Supplemental Information

N-MYRISTOYLATED c-ABL TYROSINE KINASE LOCALIZES TO THE ENDOPLASMIC RETICULUM UPON BINDING TO AN ALLOSTERIC INHIBITOR

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Running Head: Allosteric inhibitor of Abl kinases

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Materials and Methods

Protein Constructs. The Src-family members Hck, Src, Lyn, and Lck were expressed in Sf9 insect cells in their downregulated conformations and purified to homogeneity as described elsewhere (1-3).

In Vitro Tyrosine Kinase Assay. In vitro kinase assays were performed according to two different formats; [1] Continuous spectrophotometric assays: Activity of the protein kinases toward Abl substrate peptide (4) (sequence: EAIYAAPFAKKK) was determined as described previously (5,6). [2] FRET-based Z'-LYTE kinase assay (Invitrogen): Each kinase was pre-incubated with inhibitors for 30 min in kinase buffer (20 mM HEPES [pH 7.4], 50 mM NaCl, 0.1% CHAPS, 10 mM $MgCl_2$, 1 mM DTT) followed by incubation with ATP (50 μM final) and Tyr-2 peptide (2 μM final) for 1 hr at room temperature. Development reagent, containing a protease selective for the non-phosphorylated peptide, was then added and the reaction was incubated for an additional 1 hr. Reactions were terminated with the proprietary stop reagent, and coumarin fluorescence as well as the Tyr-2 peptide FRET signal were assessed on a Gemini XS microplate spectrofluorometer (Molecular Devices). Reactions containing kinases and substrate peptide in the absence of ATP served as 0% phosphorylation controls, whereas a stoichiometrically phosphorylated peptide was used as a 100% phosphorylation control. Raw fluorescence values were corrected for background, and reaction endpoints were calculated as emission ratios of coumarin fluorescence divided by the FRET signal. These ratios were then normalized to the ratio obtained with the 100% phosphorylation control. Each condition was assayed in quadruplicate, and results are presented as the mean ± S.D.

Isolation of Microsome Fraction. The polyclonal population of cells (5×10^6) expressing c-Abl^{WT} were plated onto 10 cm culture dishes in DMEM containing 10% FBS. After a 17 hr incubation, the cells were treated with either DMSO or GNF-2 for 1 hr. The cells were washed in ice-cold PBS, suspended in hypotonic buffer (10 mM Tris-Cl [pH 7.4], 0.2 mM $MgCl_2$, 5 mM KCl, protease inhibitor cocktail), subject to lysis in Dounce homogenizer, and adjusted to isotonic conditions by adding 140 mM KCl. After centrifugation at 12,000 × g for 15 min, the

supernatant (post mitochondrial fraction) was centrifuged at 100,000 × g for 1 hr. The pellets were used for microsome fractions and analyzed by western blot.

References

1. Trible, R. P., Emert-Sedlak, L., and Smithgall, T. E. (2006) *J Biol Chem* **281**(37), 27029-27038
2. Mitchell, J. L., Trible, R. P., Emert-Sedlak, L. A., Weis, D. D., Lerner, E. C., Applen, J. J., Sefton, B. M., Smithgall, T. E., and Engen, J. R. (2007) *J Mol Biol* **366**(4), 1282-1293
3. Chen, S., Dumitrescu, T. P., Smithgall, T. E., and Engen, J. R. (2008) *Biochemistry* **47**(21), 5795-5803
4. Songyang, Z., Carraway, K. L., 3rd, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., and et al. (1995) *Nature* **373**(6514), 536-539
5. Barker, S. C., Kassel, D. B., Weigl, D., Huang, X., Luther, M. A., and Knight, W. B. (1995) *Biochemistry* **34**(45), 14843-14851
6. Seeliger, M. A., Young, M., Henderson, M. N., Pellicena, P., King, D. S., Falick, A. M., and Kuriyan, J. (2005) *Protein Sci* **14**(12), 3135-3139

Supplemental Figure Legends

Supplemental Fig. 1. Biochemical characterization of the effect of Brij 35 on the kinase activity of Abl using a continuous spectrophotometric assay. The recombinant Abl (amino acids 65-534) was diluted to a final concentration of 50 nM in kinase buffer (50 mM Tris-Cl [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$) in the absence or presence of 0.1% Brij 35. Kinase activity was measured at a concentration of 300 μM substrate and increasing concentrations of ATP (*A*), or increasing concentrations of peptide substrate and 500 μM of ATP (*B*) for 20 min, and expressed as pmol/pmol**.**min. Data were fit using GraphPad Prism (GraphPad Software).

