adaptor proteins, our aim is to globally analyze the interconnected function of these proteins in BCR-ABL1 signaling. Here, we start this systems-level analysis by defining the network of physical interactions and the phosphorylation states of adaptor proteins as proximal components of the BCR-ABL1 signaling network. Through this systems biology approach, we demonstrate that insights on multiple levels of the network can be obtained – ranging from the global network level, to the protein family level, and down to the individual protein level.

Results

The proximal BCR-ABL1 phospho-complexome as the basis for systems-level leukemic signaling investigations

Adaptor proteins fulfill their functions as scaffolds for the formation of protein complexes and are regulated by phosphorylation. To analyze their function in a comprehensive fashion, we devised a quantitative mass-spectrometry (MS) based phospho-complexome enrichment method (Figure 1, Supplementary Figure 1). We selected 15 adaptor proteins with relevance to BCR-ABL1 signaling (Table 1) and purified protein complexes of tagged versions of these proteins from BCR-ABL1-expressing BaF3 cells. BaF3 cells, murine pro-B cells, represent an informative leukemia model cell line (Daley & Baltimore, 1988; Skaggs et al., 2006; Mullighan et al., 2009) and serve as the common and well-controlled reference system for our systems biology analyses: they offer extensive control over the leukemia genotype (including the primary oncogene) and provide a common molecular context, which aides large-scale quantitative data integration.

With this complex-purification approach, we defined a protein interaction network with 76 interactions between 67 proteins (Figure 2, Supplementary Table 2). Of the identified 76 interactions, 28 have been reported in protein interaction databases and 13 of these interactions have been described in the BCR-ABL1 context previously (Supplementary Text). Strikingly, in an extensive set of 1 639 interactions listed in databases for the select adaptor proteins, the interactions reproduced in our study have a higher literature support: on average 4.2 publications for the reproduced interactions compared to 1.3 publications for the interactions in the whole set. The more restrictive nature of our dataset (compared to the full 1 639 potential interactions in the databases) and its higher quality illustrate the value of such systematic *context-dependent* interaction studies as a basis for further systems-level investigations.

The phosphorylation and activation state of the proximal BCR-ABL1 complexome

Our method provides information on the phosphorylation states of the adaptor protein complexes and thus on their activation states (Figure 3A, Supplementary Table 3). For an overview, we overlaid the phosphorylation site information on the domain structures of the complex components (Figure 3B and Supplementary Table 4). Overall, a high number of the identified phosphorylation sites were located outside of known domain boundaries –in potentially unstructured regions–, which is consistent with the realized importance of linear motifs for protein-protein binding and assembly of signaling complexes (Neduva & Russell, 2006; Diella et al., 2008). Dok1, Nedd9, and Gab2 show an especially high number of phosphorylation sites clustered in regions outside of protein domains that overlap with regions predicted to be unstructured (Figure 3B), which likely reflects their function as central signaling scaffolds (O'Neill et al., 2007; Niki et al., 2004; Di Cristofano et al., 2001).

A phosphorylation site within an enzymatically active domain was identified only for the Tec and Lrrk1 kinases. Both of these phosphorylation sites lie within the activation loop of these kinases and thus are likely to affect their activity of these kinases. The Tec phosphorylation site Tyr-518 was identified in the Dok1 purification and corresponds to the activating phosphorylation of Src family kinases. Thus, Dok1 forms a complex with enzymatically active Tec kinase.

For a more comprehensive overview, we integrated our experimental phospho-complexome with phosphorylation site annotations from the PhosphoSite database (Hornbeck et al., 2004) (Figure 4A). This representation summarizes the role of phosphorylation sites in the proximal BCR-ABL1 signaling network. At the same time, however, it also illustrates the sparseness of available information on the functional roles of individual phosphorylation sites as less than 20% of the identified phosphorylation sites have an annotation.

The modular and layered structure of the BCR-ABL1 network

We sought to define the high-level organization of the BCR-ABL1 network. We started with the identification of protein complexes/clusters in the network and found that three distinct adaptor protein complexes were directly linked to BCR-ABL1 (the Grb2/Gab2/Shc1, Dok1/2, and Crk complex) (Figure 4C, Supplementary Figure 3).

Next, we compared the organization of the experimental network and the (partially) complementary gene product association network provided by the STRING database (Jensen et al., 2009). The STRING database predicts functional gene associations using several bioinformatics methods including text mining and genomic context methods. In the STRING database, 46 of the 68 proteins in our experimental network form a tightly connected network (Figure 4B). At its core, we rediscover the components of the three BCR-ABL1-bound complexes (Grb2, Gab2, Shc1, Dok1, Dok2, and Crk), whereas other adaptor proteins such as Nedd9, CD2AP, Abi1, Stam2, and Itsn2 are more peripheral in this network. To further complete the network, we used literature information to functionally annotate the more peripheral complexes (Figure 4C).

To investigate how protein functions distribute in the network, we conducted a network enrichment analysis of gene ontology (GO) terms and protein domains (Titz et al., 2008).

This analysis assesses enriched properties in the local environment of each protein and, for example, identified the known negative regulatory role of Dok1 in proliferation and a functional association of CrkI with small GTPases. Interestingly, in this analysis we also find evidence for a distinct role of Shc1 at the interface between positive and negative regulatory regions of this network, which is consistent with two opposing findings for Shc1 – its negative role in cytokine regulated survival and proliferation (Ramshaw et al., 2007), and its positive effect on the leukemic potency of BCR-ABL1 transformed cells (Goga et al., 1995) (Supplementary Table 8, Supplementary Text).

Based on these network analyses, we conclude that the proximal BCR-ABL1 signaling network has a modular and layered structure with an inner core of three complexes, which tightly associate with BCR-ABL1 (Figure 4C). These inner core adaptor protein complexes also demonstrate a functional involvement in leukemic transformation. Specifically, the Grb2/Gab2/Shc1 subcomplex has a positive effect on transformation (Goga et al., 1995; Sattler et al., 2002; Johnson et al., 2009), whereas the Dok1/Dok2 complex has a negative effect (Di Cristofano et al., 2001; Niki et al., 2004; Yasuda et al., 2004). No direct role for Crk in transformation has been shown, but it has been implicated in the compensation for the knockout of CrkL in a BCR-ABL1-driven leukemia (Hemmeryckx et al., 2002).

“Interaction directionality” as a reflection of interaction dynamics

Phosphorylation-dependent interactions such as the interaction between a phosphorylated-Tyr peptide and a SH2 domain can be regarded as directed interactions pointing from the phospho-binding domain to the phosphorylated epitope (Figure 5A). From the perspective of phospho-interaction directionality, two principal categories for protein complex assembly exist: inward- and outward-guided assembly. In the inward case, when a localized, phosphorylation-dependent signaling complex is formed in the cell, many individual steps of complex formation involve the phosphorylation of an already bound component followed by the binding of a subsequent component via its phospho-dependent interaction domain. This assembly mechanism allows for a directed “inward” recruitment of additional complex components. In the outward case, phosphorylation of a protein results in recruitment of that protein outward to one of multiple docking sites, potentially on alternative complex assemblies which may be located in other subcellular regions (Figure 5A). The inward directional, step-wise, and phosphorylation-dependent assembly of protein complexes is well described for a number of signaling pathways. In insulin-receptor signaling, the receptor is phosphorylated first, which leads to the recruitment of the adaptor IRS-1 via its PTB domain (Eck et al., 1996). Next, IRS-1 becomes phosphorylated and recruits the adaptor protein Grb-2 via its SH2 domain (Skolnik et al., 1993). Note that in many cases the formation of signaling complexes also involves the formation of interactions that are not directed by phosphorylation such as the SH3-domain dependent interaction between Grb2 and Sos1 in the aforementioned pathway.

Here we analyzed the phospho-interaction directionality of our compiled phospho-complexome to obtain further insights into the assembly principles of the proximal BCR-ABL1-dependent signaling network (Figure 5B). For this, we integrated our experimental results with information on phospho-dependent binding epitopes from the PhosphoSite

database (Hornbeck et al., 2004) or manually curated from the literature (Supplementary Table 9). The general idea was to join the “static” protein interaction dataset and the “static” phospho-dependent binding epitope dataset to infer the “dynamic” assembly of protein complexes. Overall, 15 of the interactions with known phospho-dependent directionality point inward (to BCR-ABL1), whereas only 4 interactions point outward. Interestingly, differences between the inner core complexes are observed. The Dok and Grb2/Gab2/Shc1 complexes mainly show interactions with an inward directionality, whereas the CrkI complex shows more outward directionality. This difference could be explained by the recruitment of CrkI to an alternative core – the Nedd9 complex – which has been described as a metastatic “hub” for cancer signaling, can be phosphorylated by Src family kinases, and thus could function as an independent signaling core (O’Neill et al., 2007). In addition, Ship-1 and Shc1 appear to interact via bi-directional interactions. Strikingly, the bi-directionality of these interactions coincides with the network position of these proteins between the overall more growth-promoting Grb2/Gab2/Shc1 complex and the overall more growth-inhibiting Dok complex, consistent with a unique dual role of these proteins in this interaction subnetwork.

