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Imatinib dependent tyrosine phosphorylation profiling of Bcr-Abl positive chronic myeloid leukemia cells

Bcr-Abl is the major cause and pathogenetic principle of chronic myeloid leukemia (CML). Bcr-Abl results from a chromosomal translocation that fuses the bcr and the abl genes, thereby generating a constitutively active tyrosine kinase, which stimulates several signaling networks required for proliferation and survival. Bcr-Abl's oncogenic properties comprise both functions as a kinase and as a scaffold protein ¹. A number of Bcr-Abl interaction partners and downstream effectors have been described, improving our understanding of the signaling networks deranged in CML. Brehme et al. recently defined the "core-interactome of Bcr-Abl", identifying seven major interaction partners of Bcr-Abl (GRB2, Shc1, Crk, c-Cbl, p85, Sts-1, and SHIP-2) ².

The introduction of the Bcr-Abl tyrosine kinase inhibitor (TKI) Imatinib (Gleevec) in the clinic has been a landmark in the treatment of CML ³. However, the development of Imatinib resistance poses major challenges to the clinical management of CML, and although second generation TKIs can block many Imatinib resistant mutants they are ineffective against the common T315I mutation ⁴. An alternative strategy is to circumvent Imatinib resistance by targeting Bcr-Abl downstream pathways essential for transformation. Towards this aim it is important to fully understand these pathways. Large-scale (phospho)proteomics experiments have addressed the phosphoproteome of Bcr-Abl positive cells, leading to the identification of an impressive number of serine/threonine-phosphorylated sites ⁵⁻⁷, although tyrosine-phosphorylation events remained underrepresented. Importantly, many of the protein-protein interactions in the oncogenic Bcr-Abl network are dependent on tyrosine phosphorylation, either directly or indirectly downstream of Bcr-Abl. ⁸

25 Here, we have enriched and identified phosphotyrosine peptides by quantitative mass
26 spectrometry (qMS)^{9,10} in order to examine the effect of Imatinib in the CML blast crisis cell
27 line K562 (See Supplementary Materials for experimental details). First, Western blotting was
28 used to investigate the phosphorylation status and potential degradation of several known key
29 proteins involved in Bcr-Abl signaling in K562 cells in response to treatment with Imatinib.
30 Although Imatinib strongly reduced tyrosine phosphorylation none of the five investigated
31 proteins were degraded (Fig. 1A). For qMS studies we treated cells with Imatinib as used in
32 previous studies^{5-7,11} (i.e. 0, 1, and 10 μ M for 4 hours, Fig. 1B). Cell lysates were digested
33 with the proteases LysC and trypsin, followed by stable isotope dimethyl labeling of the
34 cleaved peptides⁹. The labeling regime allows to distinguish three peptide pools (light,
35 intermediate and heavy), which were mixed in equal concentrations. Tyrosine phosphorylated
36 peptides were enriched by immunoprecipitation and analyzed by LC-MS. Analysis of the
37 quantitative changes in tyrosine phosphorylation yielded 201 unique quantifiable
38 phosphotyrosine peptide triplets (light-intermediate-heavy) on about 140 proteins, exceeding
39 by far all previous reports (Table S1)^{6,11}. Of these, 87 peptides showed at least a 2-fold
40 down-regulation after treatment with Imatinib (Table S2 and Fig. 1C).

41 Imatinib significantly decreased the tyrosine phosphorylation of many peptides originating
42 from Bcr-Abl and its core interactors (c-Cbl, CrkL and SHIP-2, Fig. 1C). Furthermore, a
43 significant number of proteins that have been shown to play pivotal roles in Bcr-Abl
44 dependent signaling (Gab1, Gab2, Shc1, Crk, ERK-2, STAT5A/B and Yes) displayed reduced
45 tyrosine phosphorylation, often on multiple sites per protein. Also, several Src Family Kinase
46 substrates exhibited reduced tyrosine phosphorylation, e.g. Cortactin, Catenin delta-1, nPKC-
47 delta and Paxillin. Finally, Imatinib reduced the tyrosine phosphorylation of several proteins
48 involved in cytoskeletal regulation, such as MEMO1, Intersectin-2, Catenin delta-1, HEPL,