Supplemental Fig. 2. Biochemical characterization of GNF-2 with a continuous spectrophotometric assay. The recombinant Abl (amino acids 65-534) was diluted to a final concentration of 50 nM in kinase buffer (50 mM Tris-Cl [pH 7.4], 50 mM KCl, 10 mM $MgCl_2$) in the absence or presence of GNF-2 (1 μM). Kinase activity was measured at 300 μM concentration of the peptide substrate and increasing concentrations of ATP (*A*), or increasing concentrations of peptide substrate and 500 μM of ATP (*B*) for 20 min, and expressed as pmol/pmol**.**min. Data were fit using GraphPad Prism (GraphPad Software).

Supplemental Fig. 3. GNF-2 fails to interact with c-Abl^{A356N}. Equal amounts of lysates (1 mg of each protein) from *abl*$^{-/-}$ *arg*$^{-/-}$ cells reconstituted with either WT or A356N c-Abl were incubated with Sepharose-immobilized GNF-2, and the bound proteins were analyzed by western blot with an anti-Abl antibody.

Supplemental Fig. 4. Quantification of the western blot results shown in Fig. 4. The intensity of each band was measured with 'Image J'.

Supplemental Fig. 5. (*A*) Abl deficient cells were plated on a coverslip and processed for indirect immunofluorescence using an anti-Abl antibody (8E9) and Alexa Fluor 488 goat anti-mouse IgG. DAPI was used to stain the nucleus. (*B*) Western blot analysis of the post mitochondrial fractions and microsome fractions. The *abl*$^{-/-}$*arg*$^{-/-}$ 3T3 cells reconstituted with c-Abl^{WT} were treated with either DMSO or GNF-2 for 1 hr, and the indicated fractions were

isolated. Equivalent amounts of proteins were analyzed by western blot with an anti-Abl antibody or an anti-PDI antibody. The intensity of each band was measured with 'Image J' and shown as fold differences.

Supplemental Fig. 6. GNF-2 does not inhibit Src-family kinase activity *in vitro*. Recombinant Hck (*A*), Lyn (*B*), Lck (*C*), and Src (*D*) were assayed using the peptide substrate Tyr-2 and a FRET-based kinase assay as described elsewhere (2, 3). The potent Src-family kinase inhibitor dasatinib (0.5 μM) was included as a positive control. Recombinant Abl (83-534) (*E*) was also assayed in the presence of the same concentrations of GNF-2 to demonstrate the efficacy of the compound in this assay. Each condition was repeated in quadruplicate and the extent of phosphorylation is expressed as the mean percent phosphorylation relative to a control phosphopeptide ± S.D.

Supplemental Fig. 7. The view of myristate-binding site in Abl showing differences between Abl and Arg.

Supporting Information Fig. 1

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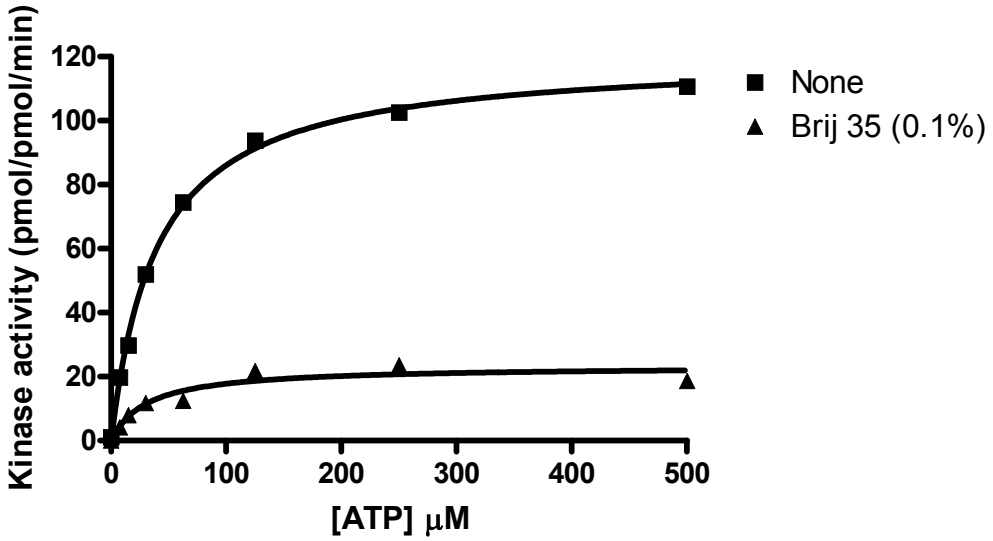
A



