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Supplementary Materials for

Lysine Methylation Promotes VEGFR-2 Activation and Angiogenesis

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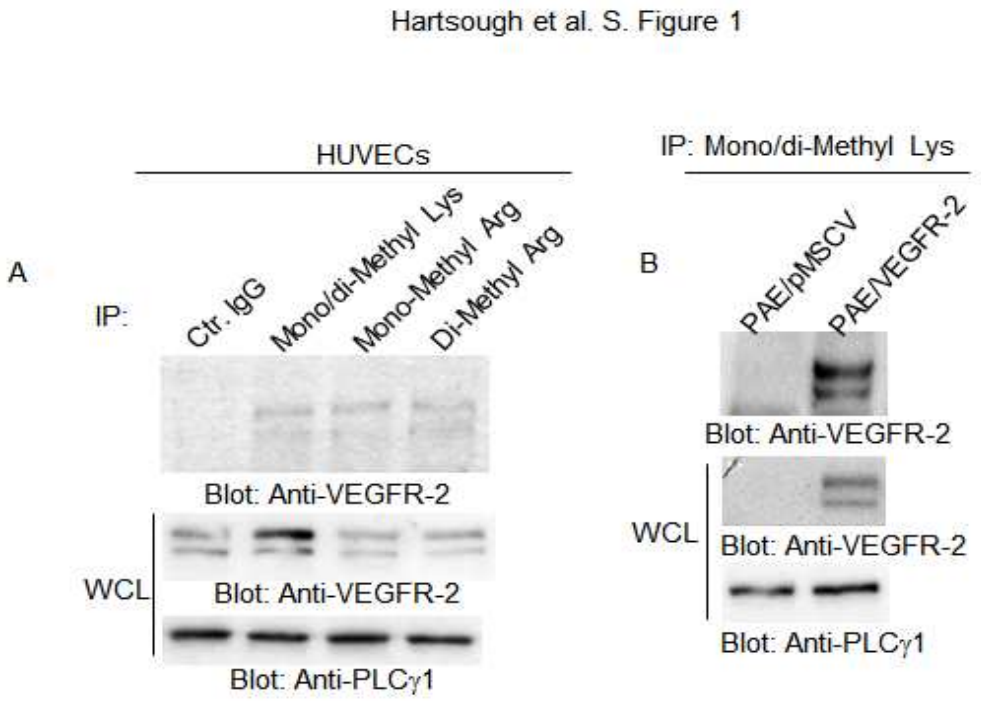
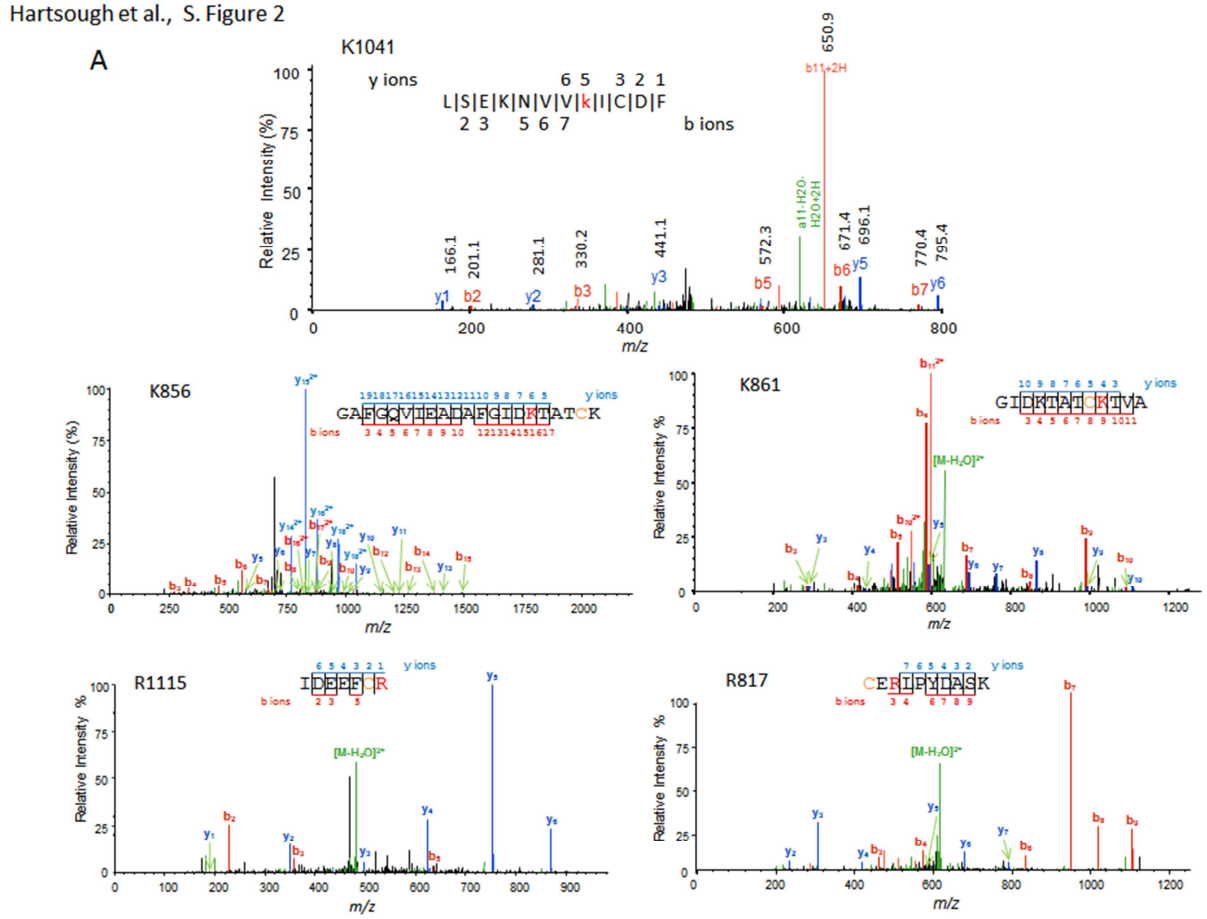
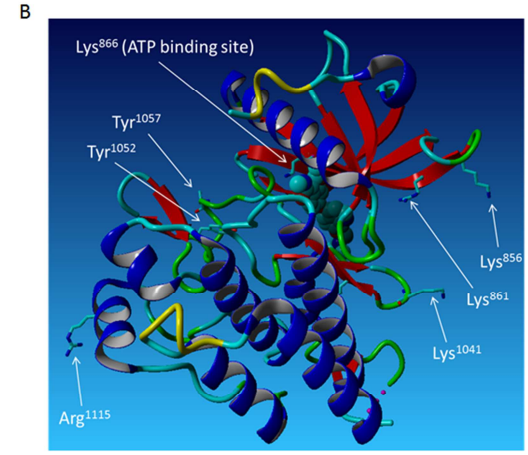