49 GRF-1, Centaurin delta 2, and Plakophilin, which mostly have not been previously linked to
50 Bcr-Abl signaling.

51 A motif analysis ¹² on the sequences of significantly down-regulated tyrosine phosphorylated
52 sites, revealed a distinct, 10-fold enriched motif, YxxP (Fig. 1D and 1E). In total, the tyrosine
53 phosphorylation of 80% (23 out of 29) of the peptides harboring the YxxP motif was
54 significantly down-regulated upon Imatinib treatment. The YxxP motif resembles a classic
55 binding site for SH2-domains and consensus target sequence for phosphorylation by Bcr-Abl
56 ¹³. Peptides found in our screen containing this motif include Bcr-Abl (Tyr 115 and 128), the
57 regulatory phosphorylation sites of the transcription factors STAT5A and B (Tyr 682 and 699,
58 respectively) ¹⁴, several sites in the adaptor proteins Gab1 (Tyr 259, 373, and 406) and Gab2
59 (Tyr 266, and 409) and all four phospho-tyrosine containing peptides of the docking protein
60 HEPL (Tyr 174, 195, 244, and 329), indicating that the latter may represent a new substrate of
61 Bcr-Abl.

62 Most of the detected tyrosine phosphorylation sites on Bcr-Abl were completely abolished by
63 Imatinib. Several of these tyrosine residues have been linked to activating signaling pathways
64 downstream of Bcr-Abl ¹⁵. In contrast, two Tyrosine residues on Bcr-Abl (Tyr 253 and 257)
65 were insensitive to Imatinib, suggesting that they may not be essential for downstream
66 oncogenic signaling.

67 Several members of the Bcr-Abl core proteome ² are adaptor/scaffold proteins, required for
68 the generation of SH2- or PTB-domain binding sites that lead to the formation of tightly
69 regulated protein interactions ¹⁶. Several of these peptides also contain the consensus
70 sequence YxxP of the enriched phosphorylation motif (Fig. 1C, D). As these proteins bind to
71 Bcr-Abl, they are likely direct Bcr-Abl substrates, and our analysis has detected known as well

72 as novel phosphorylation sites. The small adaptor protein GRB2, member of the Bcr-Abl core
73 proteome, plays a key role in Bcr-Abl signaling. Its binding to phosphorylated tyrosine 177 on
74 Bcr-Abl was shown to be required for activation of the Raf-MEK-ERK pathway in leukemic
75 cells^{8,17}. Two of the main binding partners of GRB2 are the docking proteins Gab1 and Gab2.
76 The interaction between these proteins is mediated by the association of the SH3 domain of
77 GRB2 to the proline rich regions in both Gab proteins (Fig. S1A). The observed tyrosine
78 phosphorylations of both Gab1 and 2 are downregulated by Imatinib. Therefore, we
79 quantitatively evaluated the changes in the Gab2 interactome in response to Imatinib
80 treatment. Parallel FLAG-tagged immuno-precipitations from K562 cells transfected with
81 mock or FLAG-Gab2 treated with or without 10 μ M Imatinib were performed. This affinity
82 based LC-MS approach revealed a severe alteration in the Gab2 interactome after Imatinib
83 treatment (Supplemental Fig. 1B, C). Whereas Grb2 remained tightly bound, interactions
84 between Gab2 and Shp2 as well as with components of the Bcr-Abl core-interactome (e.g.
85 Bcr-Abl, SHIP-2 and Shc1) were severely disrupted, suggesting that the Gab2 interactome is
86 dependent on the tyrosine phosphorylation state of this protein. Furthermore, the intracellular
87 localizations of Gab2 and the closely related Gab1 protein were evaluated by confocal
88 microscopy using GFP-tagged constructs of *wild type* proteins and mutants, where Imatinib-
89 regulated phosphotyrosine residues were changed to phenylalanine. *Wildtype* GFP-Gab2
90 smoothly stained the circumference of the cell, whereas the mutant protein showed a severe
91 alteration in localization with speckled staining of the cell periphery and cytosolic staining
92 (Fig. 2B and Suppl. Fig. 2). Similar changes in localization were observed with wildtype and
93 mutant GFP-Gab1. Thus, tyrosine phosphorylation of Gab1 and Gab2 and the subsequent
94 ability to promote the formation of a complex with Bcr-Abl and other core members appears
95 to be required for appropriate membrane localization. These observations strongly advocate

96 an involvement of Gab2 in the assembly of the Bcr-Abl signaling network at the plasma
97 membrane and the ability of the core proteome to transform cells.