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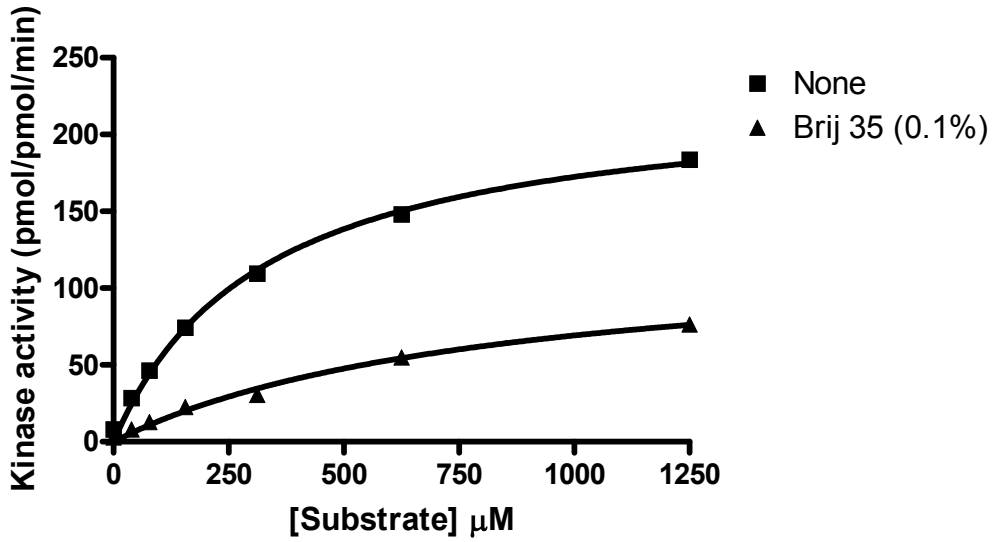

Supporting Information Fig. 2

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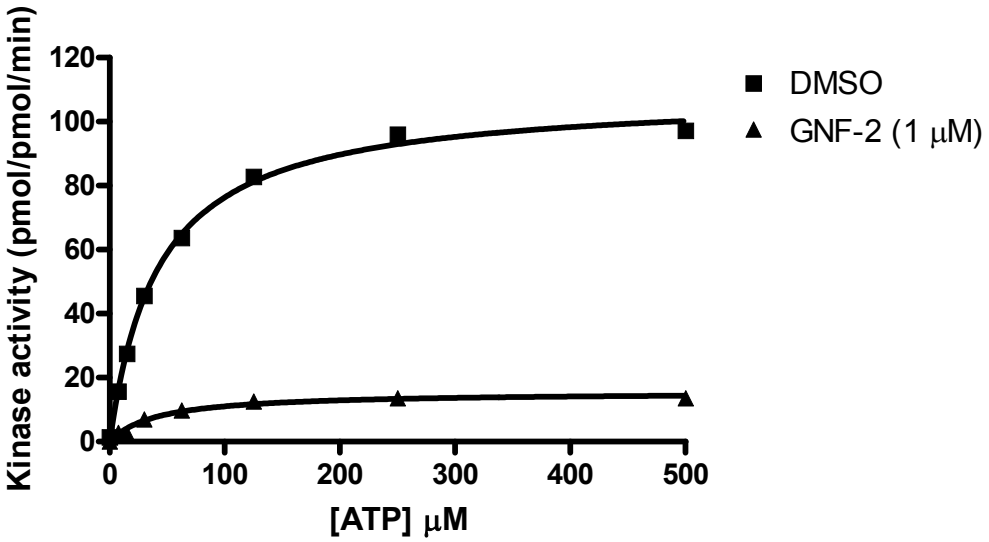
A



