Supplementary Data

Expression of a Src-Family Kinase in CML Cells Induces Resistance to Imatinib in a Kinase-Dependent Manner

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Z'-Lyte kinase assay principle and data analysis

The Z'-Lyte assay (Invitrogen) employs a Fluorescence Resonance Energy Transfer (FRET)-peptide substrate (Tyr2) with differential sensitivity to proteolytic cleavage depending on the phosphorylation state of a single tyrosine residue within the peptide sequence. The unphosphorylated peptide is cleaved during the development step, which disrupts FRET between the donor (coumarin) and acceptor (fluorescein). Peptide phosphorylation, however, prevents protease cleavage.

Phosphorylation reactions (10 µl) were conducted in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01 % Brij-35) in 386-well plates. Assay conditions were first optimized to determine the amount of each kinase required to phosphorylate 60-70% of the Tyr2 peptide. To assess inhibitor effects on kinase activity, purified recombinant Hck-YEEI (50 ng), Hck-T338A (60 ng), and Ncap-c-Abl (100 ng) were incubated for 1 h at room temperature with 100 µM ATP and 1 µM Tyr2 peptide substrate. Then, the development reagent containing the protease that selectively digests the non-phosphorylated peptide was added and the reaction was incubated for an additional 60 min at room temperature. At the end of the incubation period, the reaction was terminated with the proprietary stop reagent. Fluorescence was assessed at an excitation wavelength of 400 nm; coumarin fluorescence and the fluorescein FRET signal were measured at 445 nm and 520 nm, respectively. The phosphorylation reactions are quantitated by calculating the ratio between the donor (coumarin) and acceptor (fluorescein) emission intensities:

$$\text{Emission ratio} = \frac{\text{Coumarin Emission (445 nm)}}{\text{Fluorescein Emission (520 nm)}}$$

Reactions containing the Tyr2 peptide substrate and kinase in the absence of ATP served as 0% phosphorylation controls, whereas stoichiometrically phosphorylated Tyr2 peptide was used as a 100% phosphorylation control. To calculate inhibitor effects (percent inhibition), DMSO was used as 0% inhibition control, whereas reactions containing the unphosphorylated peptide and kinase in the absence of ATP served as 100% inhibition control. The emission ratio remains low when the FRET peptide is phosphorylated (i.e. no kinase inhibition) and is high when the FRET peptide is not phosphorylated (i.e. kinase inhibition). This "ratiometric" method allows for tight control of well-to-well variations in substrate peptide concentrations. As a result, the assay consistently yields Z'-factors above 0.7 even if the substrate is phosphorylated at low levels.

Figure S1. Inhibition of Ncap-c-Abl protein-tyrosine kinase activity by imatinib: Example of raw data from the Z'Lyte assay. Recombinant Ncap-c-Abl was assayed using the Z'-Lyte method as described above in the presence of imatinib with DMSO as negative control. Each condition was assayed in quadruplicate, and the mean emission ratios from a representative experiment are shown ± S.D. The 100% inhibition control ratio is shown on the left (No ATP), followed by ratios determined in the presence of imatinib ranging from 1 nM to 10 μM in half-log steps. Bars showing the emission ratios obtained with the 0% inhibition control (DMSO only; no drug) and with the stoichiometrically phosphorylated control peptide in the absence of Hck or ATP (pTyr Con) are shown on the right. The transformed data are shown in Figure 1D of the main paper.