98 Several affected phosphotyrosine proteins identified in our screen can be linked to
99 cytoskeletal processes, like regulation of the actin cytoskeleton, cell migration or invasion.
100 Amongst these, HEPL, a member of the p130^{CAS} family of scaffold proteins that is involved
101 in cell migration ¹⁸, showed dephosphorylation of four different sites in response to Imatinib
102 treatment (Fig. 1C). Notably, HEPL was identified as a binding partner of CrkL in K562 cells
103 ². The closely related family members p130^{CAS}/BCAR1 and HEF1/CAS-L have both been
104 identified as binding partners of CrkL (and other Crk family members) and phosphorylation
105 targets of Bcr-Abl ¹⁸. In addition, we also identified CD2-associated protein, TRIP-6,
106 Intersectin-2, and L-Plastin, all scaffold proteins involved in the modulation of the actin
107 cytoskeleton, as being dephosphorylated by Imatinib treatment in our screen. These findings
108 are in agreement with the literature suggesting that Bcr-Abl can affect the cytoskeleton and
109 cell motility downstream of the core signalling complex ¹⁹, our data open new avenues of
110 investigations to follow up the role of cytoskeletal changes in Bcr-Abl transformation.

111 In summary, to probe the effect of Imatinib on the tyrosine kinase Bcr-Abl positive cells we
112 applied a targeted quantitative mass spectrometry based proteomics approach. Since tyrosine
113 phosphorylation is typically underrepresented in shot-gun phosphoproteome analysis, we used
114 phosphotyrosine specific immuno-capturing to systematically screen for Imatinib dependent
115 tyrosine phosphorylation events in K562 cells. Our data reveal that several core Bcr-Abl
116 interactors are putative direct substrates. In following up a novel Imatinib inhibited target, the
117 adaptor protein Gab2 was found to regulate localization of the Bcr-Abl core complex to the
118 plasma membrane in a phosphorylation dependent manner enabling us to add crucial new
119 dimensions of detail to the Bcr-Abl signaling network.

120

121 *Letter to the Editor*

122

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124 Leukemia (CML)

125

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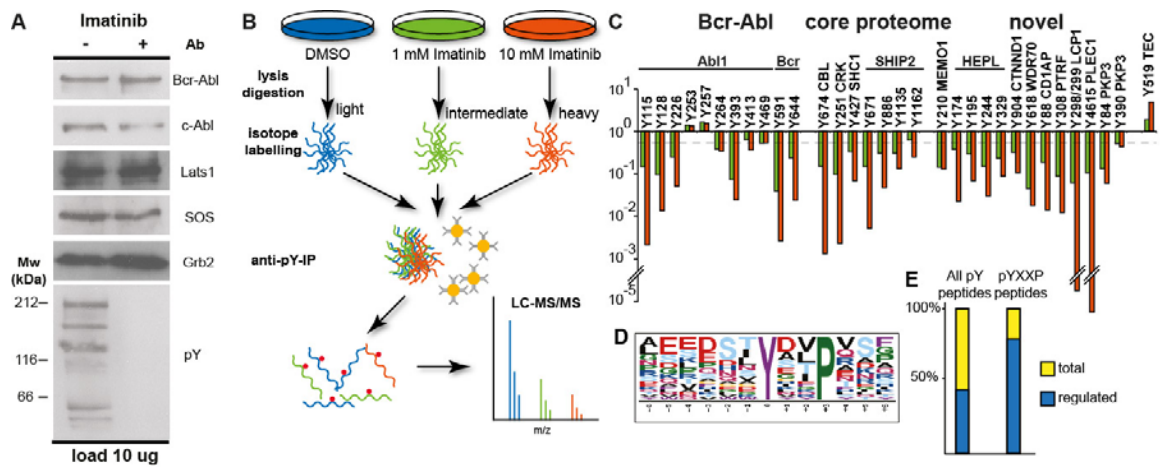
153 Supplementary information is available at the Leukemia website