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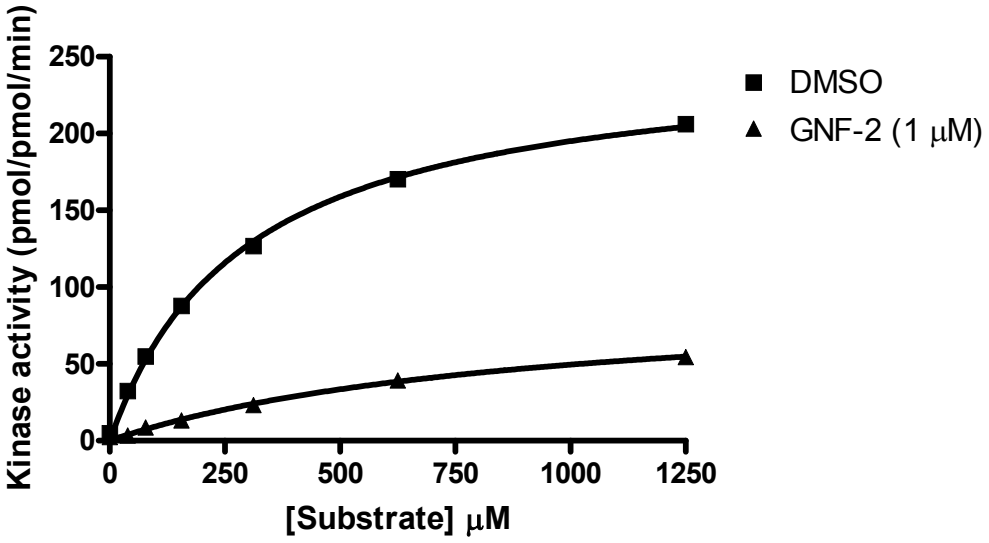

Supporting Information Fig. 3

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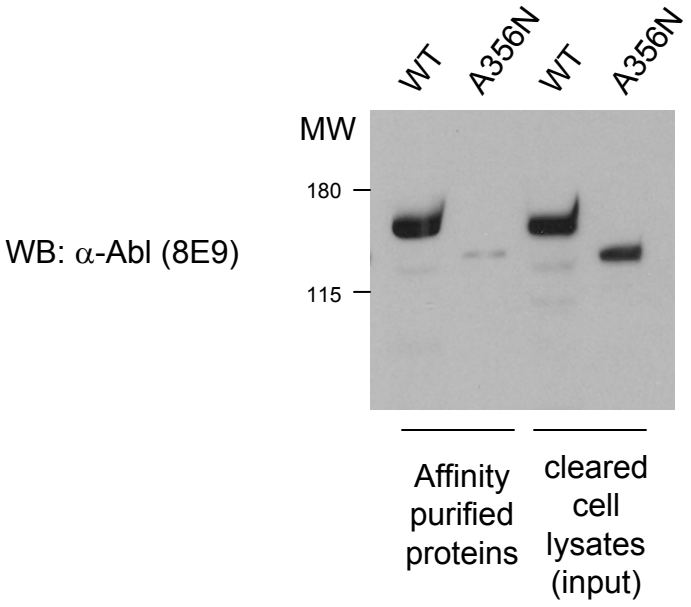