Differential roles of adaptor protein family members in the inner core complexes and a novel level of regulation for the CrkII protein

We analyzed the role of adaptor protein families in the Dok and Crk inner core complexes in more detail. We sought to define, whether these protein complexes are distinctly regulated by different adaptor protein family members (Supplementary Text). For the Dok family we compared Dok1 and Dok2, which are often assumed to have highly overlapping functions (Niki et al., 2004; Yasuda et al., 2004, 2007), but have only 48% sequence similarity, carry distinct phosphorylation sites, and show phosphorylation differences in platelets (Hughan & Watson, 2007). Here, we confirm a largely overlapping protein interaction profile of Dok1 and Dok2 (Figure 2A). However, we also observed differences. For example, only Dok1 was found to bind to the Csk kinase. We quantitatively followed up on this result and confirmed a preferential binding of Dok1 to Csk, whereas Dok2 bound preferentially to the RasGap1 effector protein (Figure 6A).

For the Crk complex we compared the adaptor protein family proteins CrkI, CrkII, and CrkL (CrkI is a truncated splicing isoform of CrkII). Both CrkII and CrkL carry an inhibitory Tyr-phosphorylation (Tyr221 and Tyr207, Figure 3A and 4A), which induces a closed inactive conformation (Kobashigawa et al., 2007). This inactive conformation, which inhibits the binding of effector proteins, is likely reflected by the different binding patterns of CrkI and CrkII in our interaction dataset. Strikingly, we observe that CrkII not only lacks binding to certain effector proteins, but at the same time, demonstrates binding to 14-3-3 proteins that CrkI does not (Figure 2A and Figure 6B). 14-3-3 proteins regulate signal transduction processes by binding to phosphorylated Ser and Thr residues and have been recognized as important signal integrators that impact cancer development (Morrison, 2009). We hypothesize that the binding to 14-3-3 proteins adds another level of (possibly negative) regulation on top of the negative regulation of CrkII by Tyr221 phosphorylation.

To test whether these two levels of CrkII regulation are mechanistically separable, we treated CrkII-bait expressing cells with increasing concentrations of the BCR-ABL1/SFK kinase inhibitor dasatinib and measured the effect on binding and Tyr-phosphorylation (Figure 6C/D). We confirmed the published observation that the inhibitory phosphorylation site on CrkL/CrkII only responds to treatment with 100 nM dasatinib, whereas the bulk of Tyr phosphorylation is already suppressed at 5 nM dasatinib (Shah et al., 2008). At 100 nM dasatinib we observed that the Tyr dephosphorylation of CrkII coincides with the loss of Cbl binding. 14-3-3 proteins, however, remained bound to CrkII even at the highest dasatinib concentration. We conclude that inhibitory Tyr-phosphorylation and 14-3-3 binding represent two independent and separable mechanisms of CrkII regulation based on their distinct dasatinib dose-response patterns.

In summary, for both the Dok and the Crk complex, we identified functional differences for adaptor protein family members and found a candidate regulatory mechanism for CrkII mediated by 14-3-3 protein binding.

Identification of additional components of the proximal BCR-ABL1 network

We found several novel or less well-known components in the BCR-ABL1 signaling network (Figure 2A). For further investigations, we selected the kinases Pragmin and Lrrk1 as novel components of the Crk and the Grb2/Gab2/Shc1 inner core complexes. Pragmin (Sgk223) was recently described as an effector of the Rnd2 GTPase, which stimulates RhoA activity (Tanaka et al., 2006). In a kinetics experiment we observed the rapid dephosphorylation of Pragmin and its paralog Sgk269 upon Gleevec treatment (Supplementary Figure 4A). We also found that Pragmin forms a protein complex with CrkI and Csk. Interestingly, CrkI has been functionally associated with small GTPase signaling (Supplementary Table 8) (Feller, 2001). Thus, the identified interaction might form the basis for a functional link between BCR-ABL1, CrkI, Pragmin, and RhoA signaling, which is consistent with the documented role of v-Crk in RhoA activation (Altun-Gultekin et al., 1998).

Lrrk1 as a component of the Grb2/Gab2/Shc1 signal integrator complex

The kinase Lrrk1 has an unusual structure with a central GTPase domain, which regulates its kinase activity (Korr et al., 2006), and it has been identified as a novel growth regulatory factor in a large-scale screen (Harada et al., 2005). Its paralog Lrrk2/Dardarin has been extensively studied since its gene is linked to both familiar and sporadic Parkinson’s disease (Paisán-Ruíz et al., 2004; Gilks et al., 2005). However, the molecular function of Lrrk1 and Lrrk2 remains elusive. A high similarity of Lrrk kinases to the Mapkkk-family of Ser-/Thr-kinases has been recognized and Lrrk2 can phosphorylate Mapkkk-substrates *in vitro*. However, the *in vivo* relevance of this phosphorylation is unclear (Gloeckner et al., 2009).

In our studies, we discovered Lrrk1 as a protein interacting with Grb2 and Shc1 (Figure 2A) and assigned it to the Grb2/Gab2/Shc1 inner core complex (Figure 4C). A recent independent large-scale interaction study also found support for the Lrrk1-Grb2 interaction (Brehme et al., 2009). To further validate this finding and to gain additional functional insights, we first conducted the reciprocal complex purification with Lrrk1 and three Lrrk1

fragments as the baits (Figure 7A, Supplementary Table 10). These reciprocal complex purification experiments confirmed the participation of Lrrk1 in the core complex (e.g., Grb2, Shc1, Gab2, and Ptpn11), mapped its interaction with the core complex to the n-terminus of Lrrk1, and implicated the extended Lrrk1 complex in the proximal regulation of Map-kinase signaling via Sos1/2, Rsu1 (ras suppressor protein 1), and Map4k1.

Lrrk1's activity is regulated by its GTPase domain (Korr et al., 2006). We used our phospho-complexome approach to identify candidate sites for additional regulation by phosphorylation. We found two tyrosine phosphorylation sites for Lrrk1—one site is located in the n-terminal ankyrin domain, pY94, and another site located within the kinase domain, pY1453 (Fig. 7A). Whereas the pY94 site might regulate protein binding, we focused on pY1453 and found bioinformatic evidence that its presence in the kinase domain regulates the kinase activity of Lrrk1 (Supplementary Text).

The Grb2/Gab2/Shc1/Lrrk1 signal integrator complex is functionally involved in the balance of cellular stress responses

Next, to obtain information on how Lrrk1 functionally affects proximal BCR-ABL1 signaling, we conducted a quantitative, label-free mass-spectrometry-based tyrosine phospho-proteomics experiment (Figure 7B–E). Interestingly, over-expressing Lrrk1 mainly affects the tyrosine phosphorylation status of two proteins: Shc1 and Centd2 (Arap1). Phosphorylated Shc1-Tyr423 induces the Shc1 interaction with Grb2 (Figure 4A) (Faisal et al., 2004; Sasaoka et al., 2001) and the increased phosphorylation may be explained by the stabilization of this complex by Lrrk1 over-expression. Centd2 (Arap1) showed an increased phosphorylation on two sites: Tyr753 and Tyr977. Centd2 was identified as a regulator of small GTPases with both an Arf GAP and a Rho GAP domain (Miura et al., 2002), which participates in the regulation of EGF receptor signaling (Daniele et al., 2008; Yoon et al., 2008). We hypothesize that the functional phosphorylation-based link between Lrrk1 and Centd2 points to a regulatory interaction between the small-GTPase regulator Centd2 and Lrrk1, which carries a small GTPase domain.

Finally, we analyzed the functional role of the Lrrk1 complex on downstream signaling and phenotypic response. We disrupted the function of the Lrrk1 complex by over-expressing the n-terminus of Lrrk1 and monitored the phosphorylation response of Erk and Jnk upon three different cellular stresses: DNA damage (etoposide), inhibition of protein synthesis (anisomycin), and inhibition of the electron transport chain (rotenone) (Figure 8A–C). Consistently, we observed that disruption of the Lrrk1 complex increases the response by Erk, whereas the Jnk response is somewhat reduced. Thus, the Lrrk1 complex possibly regulates the Erk/Jnk balance upon cellular stresses. The DNA topoisomerase II inhibitor etoposide is used as a chemotherapeutic drug in childhood acute lymphoblastic leukemia (Pui et al., 2007; Raetz et al., 2008). We selected etoposide to test for phenotypic consequences of this imbalanced stress response and found an impaired ability of BCR-ABL1-expressing cells to cope with etoposide-induced cellular stress, when the Lrrk1 complex is perturbed by over-expression of the n-terminus of Lrrk1 (Figure 8D).

Discussion

BCR-ABL1 induces cellular responses and causes leukemic transformation through a complex interconnected signaling network (Wong et al., 2004). A comprehensive system-level model of the BCR-ABL1 proximal signaling network is needed for further insights into leukemic transformation. As a step towards this goal, we present an integrated phospho-complexome analysis of proximal BCR-ABL1 signaling and obtain results on multiple levels of detail of this network.

First, we devised a quantitative mass-spectrometry-based method (Figure 1, Supplementary Figure 1), which supports the identification of specific protein interactions (Figure 2B) and allows for comparisons between different complex purifications (Figure 6). Our method is similar in purpose to other recent quantitative affinity-purification/MS approaches (Trinkle-Mulcahy et al., 2008; Blagoev et al., 2003; Goudreault et al., 2009). The label-free quantitation method used for our method does not depend on the incorporation of specific isotope labels and does not strictly limit the number of samples for comparisons. Also, we added an iron-metal-affinity phospho-peptide enrichment step to the method. Similar to a different published approach (Pflieger et al., 2008), this allows the concurrent analysis of the phosphorylation states of the protein complexes and subsequently allows for inference of the activation states of the purified protein complexes (Figure 3, Figure 4A). Our resulting integrated phospho-complexome analysis approach is versatile and can be easily adapted for the study of proximal signaling networks of other oncogenic kinases.