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155 **Figure Legends**

156 **Figure 1.** (A) Experimental Setup K562 cells were treated with Imatinib for 4 hours. Western
157 blotting was performed to assess the abundance of several key proteins of Bcr Abl dependent
158 signaling, as well as the effect of Imatinib treatment on total phosphotyrosine levels. (B)
159 Overview of the quantitative proteomics workflow. Cells were treated with different doses of
160 Imatinib (0, 1 and 10 μ M) for 4 hours, followed by cell lysis and protein digestion. The
161 peptides from each Imatinib treatment were then differentially labeled using stable isotope
162 dimethyl labeling. The three differentially labeled digests were combined, followed by
163 simultaneous enrichment of tyrosine phosphorylated peptides using immobilized
164 phosphotyrosine specific antibodies. The enriched fraction was analyzed by RP LC-MS and
165 resulting changes quantified. C) Quantitative profiles of site-specific tyrosine phosphorylation
166 upon Imatinib inhibition of Bcr-Abl. The changes in tyrosine phosphorylation versus the
167 control are represented in log scale D) Peptides showing a more than 2-fold decrease in
168 tyrosine phosphorylation upon treatment with Imatinib were subjected to motif analysis using
169 the Motif-X algorithm. The YxxP motif shown was significantly overrepresented in the
170 dataset. E) Comparison of the frequency of the motif YxxP between downregulated
171 phosphotyrsone peptides (23) versus all phosphotyrosine peptides (29/203).

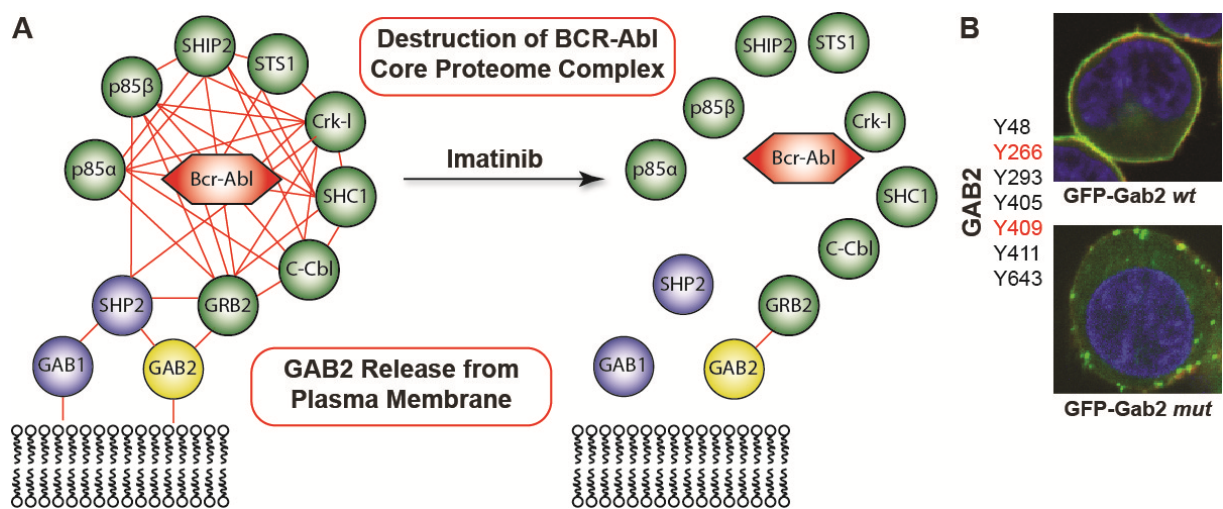
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174 **Figure 2 (A)** Imatinib treatment of K562 cells leads to destruction of the core Bcr-Abl
 175 complex (green, adapted from Brehme et al.). A small selection of peripheral interactors are
 176 depicted in blue. GAB2 is used as flag-tagged entry point for interaction analysis upon
 177 imatinib treatment. LC-MS/MS revealed release of GAB2 from the Bcr-Abl proteome,
 178 except GRB2. **(B)** Mutation of all regulated GAB2 tyrosine phosphorylated residues to
 179 phenylalanine reveals abolishment of membrane localization of both GAB2 and likely also
 180 the Bcr-Abl Core interactome.

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