Figure S1. Validation of the anti–methyl-Lys and anti–methyl-Arg antibodies. (A) Methyl-Lys/Arg immunoprecipitates from HUVECs were immunoblotted with anti-VEGFR-2 antibody. Whole cell lysates (WCL) were blotted for VEGFR-2 and PLCγ1 as the loading control. (B) Methyl-Lys/Arg immunoprecipitates from PAE cells expressing empty vector (pMSCV) or VEGFR-2 were blotted with anti–VEGFR-2 antibody. WCL were blotted for VEGFR-2 and PLCγ1 as the loading control. The data is representative of two independent experiments.

Hartsough et al., S. Figure 2





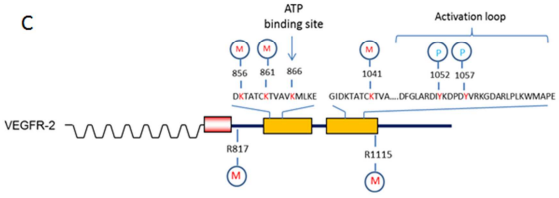


Figure S2. Mass spectra of methylated VEGFR-2 peptides and their locations. PAE cells expressing VEGFR-2 were immunoprecipitated with anti-VEGFR-2 antibody and subjected to mass spectrometry analysis as described to materials and methods section. Methylated peptides and their position are shown. The data is representative of three independent experiments. (B) The crystal structure of VEGFR-2 and the location of the methylation sites including Lys^{1041}, Lys^{866}, Lys^{856} and Arg^{1115} are shown. Also Lys861, an ATP binding site and the location of Tyr^{1052} and Tyr^{1057} (tyrosine autophosphorylation sites) are shown. (C) Schematic of VEGFR-2 protein and the location of methylation sites, ATP binding site and tyrosine autophosphorylation sites are shown. (D) In vitro methylation of Lys^{856}-Lys^{861}, Lys^{1041} and Arg^{1115} peptides is shown. PAE cell homogenate used as source of methyltransferases. The blot is representative of two independent experiments.