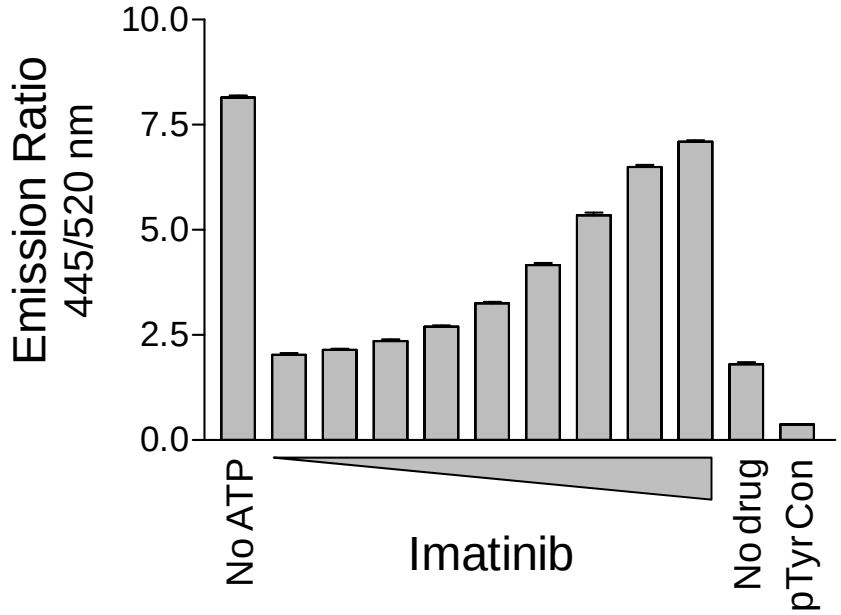
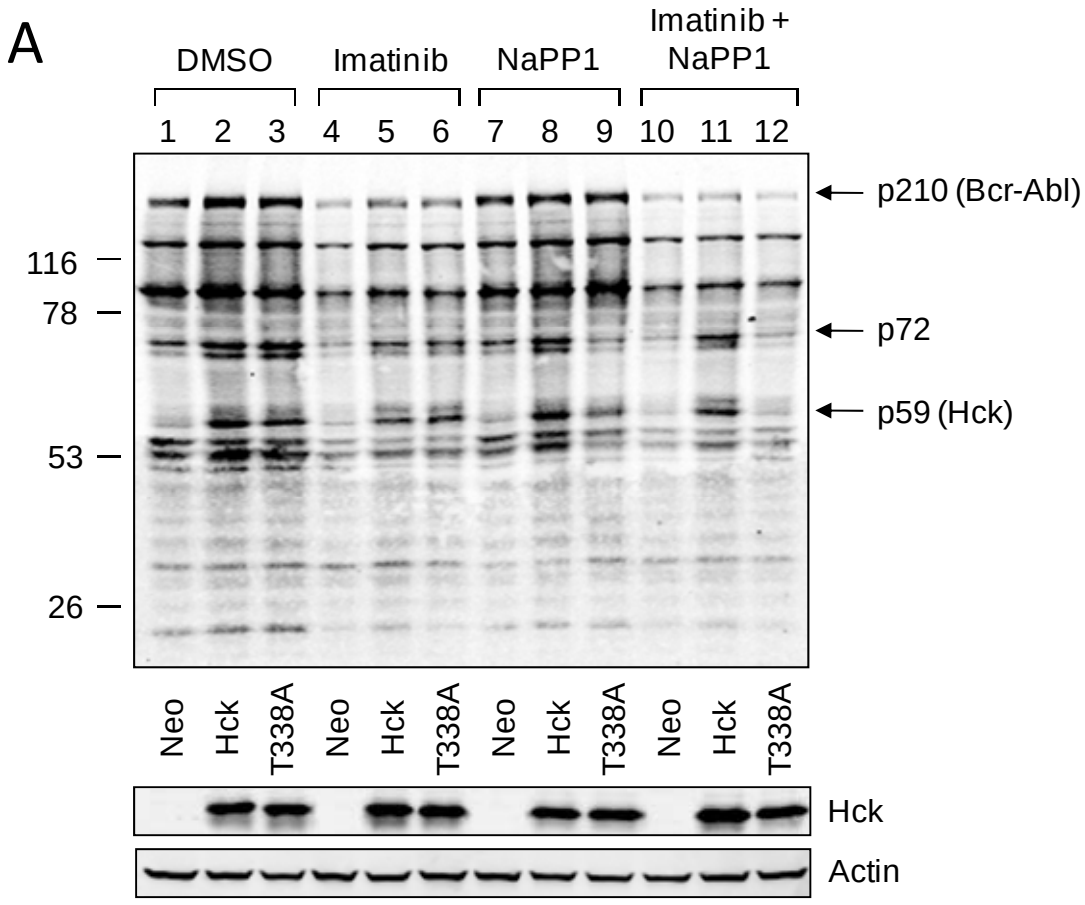
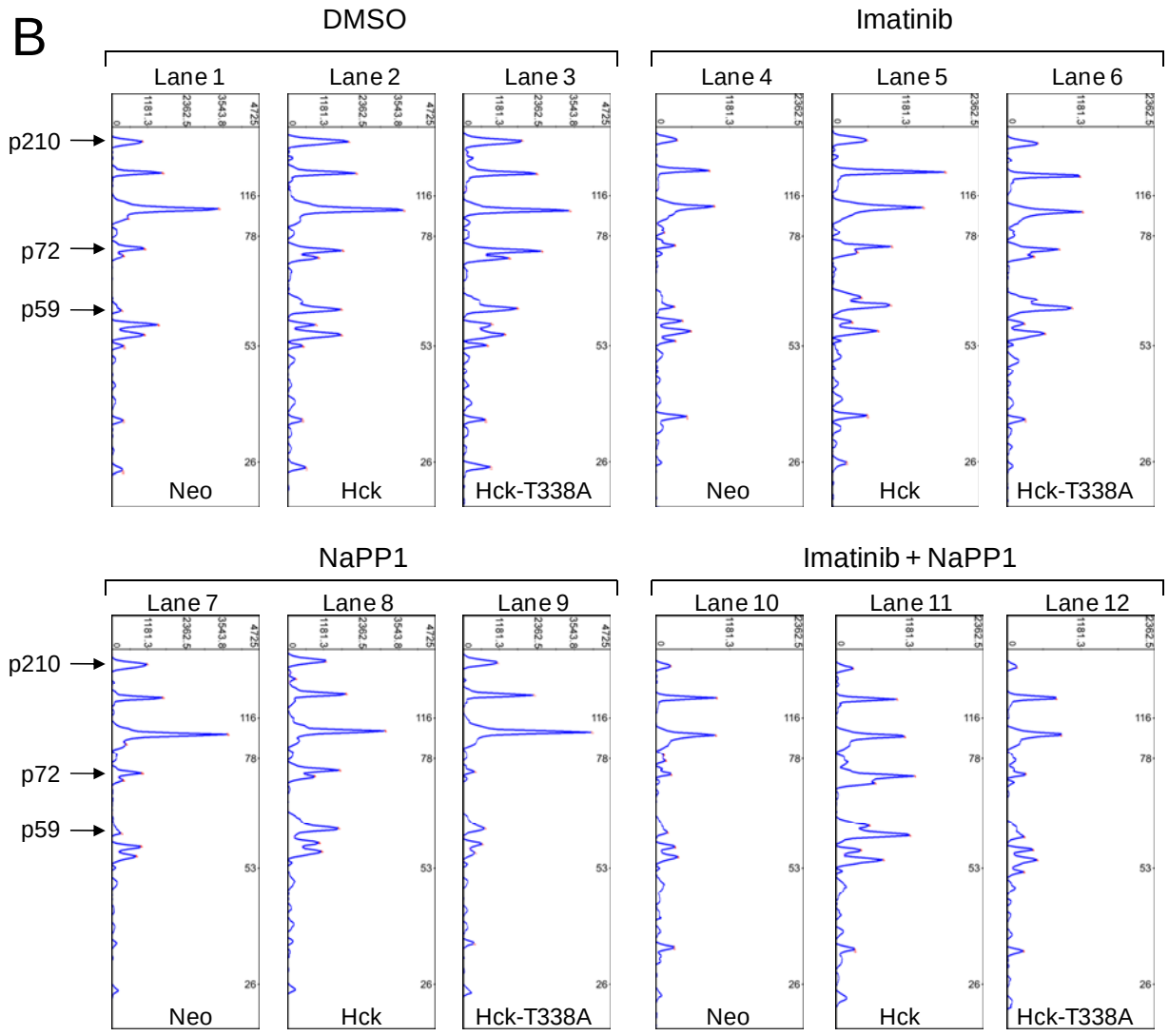