We employed this method for the analysis of proximal BCR-ABL1 signaling. On the global network level, we observe the modular and layered organization of this network: the adaptor protein complexes are organized into an inner core of three adaptor complexes with functions closely related to leukemic transformation (both positive and negative); and more distant adaptor protein complexes, which fulfill extended functions such as actin regulation (Figure 4C). Modularity has been realized as an important feature of biological networks (Spirin & Mirny, 2003; Hartwell et al., 1999), including cancer signaling networks (Taylor et al., 2009), and it is the basis for the pathway-centered dissection of signaling networks. Our analysis supports the biological significance of the observed modularity and provides a conceptual framework for the extended analysis of this and other oncogenic signaling networks.

As a tool for the analysis of oncogenic signaling networks, we further introduced the concept of an “interaction directionality analysis” (Figure 5). This approach allows for the inference of interaction dynamics from a static protein network by evaluating and summarizing the phosphorylation-dependent interaction epitopes of an interaction subnetwork. Our application of this perspective reveals that the anticipated step-wise, inward assembly of BCR-ABL1-related phospho-protein complexes (starting with the autophosphorylation of BCR-ABL1) is reflected within the Dok and Grb2 core sub-modules of the proximal BCR-ABL1 network. Moreover, our analysis also points to the less conventional, outward-biased CrkI sub-module, which may reflect an alternative complex assembly with Nedd9 at the core.

Two of the identified core complexes are centered on adaptor protein families with multiple members (the Dok and Crk family). Co-expression of adaptor protein family members can contribute to the robustness of oncogenic signaling networks and members of the Dok and the Crk family have been found to compensate for each other in leukemic transformation (Hemmeryckx et al., 2002; Yasuda et al., 2004). Here, we identify both overlapping and differential binding patterns within the Dok and the Crk adaptor protein families and identify a novel level of regulation for the CrkII protein by 14-3-3 protein binding. These findings provide insights into the mechanisms by which protein family members can compensate for each other in oncogenic signaling networks and can help guide efforts to explain the observed differential mutation frequencies of protein family members in human cancers (Berger et al., 2010).

Finally, our study yields details on individual core complex components: (1) We find Shc1 at the interface of positive and negative regulation of leukemic transformation, which is consistent with reports that Shc1 can have both positive and negative functions in signaling (Goga et al., 1995; Ramshaw et al., 2007) (Figure 4C, Figure 5B); (2) We provide evidence for a potential role of the kinase Pragmin at the interface of Crk and RhoA signaling (Tanaka et al., 2006); (3) We followed-up on the function of the Lrrk1 kinase as a component of the Grb2/Gab2/Shc1 core complex and experimentally identified a possible function for Lrrk1 in influencing the balance of Map-kinase signaling as a part of a cellular stress response (Figure 7, Figure 8). We hypothesize that Lrrk1 might influence this balance both through its enzymatic activity – Lrrk1's paralog, Lrrk2, has been shown to phosphorylate MKK4/7 upstream of Jnk (Gloeckner et al., 2009) – and through its structural role in the Grb2/Gab2/Shc1 core complex – as suggested by the stabilization of Shc1 phosphorylation. Based on our results, the Lrrk family protein domain structure, and literature information (mainly for Lrrk2) (Korr et al., 2006; Gloeckner et al., 2009; Saha et al., 2009; Liou et al., 2008; Heo et al., 2010; Sämann et al., 2009), we hypothesize that both Lrrk1 and its close paralog Lrrk2 participate as components of extensive signaling complexes in the signal integration of multiple input signals from stress, growth, and cytoskeleton-based signaling cues. The misregulation of these responses could potentially modulate the response to chemotherapeutic drugs and other stresses as indicated by our etoposide results.

Our study "stands on the shoulder of giants:" a comprehensive systems-level view cannot be reached without integrating knowledge obtained previously on multiple levels of the system. At the same time, however, the availability of context-dependent, experimentally defined protein networks is essential. For a true understanding of leukemic transformation in its whole complexity and for the rational development of combination therapies, the integration of large- and small-scale experiments into a systems-level view will be crucial. Here we take a first step towards this goal and present an integrated phospho-complexome view of BCR-ABL1 signaling. Although we obtain insights on different levels of the network, the overall systems-level information remains sparse. For example many phosphorylation sites lack functional annotation. Even for a very well studied system, such as BCR-ABL1 signaling, future studies on all levels are required to reach a true mechanistic understanding that spans from the network level down to individual phosphorylation-sites.

Materials & Methods

A more comprehensive description of these methods is provided in the Supplementary Text. The protein interactions from this study have been submitted to the IMEx (http://imex.sf.net) consortium through IntAct (Kerrien et al., 2007) and assigned the identifier IM-8314.

Cloning and creation of stable cell lines

The select 15 adaptor proteins and the kinases Pragmin and LRRK1 were cloned into the pCeMM-NTAP(GS)-Gw (Bürckstümmer et al., 2006) vector using the Gateway system (Invitrogen, Carlsbad, CA, USA) (Supplementary Table 1). In this vector, proteins are n-terminally linked to a TAP-tag (tandem-affinity-purification tag) consisting of an IgG-binding protein G and a streptavidin-binding SBP tag (Figure 1A). BaF3 cells, which over-express both BCR-ABL1 (p210 isoform) (BaF3/p210 cells) and an adaptor or kinase were obtained by retroviral infection as described previously (Burgess et al., 2005). Expression of TAP-tagged proteins was confirmed by Western Blot probing with normal rabbit IgGs (SC-2027, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (see Supplementary Figure 2 for an example).

Quantitative phosphorylation-complexome analysis

BaF3/p210 cells expressing TAP-tagged bait proteins were lysed and bait protein complexes were purified in a single-step purification via streptavidin beads (Pierce Streptavidin Plus UltraLink Resin, Thermo, Rockford, IL, USA) (Bürckstümmer et al., 2006) (Figure 1A). Following the trypsin digesion of the purified protein complexes (Promega, Madison, WI, USA), phosphorylated peptides were enriched using PHOS-Select iron affinity gel (Sigma Aldrich, St. Louis, MO, USA). Both the flow-through fraction and the (phospho-enriched) elution fraction were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptides were quantified using a label-free quantification method described previously (Skaggs et al., 2006; Zimman et al., 2010) (Figure 1B, Supplementary Figure 1, Supplementary Text). Scaling of the obtained *raw quantities* for each peptide yielded *relative quantities* that are less dependent on peptide detection efficiencies (Figure 1C). For interaction filtering, an *enrichment value* for each co-purified protein was defined. This value compares the *relative quantity* (median of the *relative quantities* of the individual peptides (pep)) of a co-purified protein (prey) in the actual bait purification (bp) and the (vector only) control purification (cp) and accounts for the standard deviation (SD) of the measurements (Figure 1C):

$$\text{enrichment value}_{\text{bp,prey}} = \frac{\text{median}(\text{relative quantity}_{\text{bp,pep}}) - \text{median}(\text{relative quantity}_{\text{cp,pep}})}{\text{SD}(\text{relative quantity}_{\text{bp,pep}}) + \text{SD}(\text{relative quantity}_{\text{cp,pep}})}.$$

Note that a single-step purification (rather than tandem purification) was conducted, since this resulted in higher sensitivity in initial tests, e.g., the Dok1/Csk interaction was only detected by single-step purification. At the same time, the quantitative filter procedure compensates for the higher background of the single-step purification. The quantitative measurement of the background allows for the characterization of the ‘background (non-

specific) distribution' that can be used to add confidence measures to interactors determined to be specific (Figure 1C).

Tyrosine phospho-profiling experiment

The tyrosine phospho-profile of BaF3/p210 cells and BaF3/p210 cells over-expressing the Lrrk1 kinase (or its n-terminus) were analyzed using a quantitative mass-spectrometry-based method as described previously (Skaggs et al., 2006; Zimman et al., 2010). Phospho-Shc1 (Tyr423) antibodies for Western Blot verification were obtained from Cell Signaling Technologies.

Treatment of CrkII-expressing cells with dasatinib

BaF3/p210 cells stably transfected with CrkII in the pCeMM-NTAP(GS)-Gw vector (or with the empty vector) were treated with 0, 1, 5, 10, and 100 nM dasatinib (ChemieTek, Indianapolis, IN, USA) for 30min. Cells were lysed and processed for mass-spectrometry analysis. In parallel, the cleared lysates were analyzed by Western Blot using phospho-Stat5 (Tyr694) (Cell Signaling Technologies, Danvers, MA, USA), phospho-CrkL (Tyr209) (Cell Signaling Technologies), and pan phopho-tyrosine (4G10-HRP, Millipore, Billerica, MA, USA) antibodies.