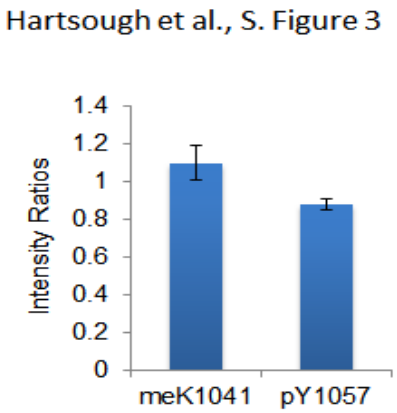


Figure S3. Ratios of methylation of Lys^{1041} and tyrosine phosphorylation of VEGFR-2: Wild-type VEGFR-2 and K1041 mutant VEGFR-2 protein samples were immunoprecipitated and then separated on SDS-PAGE and after in-gel chymotrypsin digestion the peptide fragments were submitted for LC-MS/MS analysis. The ion signal intensities of 18 normalized peptides from VEGFR-2 were identified and used for calculation. The data is representative of three independent experiments. Error bars = SD. P value >0.05

Hartsough et al., S. Figure 4

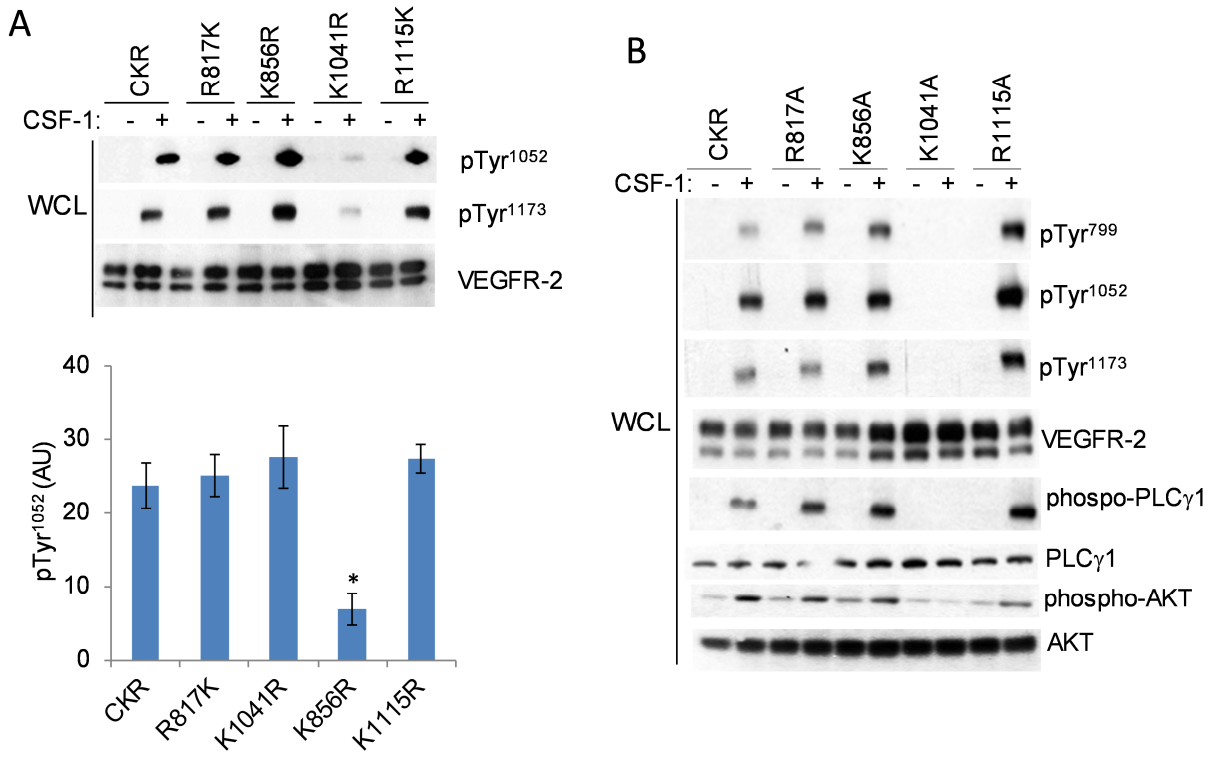


Figure S4. Phosphorylation of Lys and Arg mutant CKRs in response to ligand stimulation. (A) Serum-starved PAE cells expressing CKR, R817K, K856R, K1041R and R1115K were stimulated with CSF-1 for 10 min (+). Cell lysates were blotted for pTyr^{1052}, pTyr^{1173} and total VEGFR-2. Results are presented as induction of VEGFR-2 phosphorylation at Tyr^{1052} normalized to total VEGFR-2. Median of three independent experiments is shown. Error bars= rang. Data are means SD from three experiments. *P<0.041. (B) Serum-starved PAE cells expressing CKR, R817A, K856A, K1041A and R1115A were stimulated with CSF-1 for 10 minutes (+) or left un-stimulated (-).Whole cell lysates were blotted for pTyr^{799}-VEGFR-2, pTyr^{1052}-VEGFR-2, pTyr^{1173}-VEGFR-2 and total VEGFR-2. The same cell lysates were blotted for phospho-PLCγ1, total PLCγ1, phospho-AKT, and total AKT. The blots are representative of three independent experiments.