Figure S2. Hck or Hck-T338A overexpression in K562 cells induces elevated tyrosine phosphorylation levels of several substrates without changing the overall tyrosine phosphoprotein banding pattern. K562 cells were infected with recombinant retroviruses carrying a neomycin selection marker (Neo), wild-type Hck or Hck-T338A and selected with G418. The resulting cell populations were plated in 0.5% FBS overnight and then treated with the DMSO carrier solvent, imatinib (1 μM), NaPP1 (3 μM), or imatinib plus NaPP1 for 5 hours. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-phosphotyrosine (PY99) and anti-Hck antibodies. Immunoreactive proteins were simultaneously detected using the Odyssey Infrared Imaging System (LI-COR, Inc.). As a loading control, replicate blots were also probed with an anti-actin antibody. The entire experiment was repeated three times from independently derived cell populations, and produced comparable results in each case. (A) Representative anti-phosphotyrosine, Hck and actin immunobloblots. (B) Using the "edit size standards" feature of the Odyssey 3.0 software, the position of each molecular weight marker was used to estimate the molecular weights of each pTyr-band. The positions of the three bands showing the most prominent changes following Hck expression are indicated (p210, p72, and p59); the locations of these three phosphoproteins are also indicated on the blot shown in Part A. (C) Bargraphs show the fold changes relative to the DMSO controls of the average phosphotyrosine signal intensities ± S.D. for p210 (Bcr-Abl), p72 and p59 (Hck) from three independent experiments. For additional details, see main text.