Supporting Information Fig. 4

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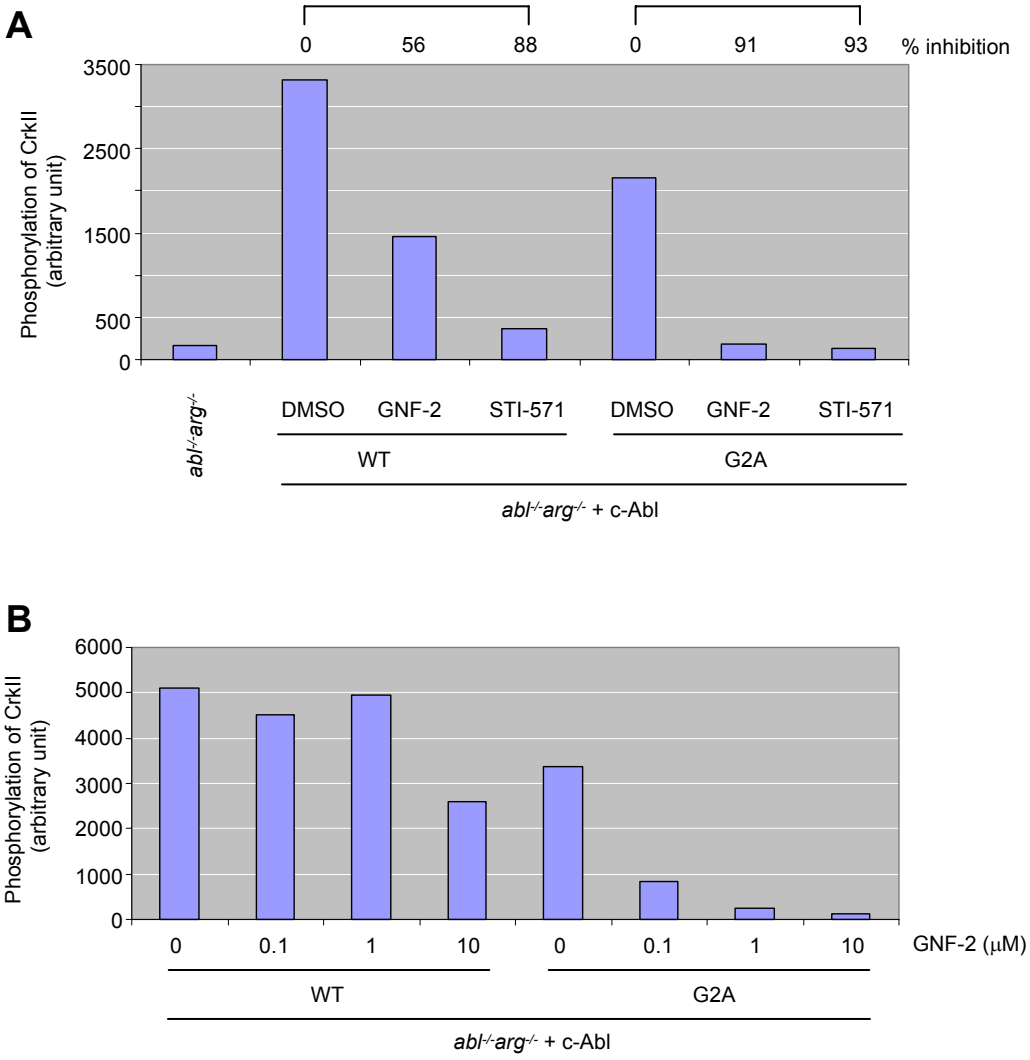

Supporting Information Fig. 5

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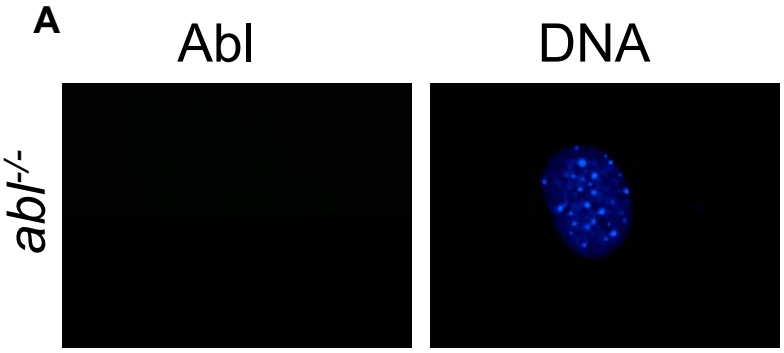