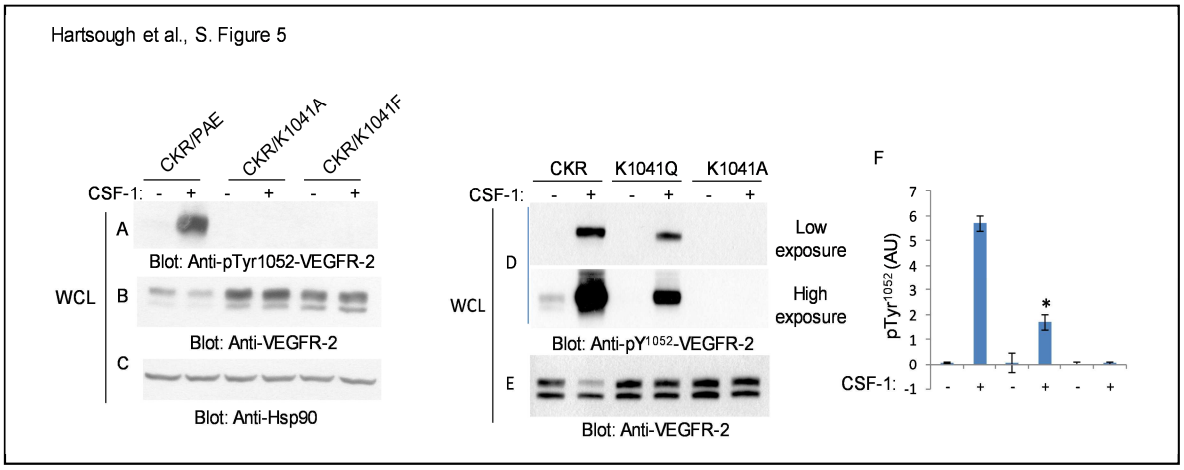


Figure S5. Mutation of Lys[1041] to Phe inhibits, whereas mutation to Gln partially preserves, phosphorylation of VEGFR-2. (A-C) Serum-starved PAE cells expressing CKR, K1041A and K1041F were stimulated with CSF-1 for 10 minutes (+) or left un-stimulated (-), cells were lysed and whole cell lysates were blotted for phospho-Y[1052]-VEGFR-2 and total VEGFR-2 and HSP90 for protein loading. The data is representative of three independent experiments. (D and E). Phosphorylation of CKR, K1041Q and K1041A and total VEGFR-2 protein levels are shown. (F) Data is presented as median of induction phosphorylation of VEGFR-2 at Tyr[1052] normalized to total VEGFR-2 from three independent experiments. Error bars= range SD. *P= 0.034.