B
DMSO
Imatinib
Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Lane 6
p210
p72
p59
Neo
Hck
Hck-T338A
NaPP1
Imatinib + NaPP1
Lane 7
Lane 8
Lane 9
Lane 10
Lane 11
Lane 12

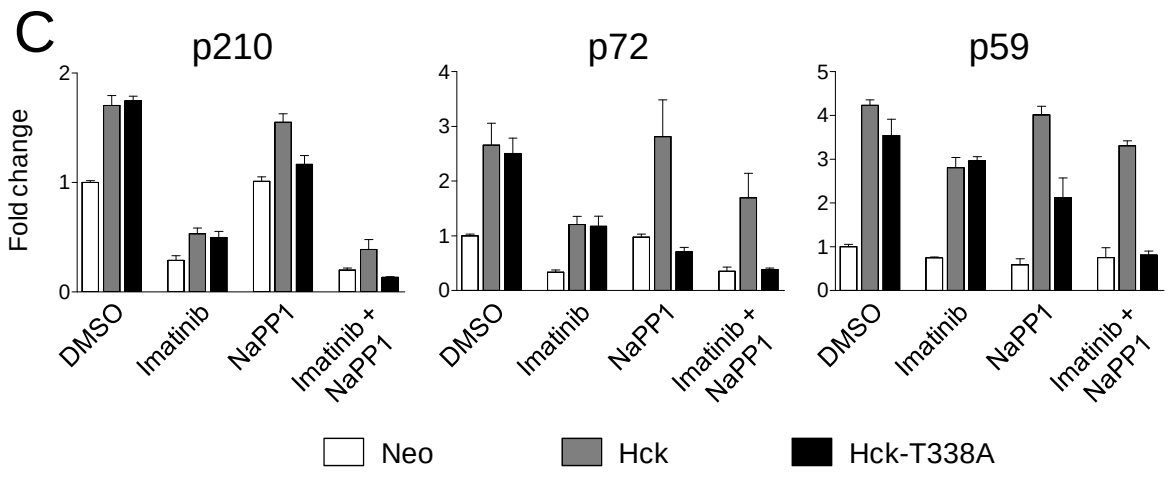

C
p210
p72
p59
Fold change
DMSO
Imatinib
NaPP1
Imatinib + NaPP1
Neo
Hck
Hck-T338A

Figure S3. Wild-type Hck or Hck-T338A overexpression increases phosphorylation of Bcr-Abl at regulatory tyrosine residues in a kinase-dependent manner. K562-Neo, K562-Hck, and K562-Hck-T338A cell populations were plated in 0.5% FBS overnight and treated with the indicated concentrations of imatinib in the presence (+) or absence (-) of NaPP1 (3 μM) for 5 hours. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with phosphospecific antibodies for Abl pY89 (A), pY245 (B), pY412 (C) and Bcr pY177 (D). As loading controls, duplicate blots were also probed with anti-Abl antibodies to determine Bcr-Abl expression levels. Western blots were analyzed using the Odyssey Infrared Imaging System. The entire experiment was repeated twice from independently derived cell populations, and representative blots are shown. Phosphotyrosine signal intensities were normalized to the levels of Bcr-Abl protein present in each lane. The resulting intensity ratios are presented in the bar graphs relative to ratios obtained from DMSO-treated K562-Neo cell populations ± S.D.