LRRK1 experiments: response to cellular stresses

To measure cellular stress responses on the signaling level, BaF3/p210 cells stably transfected with the n-terminal part of Lrrk1 in pCeMM-NTAP(GS)-Gw or with the empty vector alone were treated with 50 μM etoposide for 1 h and 3 h, with 1 μM and 10 μM rotenone for 3h, and with 50 μg/ml anisomycin for 20 min and 60 min. Phospho-Erk1/2 (Thr202/Tyr204) and phospho-Jnk (Thr183/Tyr185) antibodies were obtained from Cellular Signaling Technology.

For the cell viability assay BaF3/p210 cells stably transfected with the n-terminal part of Lrrk1 in pCeMM-NTAP(GS)-Gw or with the empty vector alone were seeded at a density of 4×10^5 cells/ml. Cells were treated with 1 μM, 5 μM, and 50 μM etoposide in triplicates. Viable cells were counted after 48h incubation using a Vi-CELL counter (Beckman Coulter, Brea, CA, USA).

Bioinformatics, network, and enrichment analysis

The Cytoscape software (Ver. 2.6.3) was used for network visualizations (Shannon et al., 2003). Data was processed and analyzed with custom software written in perl and C# making use of statistical and plotting functions of the R statistical package (R Development Core Team, 2009) and statconn connector packages (Baier & Neuwirth, 2009). A protein association network was obtained from the STRING database (Jensen et al., 2009). Information on phosphorylation sites was obtained from the PhosphoSite database (Hornbeck et al., 2004). The network enrichment analysis approach (guilt-by-association approach) that was employed to assess enriched properties in the sets of adaptor protein interactors has been described previously (Titz et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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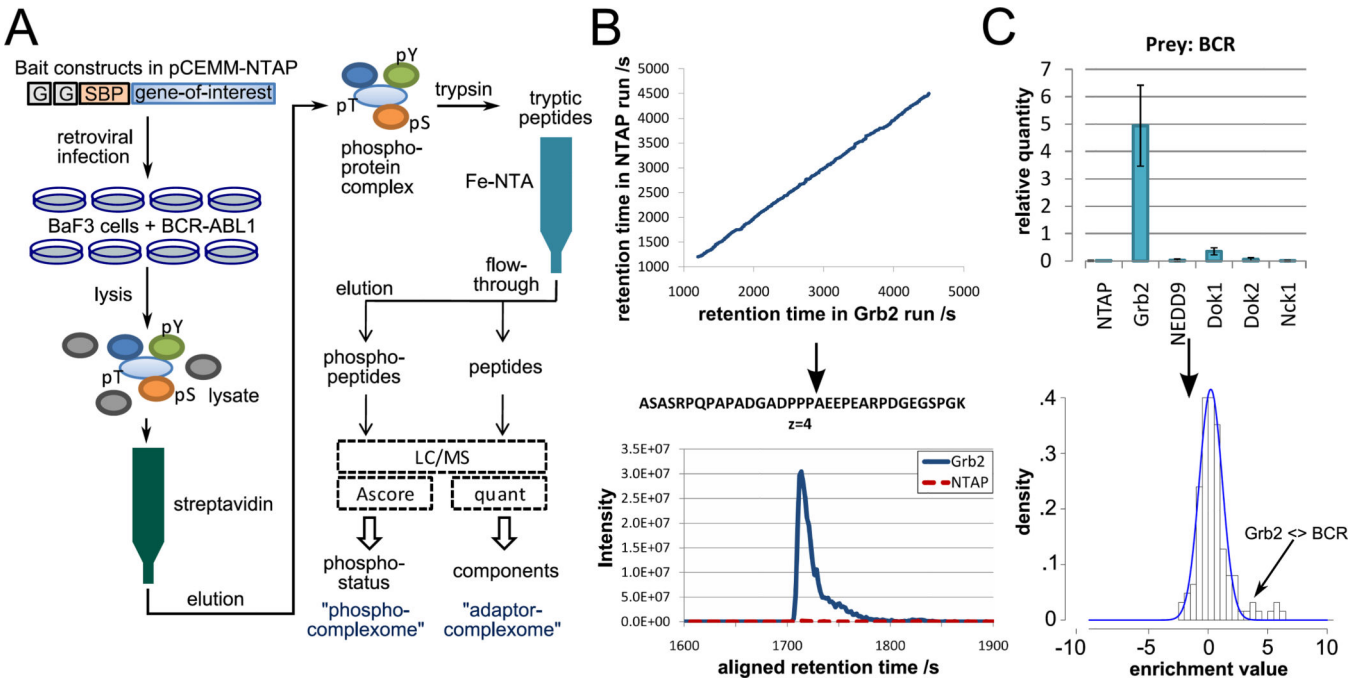

Figure 1. A Quantitative Phospho-Complex Enrichment Method

(A) Bait constructs for the genes of interests were assembled by recombinational cloning in the pCeMM-NTAP(GS)-Gw vector (Bürckstümmer et al., 2006). This vector contains two protein G tags (G) and a streptavidin-binding peptide (SBP) tag. BCR-ABL1-expressing Ba/F3 cells, murine pro-B cells, were infected with retroviral particles of these bait constructs. GFP-positive cell populations with stably integrated bait constructs were collected by flow-sorting. Cells were expanded, lysed, and phospho-protein complexes were purified via streptavidin beads. Only a single-step purification was conducted, since this yielded sufficient specificity and a higher sensitivity than a tandem-affinity purification procedure (see Methods). The purified proteins were then digested into peptides. Phospho-peptides were next enriched using an iron-affinity chromatography step (Fe-NTA). The non-phospho flow-through fraction was analyzed by mass-spectrometry and quantified for the identification of protein complex components, and the phospho-enriched elution fraction was analyzed by mass-spectrometry to obtain phosphorylation information. **(B)** For peptide quantification chromatography runs of different samples in the same batch were aligned (Prakash et al., 2006; Zimman et al., 2010): the alignment plot (upper panel) shows the corresponding retention times for mass-spectrometry runs of the Grb2 adaptor and the respective control purification (NTAP). After the alignment step the peptides were quantified by integration of the MS1 elution peaks: A comparison of the elution peaks of a BCR(−ABL1) peptide from a Grb2 purification and a control purification (NTAP) is shown (lower panel). **(C)** Multiple individual peptide quantification values were combined into a *relative quantity* value and a standard deviation for each purified protein (upper panel). The enrichment value compares this relative quantity for a co-purified protein between a given bait purification and the control purification. The distribution of all enrichment values is used to select specifically interacting proteins more confidently (lower panel).

"Grb2<>BCR" marks the enrichment value for the Grb2-BCR interaction. See Supplementary Figure 1 and Supplementary Text for more details.