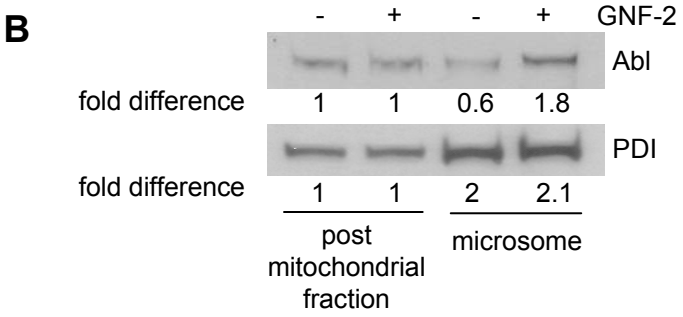

Supporting Information Fig. 6

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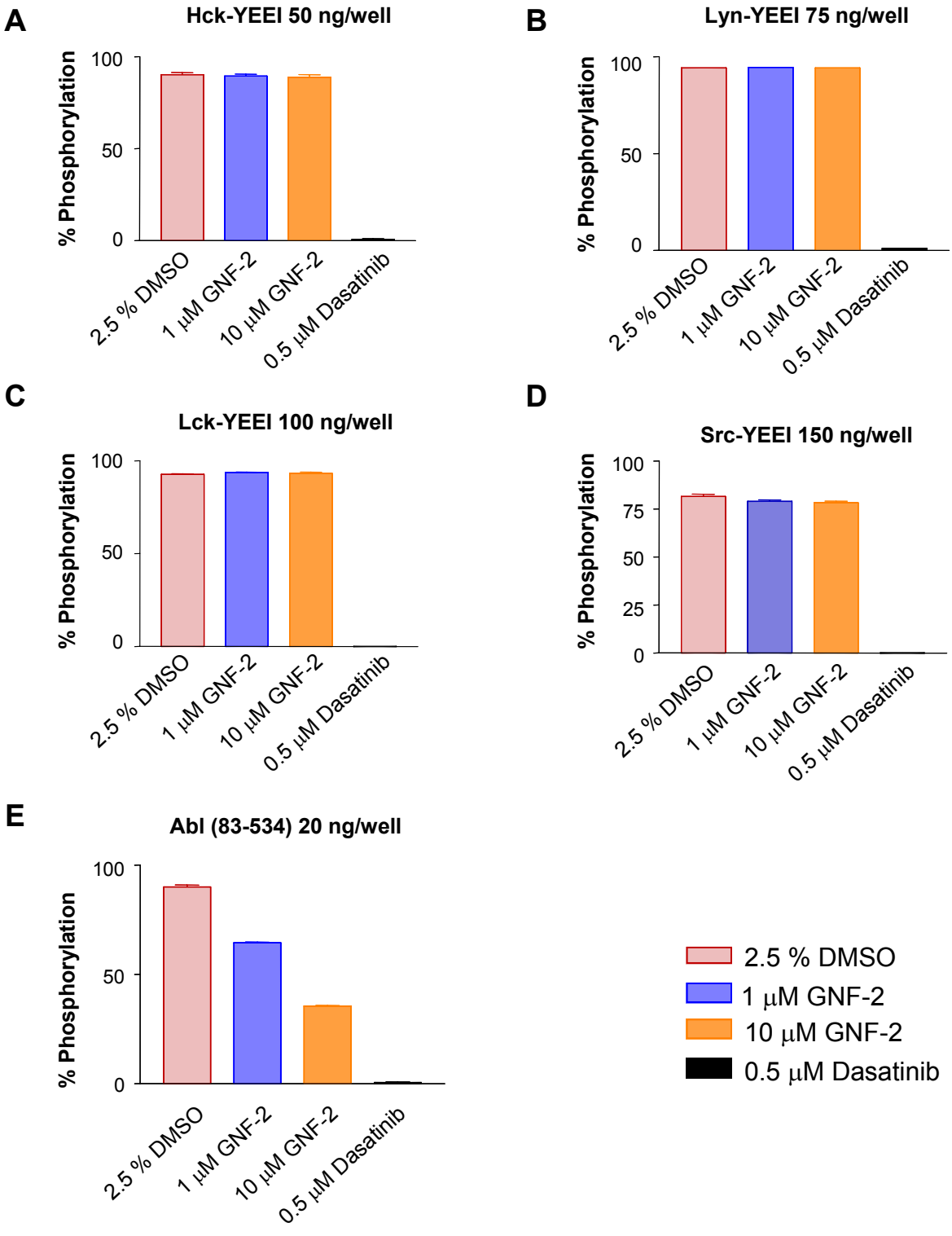

Supporting Information Fig. 7

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