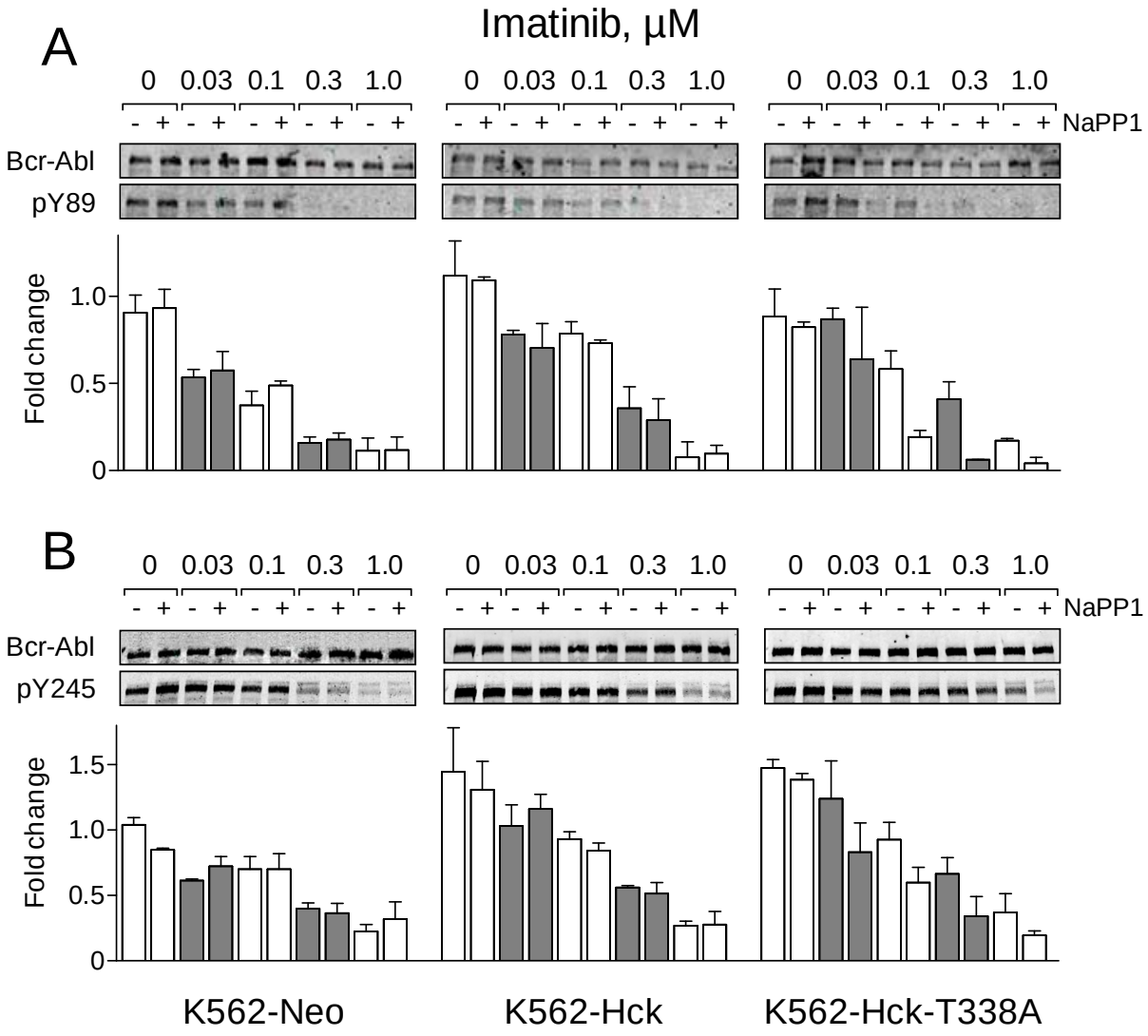


Figure S3, continued