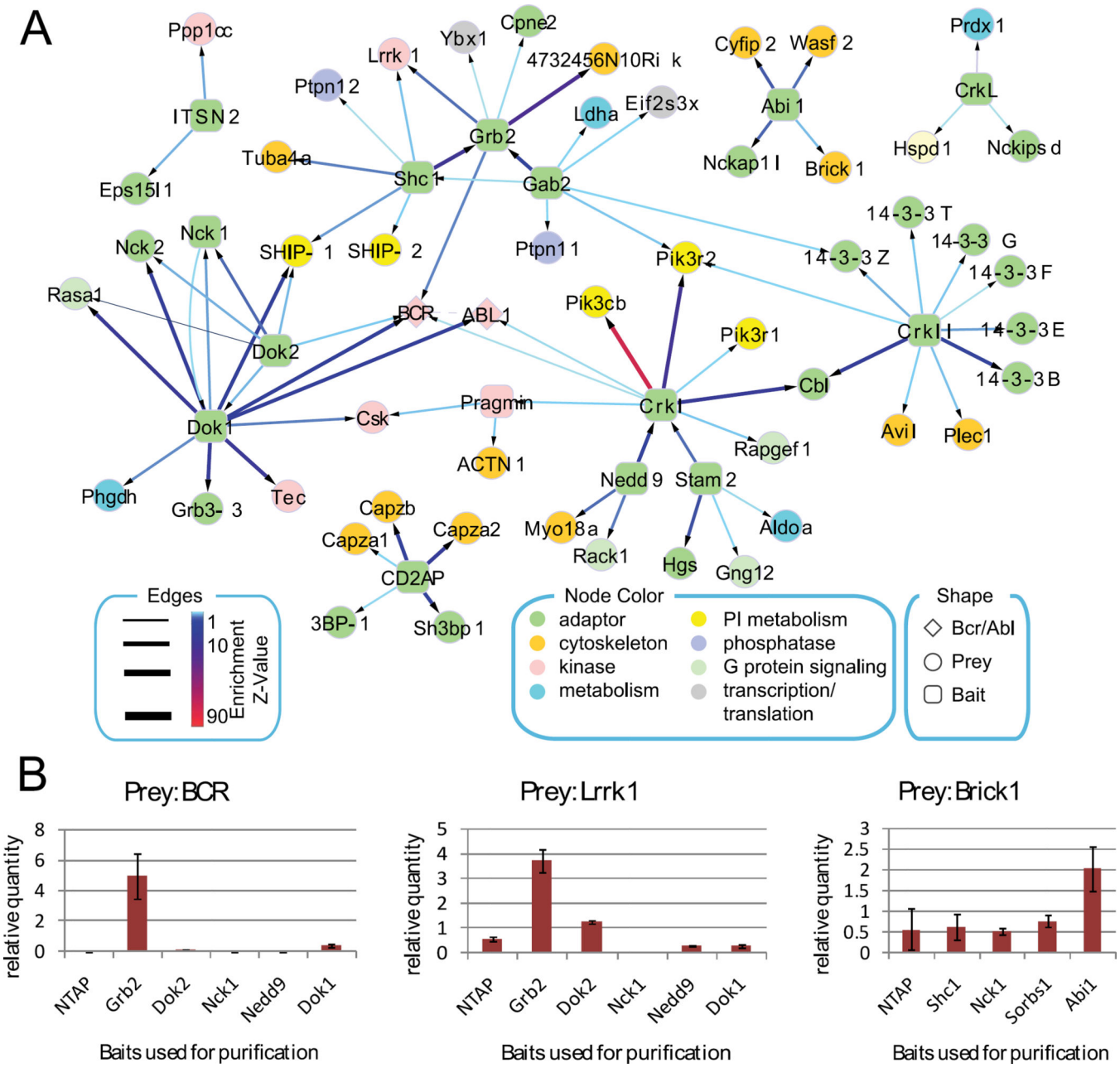

Figure 2. The Proximal Adaptor-Protein Complexome of the BCR-ABL1 Oncogene

(A) A network view of the identified adaptor protein complexes: The nodes of the network denote the proteins and the edges show the interactions between these proteins detected by our complex purification approach. The node color represents the functional class of the proteins and the node shapes their experimental roles (bait vs. prey). The edges are scaled and colored according to the enrichment value of the purification. Note that in this representation some enrichment values are derived from different purification experiments and cannot be compared directly. **(B)** Select examples of how the identification of specific interactions is supported by quantitative comparisons: within the same experimental batch these representations allow for a direct comparison of the enrichment of a given prey in

different bait purifications. Grb2 binds to BCR-ABL1 – a well-established interaction, which is mediated by phosphorylated Tyr177 of BCR-ABL1 (Million & Van Etten, 2000); Lrrk1, a protein kinase of unknown function, binds specifically to Grb2; and Brick1 is identified as an additional component of the Abi1/Wave2 complex (Gautreau et al., 2004).

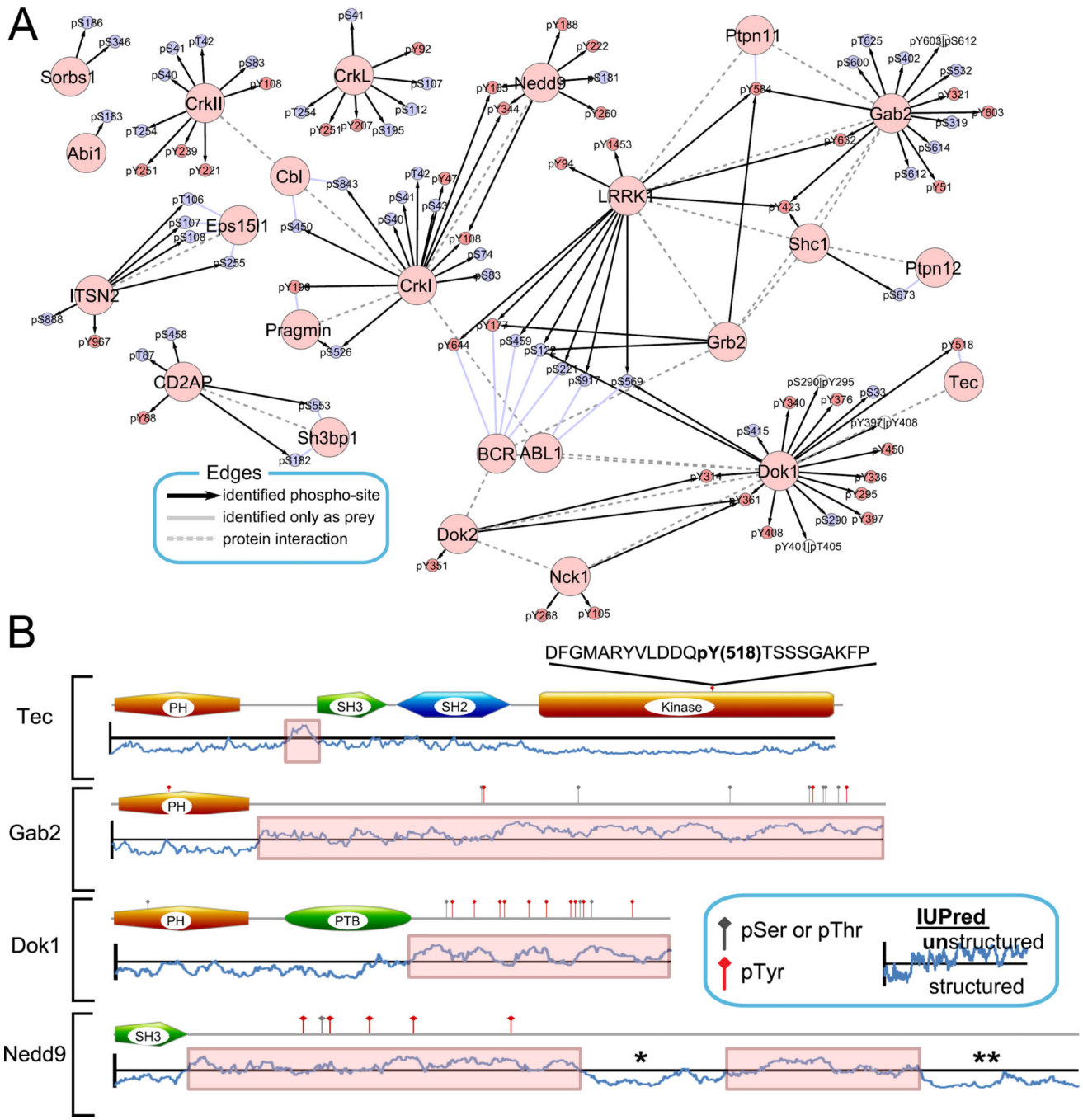

Figure 3. Phosphorylation sites detected in the purified protein complexes

(A) Network representation of the identified phosphorylation sites. Large nodes denote proteins; small nodes denote the detected phosphorylation sites of these proteins. Phosphorylation sites that belong to a certain protein are drawn close to its respective protein node. Black arrows pointing from the bait protein to a given phosphorylation site indicate the specific bait purification in which the phosphorylation site was identified. Grey lines connect proteins to their phosphorylation sites if the phosphorylation site was detected only on a prey protein. (B) Mapping of identified phosphorylation sites from our phospho-

complexome method onto the domain structure of their corresponding proteins (see Supplementary Table 4 for the full set). In addition to the domain structure, a prediction for unstructured protein regions is shown for each protein (IUPred, (Dosztanyi et al., 2005)): values above the black line (0.5) indicate an unstructured position; overall unstructured regions are labeled with a red box. The Tec phosphorylation site Y518 corresponds to the activation loop phosphorylation of SFKs. Nedd9, Dok1, and Gab2 show an especially high number of phosphorylated sites not in protein domains but rather in regions predicted to be unstructured. Note that Nedd9 contains two ambiguous regions in terms of structured predictions, which probably encode for a “serine-rich superfamily” (*) and a “DUF3513” (**) domain (Marchler-Bauer et al., 2009). Protein images were generated with the MyDomains tool (www.expasy.org/tools/mydomains).

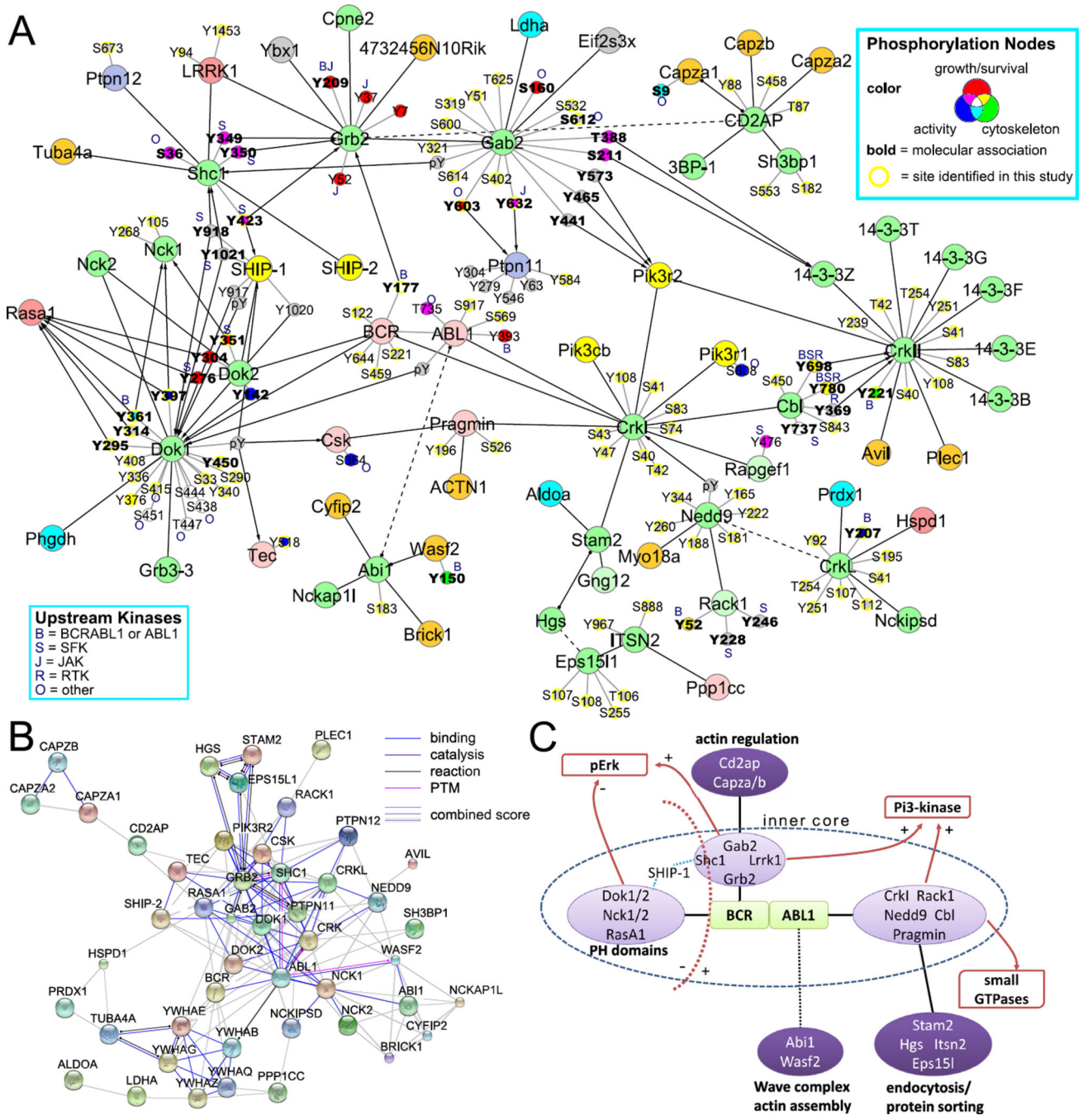

Figure 4. An extended view of the BCR-ABL1 phospho-protein network based on experiment and literature integration

(A) Overlay of our experimental results with phosphorylation site annotations obtained from the PhosphoSite database. Large nodes represent proteins; small nodes represent their phosphorylation sites. Phosphorylation sites detected in this study are denoted with a yellow circle. Detected interactions from our study are shown as the edges of the network; if available, interactions are linked to the phosphorylation sites which mediate the interaction (for some phospho-dependent interactions the specific site is unknown: “pY” nodes). The color of the phosphorylation sites denotes their functional annotation. Known upstream

kinases are indicated (see legend key, RTK = receptor tyrosine kinase). Abi1, CD2AP, and CrkL were linked to the network based on additional literature-based evidences (dotted lines, Supplementary Text). This representation yields insights into the assembly of the identified core complexes. For example, we obtain detailed information on the phosphorylation-dependent assembly of the Grb2/Gab2/Shc1 complex. BCR-pY177 is crucial for the recruitment of this complex (Million et al., 2000), this site is phosphorylated by BCR-ABL1 itself, and it has been implicated in the regulation of growth and survival. The Grb2/Gab2/Shc1 core complex is stabilized through pair-wise interactions between all three components. **(B)** String database integration. A protein association network was obtained from the STRING database with an extended “multi names” query of all protein names in the adaptor complexome of panel A (Jensen et al., 2009). **(C)** High-level summary of network organization. Three adaptor complexes form the inner core of the network and three complexes are more peripheral. Implicated regulation of pErk, Pi3-kinase, and small GTPases is indicated. Regulation can be positive (+), negative (−), or mixed (Shc1). Literature information was used to annotate the more peripheral complexes: (1) the Stam2/Hrs complex, as well as ITSN2 and Eps15l, function in the endosomal/vacuolar protein sorting pathway (Bache et al., 2003; McGavin et al., 2001; Kanazawa et al., 2003); (2) the complex formed by Cd2ap is involved in the regulation of the actin cytoskeleton (Kirsch et al., 1999); and (3) the Abi1/Wasf2(Wave2) complex regulates actin assembly (Stuart et al., 2006; Leng et al., 2005; Zipfel et al., 2006).

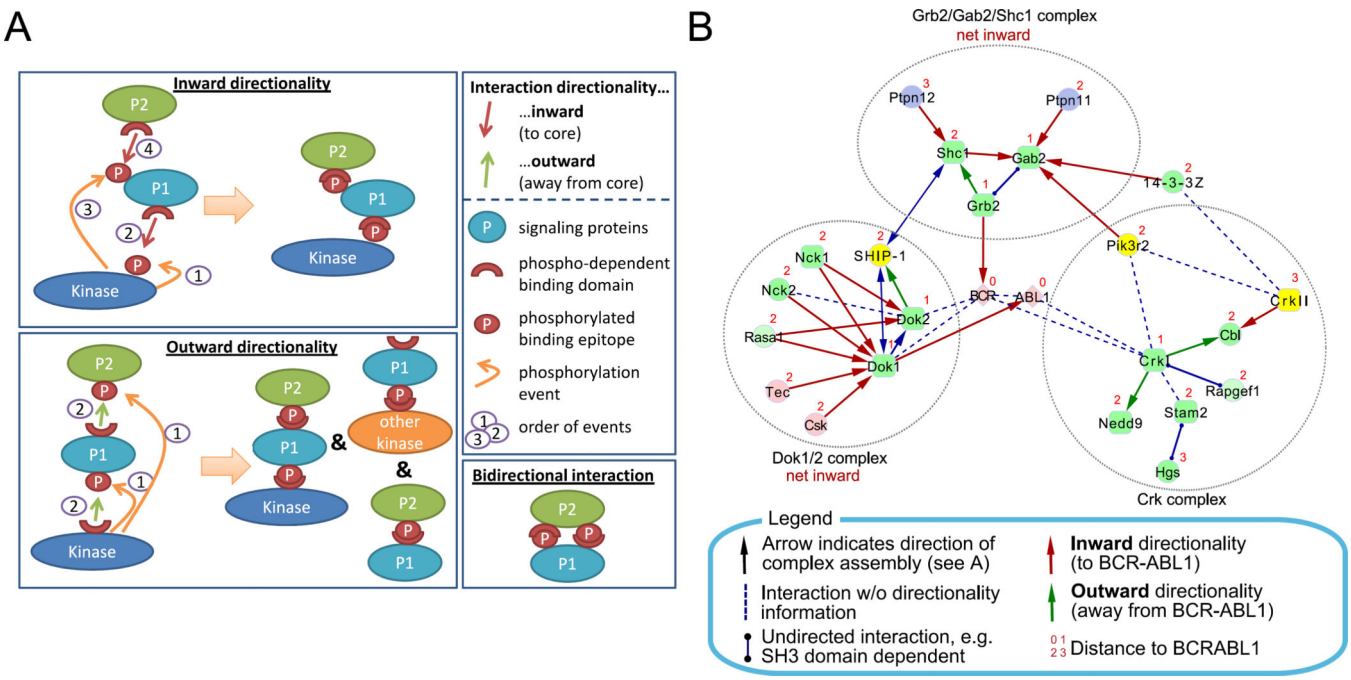

Figure 5. Interaction directionality analysis reflects step-wise assembly of the signaling network

(A) The concept of interaction directionality. For phosphorylation-dependent interactions we define the direction of the interaction pointing from the phospho-dependent binding domain to the phosphorylated binding epitope. Here we describe some illustrative examples of interaction directionality. In many cases, protein complexes are assembled on a core component. Here we represent the complex core as an autophosphorylated kinase – however, intrinsic kinase activity is not required for the core. Complex components assemble on the core through binary phospho-dependent protein interactions. We define interactions that point to the core as “inward directional” (red arrows) and interactions that point to the periphery as “outward directional” (green arrows). We show two examples for a three component complex; the first involving two “inward directional” interactions and the second involving two “outward directional” interactions. Inward directionality leads to an ordered, step-wise complex assembly on the core. Outward directionality allows alternative complex assemblies, which do not involve the core and can be located in other subcellular regions. Note that bidirectional and non-directional (i.e., non-phospho-regulated such as SH3 domain-mediated; not illustrated) interactions are also common in signaling networks. **(B)** Interaction directionality analysis of the proximal BCR-ABL1 network is consistent with the step-wise, inward assembly of the Grb2/Shc1/Gab2 and Dok complexes on the BCR-ABL1 core. This representation was generated by integrating our experimentally defined interaction dataset with information on phospho-dependent interaction epitopes from the PhosphoSite database (Hornbeck et al., 2004) or manually curated from the literature (Supplementary Table 9). Note that this information is only available for a subset of the interactions, mainly from other, non-leukemic biological systems. Thus, only the parts of the network with information on the direction of protein interactions are shown. The interaction direction is shown as arrows pointing from the phospho-dependent binding domain to the phosphorylated epitope. Red arrows denote interactions with inward and green arrows

interactions with outward directionality (with respect to BCR-ABL1). The boundaries of the three inner core complexes are indicated.

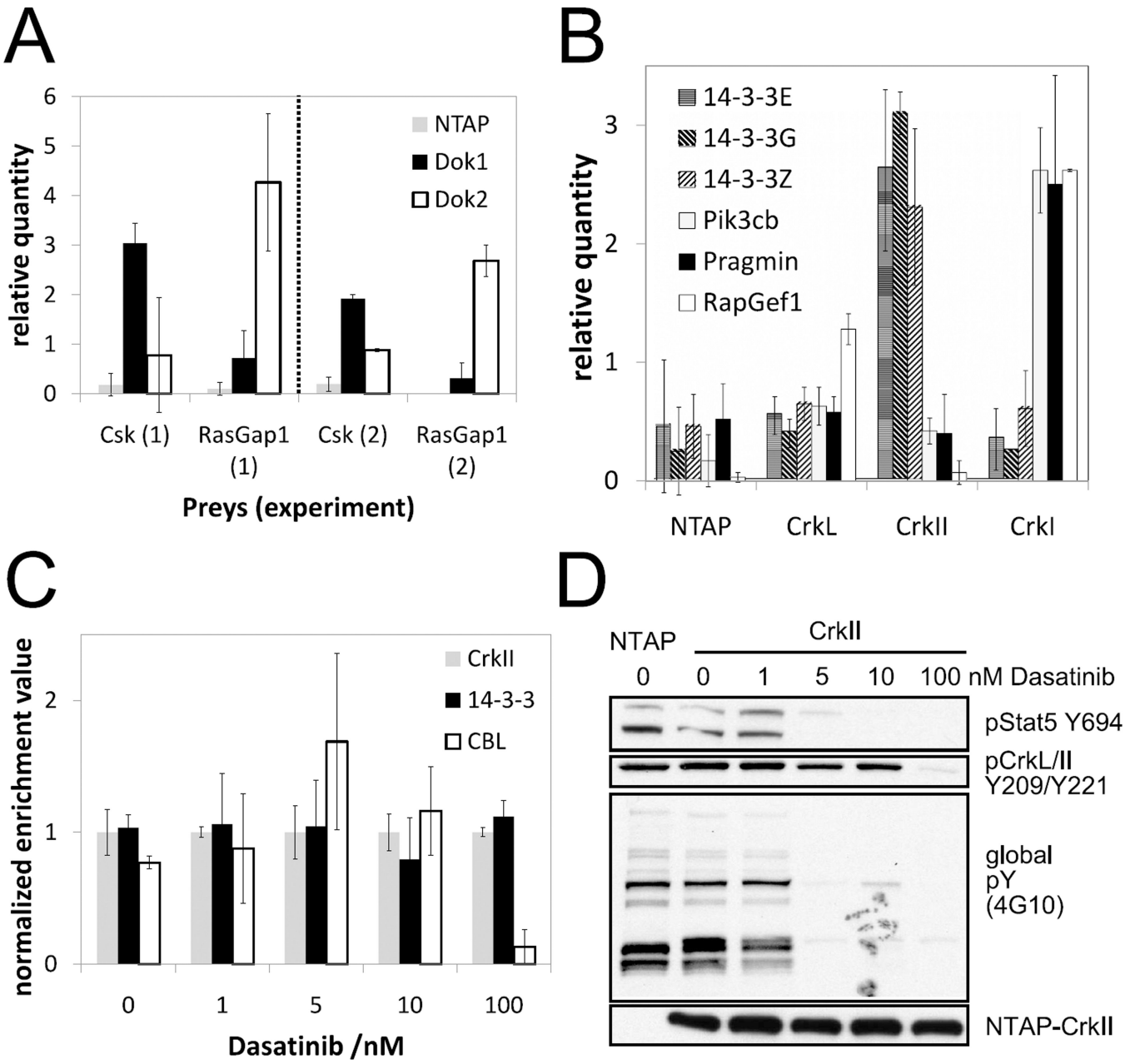

Figure 6. Differences within adaptor protein families revealed by quantitative complex purification

(A)–(C) Results from our quantitative MS-based complex purification method (same representation as in Figure 1C and Figure 2B). **(A)** Quantitative binding differences within the Dok-adaptor protein family: Both Dok1 and Dok2 bind to Csk and RasGap1 as compared to the control purification (NTAP). However, Dok1 preferentially binds Csk and Dok2 preferentially binds RasGap1. Results from two biological replicate experiments are shown (labeled as (1) and (2)). **(B)** Our quantitative MS-based complex purification method confirms the binding differences within the Crk-family seen in Figure 2A: CrkI binds to the effector proteins Pik3cb, Pragmin, and Rapgef1, whereas CrkII is found in association with 14-3-3 proteins. **(C)** Our quantitative MS-based complex purification method captures

distinct dasatinib response patterns for different CrkII interacting proteins. Dasatinib titration for the CrkII complex purification shows dissociation of Cbl at 100 nM dasatinib, whereas 14-3-3 proteins remain bound. Results from one representative experiment of two are shown. **(D)** Distinct dasatinib dose response patterns for phosphorylation events on CrkII and other proteins. Phosphorylation-specific Western Blots for experiment shown in panel C: whereas the global pY phosphorylation and the phosphorylation of Stat5 are strongly suppressed at 5 nM dasatinib, the phosphorylation of CrkL/CrkII (the antibody does not distinguish the family members) is maintained up to 10 nM dasatinib.

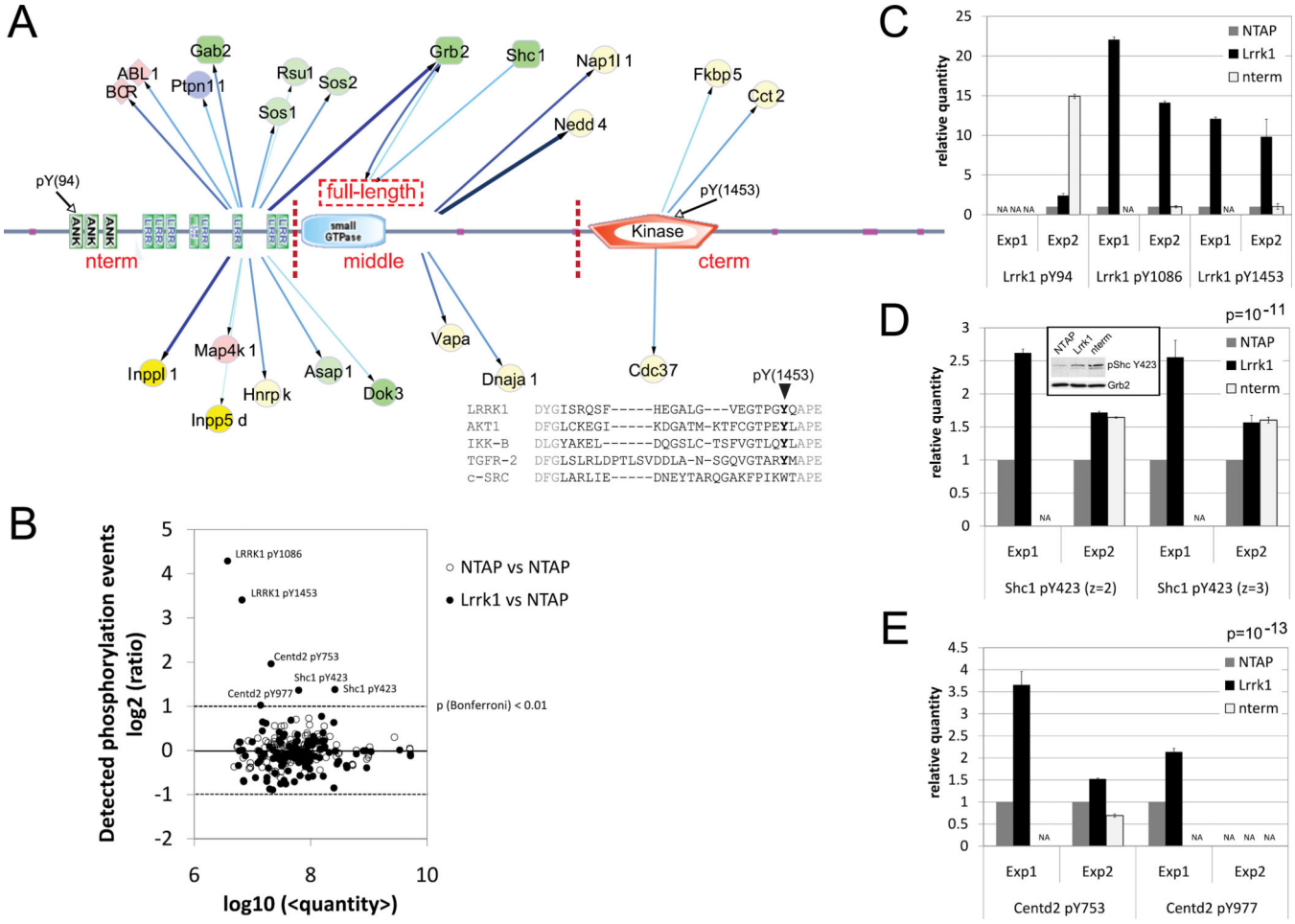

Figure 7. Lrrk1 is a novel proximal signaling component of the BCR-ABL1 network

(A) Complex purification results with Lrrk1 and Lrrk1 fragments (nterm, middle, cterm) as baits. Insert: Alignment of activation loop sequences. Phosphorylated tyrosine residue pY1453 of Lrrk1 and homologous residues are marked in bold. The interaction of Lrrk1 with the Grb2/Gab2/Shc1 core complex was mapped to the n-terminus of Lrrk1. Additional components of this core complex are detected, which were probably purified via indirect interactions: Gab2, BCR-ABL1, Ptpn11, Ship-1, and Ship-2. Another set of interacting proteins further implicates the extended core complex in the proximal regulation of Map-kinase signaling: Sos1/2, Rsu1 (ras suppressor protein 1), and Map4k1. The middle fragment of Lrrk1 associates with VAPA, the VAMP-associated protein-A, which is involved in ER-to-Golgi transport (Prosser et al., 2008). VAPA has been functionally linked to neurotransmitter release (Skehel et al., 2000, 1995) which establishes a potential link with Lrrk2's role in Parkinson's disease pathogenesis. The kinase domain (c-terminal fragment) associates with Cdc37, a kinase-specific co-chaperone, which directly binds to the kinase domain of its previously identified targets (Caplan et al., 2007). Interestingly, Lrrk2, the paralog of Lrrk1, was found in a complex with Cdc37 and chaperone inhibition was proposed as a treatment option for Parkinson's disease (Wang et al., 2008). **(B)** Global phosphoprofiling experiment for BaF3/p210 cells over-expressing LRRK1. Anti-phosphotyrosine antibody and mass spectrometry-based global phospho-proteomics was

used to characterize the influence of Lrrk1 on the BCR-ABL1 signaling network. The phospho-peptide abundance ratios for LRRK1 vs. control (NTAP) (black dots) are compared with the ratios of two control experiments (white dots). The dotted line marks a significance p-value for the upregulation of a phosphorylation-site of less than 0.01 (based on log-normal distribution of control ratios; Bonferroni corrected for multiple testing). Significantly upregulated phospho-peptides (from Lrrk1, Centd2, and Shc1) in BaF3/p210/Lrrk1 cells are labeled. (**C-E**) For the three proteins with significantely upregulated phospho-peptides, the mass-spectrometry-based quantification results in the experiment shown in panel B (Exp1) are compared with a second biologically replicated experiment (Exp2). Error bars indicate SEM of technical replicates (same sample analyzed twice by MS) within each biological replicate (independently cell culture expanded and processed samples). **(C)** Higher level of three Lrrk1 phosphorylation sites detected upon over-expression of Lrrk1 and the n-terminus of Lrrk1 (nterm). **(D)** Phosphorylation of Shc1-Y423 is induced both by over-expression of Lrrk1 and its n-terminus (nterm) (joint Bonferroni-corrected p-value for Lrrk1 effect is 10^{-11}). Induction is also visible in phospho-specific Western Blot (insert). **(E)** Two Centd2 (Arap1) phosphorylation sites are induced upon Lrrk1 over-expression (joint Bonferroni-corrected p-value for Lrrk1 effect is 10^{-13}).

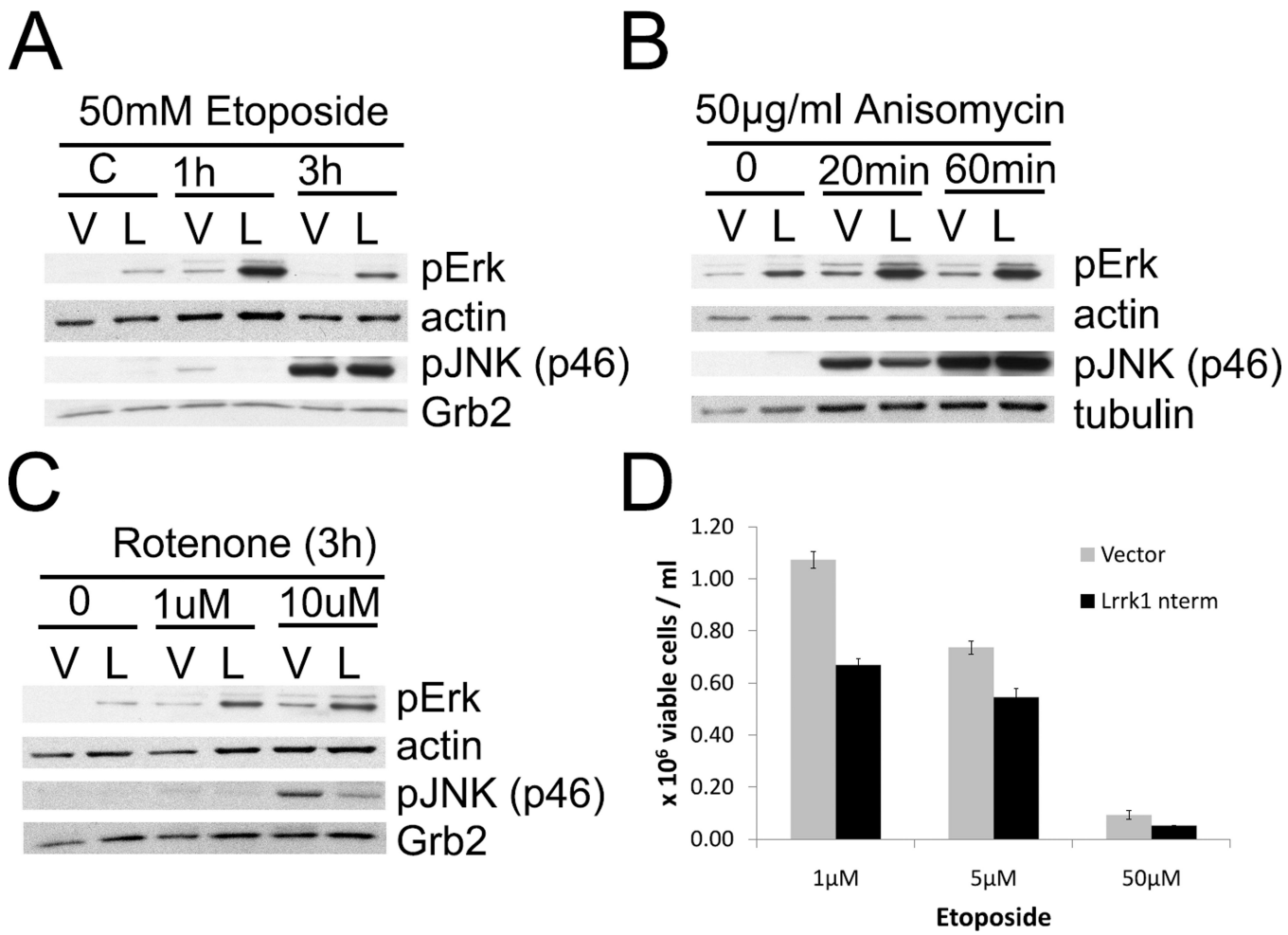

Figure 8. Functional involvement of Lrrk1 in the balance of BCR-ABL1-mediated MAP-kinase signaling

Perturbation of Lrrk1 complex leads to differential stress responses of BCR-ABL1-positive cells. BaF3/p210 cells expressing the n-terminus of Lrrk1 (L) or an empty vector (V) were treated with etoposide **(A)**, anisomycin **(B)**, and rotenone **(C)** and the differential effect on Erk (inhibitory) and Jnk (slightly activating) phosphorylation was measured by Western Blot. Note that the effect on Jnk phosphorylation is small and mainly seen for the rotenone experiment (at 10µM). **(D)** Perturbation of Lrrk1 complex results in higher sensitivity towards etoposide treatment. BaF3/p210 cells expressing the n-terminus of Lrrk1 (Lrrk1 nterm) or an empty vector (Vector) were treated with etoposide and the number of viable cells was measured after 2 days. A representative result out of two biological replicates is shown.

Table 1

The adaptor proteins selected for this study.

Family	Name	Synonyms	BCR-ABL1 Connection
Crk	**CrkI** **CrkII** **CrkL**		CrkL is phosphorylated in BCR-ABL1 expressing cells (Oda et al., 1994; Nichols et al., 1994); BCR-ABL1 causes leukemia in absence of CrkL and CrkI/II Might compensate for CrkL (Hemmeryckx et al., 2002).
Dok	**Dok1** **Dok2**	p62DOK DokR	Dok1/2 knockout accelerates BCR-ABL1-driven leukemia progression (Niki et al., 2004; Yasuda et al., 2004).
Gab	**Gab2**		Critical for transformation by BCR-ABL1 (Sattler et al., 2002).
Grb	**Grb2**		Grb2 binding site required for induction of myelogenous leukemia-like disease by BCR-ABL1 (He et al., 2002)
Nck	**Nck1**	Nck	
	Cd2ap	CMS, Mets1	Consistently phosphorylated in BCR-ABL1$^+$ cells (Goss et al., 2006).
Cas	**Nedd9**	CasL, Hef1	Phosphorylated in BCR-ABL1$^+$ cells (de Jong et al., 1997).
	Shc1	Shc, ShcA	Overexpression compensates for loss of BCR-ABL1 SH2 domain (Goga et al., 1995).
	Abi1	Nap1bp	Silencing of Abi1 affects proliferation of BCR-ABL1 transformed cells *in vivo* (Yu et al., 2008).
	Itsn2	Ese2, Sh3d1B	Phosphorylated in BCR-ABL1$^+$ cells (unpubl.)
	Stam2	Hbp	Phosphorylated in BCR-ABL1$^+$ cells (unpubl.)
	Sorbs1	Cap, Sh3d5	Phosphorylated in BCR-ABL1$^+$ cells (Skaggs et al., 2006).