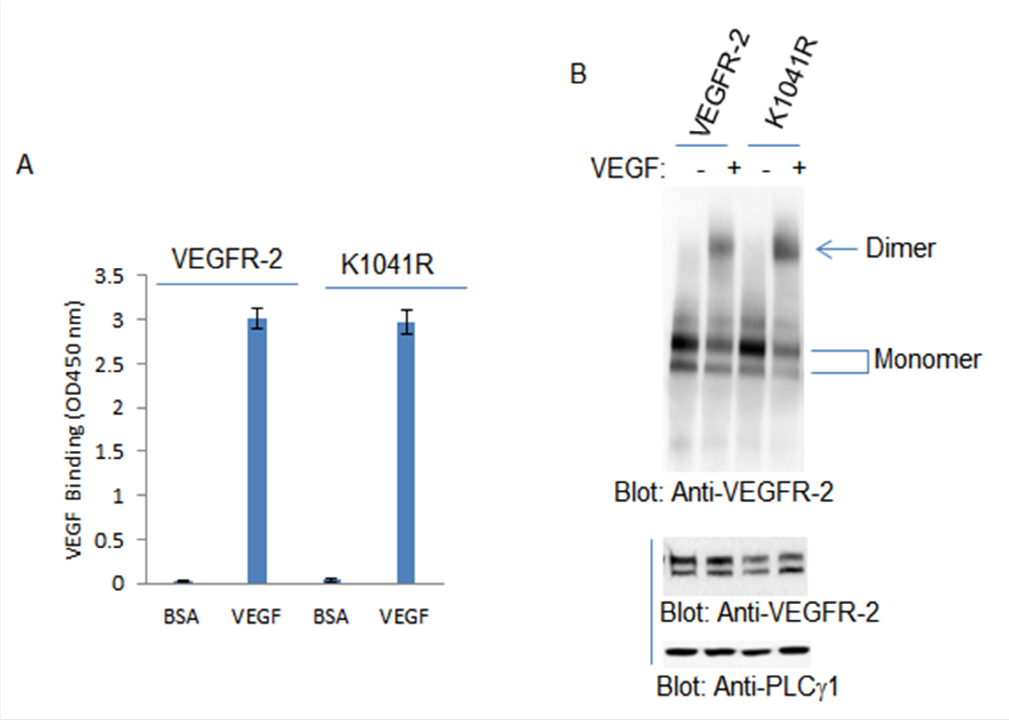


Figure S6. The K1041R mutation does not inhibit ligand binding or dimerization of VEGFR-2. (A) Equal numbers of PAE cells expressing wild-type VEGFR-2 or K1041R-VEGFR-2 were plated on 96-well plates (4 wells per group) and incubated with biotinylated VEGF or biotinylated BSA for 10 minutes in 4°C. After extensive washing the binding of VEGF to surface VEGFR-2 was determined in plate reader at OD 450nm. The data shows the means ± SD of three independent experiments.
(B) Cell lysates derived from PAE cells expressing VEGFR-2 or K1041R-VEGFR-2 that were either not stimulated (-) or stimulated with VEGF (+) resolved on 4-15% non-reducing gradient gels and blotted for VEGFR-2. Whole cell lysates from the same group were resolved on reducing gel and immunoblotted for VEGFR-2 and PLCγ1. Data are representative of two independent experiments.

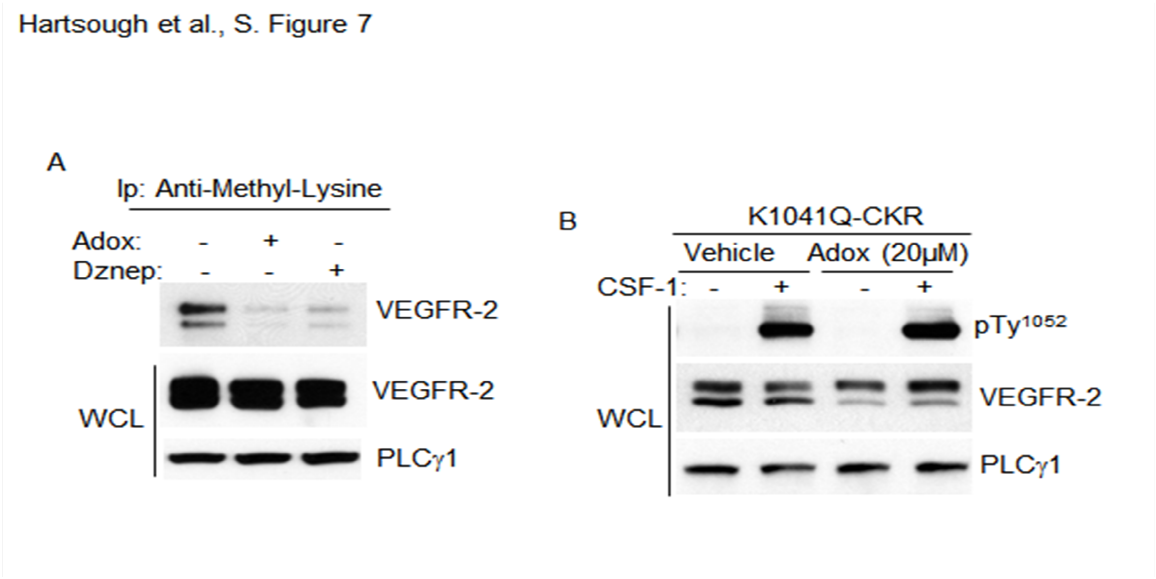


Figure S7. Adox treatment inhibits the methylation of VEGFR-2. (A) PAE cells expressing VEGFR-2 were treated with Adox to generate hypo-methylated VEGFR-2. The hypo-methylated VEGFR-2 was immunoprecipitated and blotted for VEGFR-2. Whole cell lysates (WCL) were blotted for VEGFR-2 and PLCγ1 as the loading control. The blots are representative of three independent experiments. (B) PAE cells expressing K1041Q mutant CKR were pre-treated with vehicle or Adox followed by CSF-1 stimulation (+). Lysates were blotted for anti-pTyr, total VEGFR-2 and for PLCγ1. The blots are representative of three independent experiments.

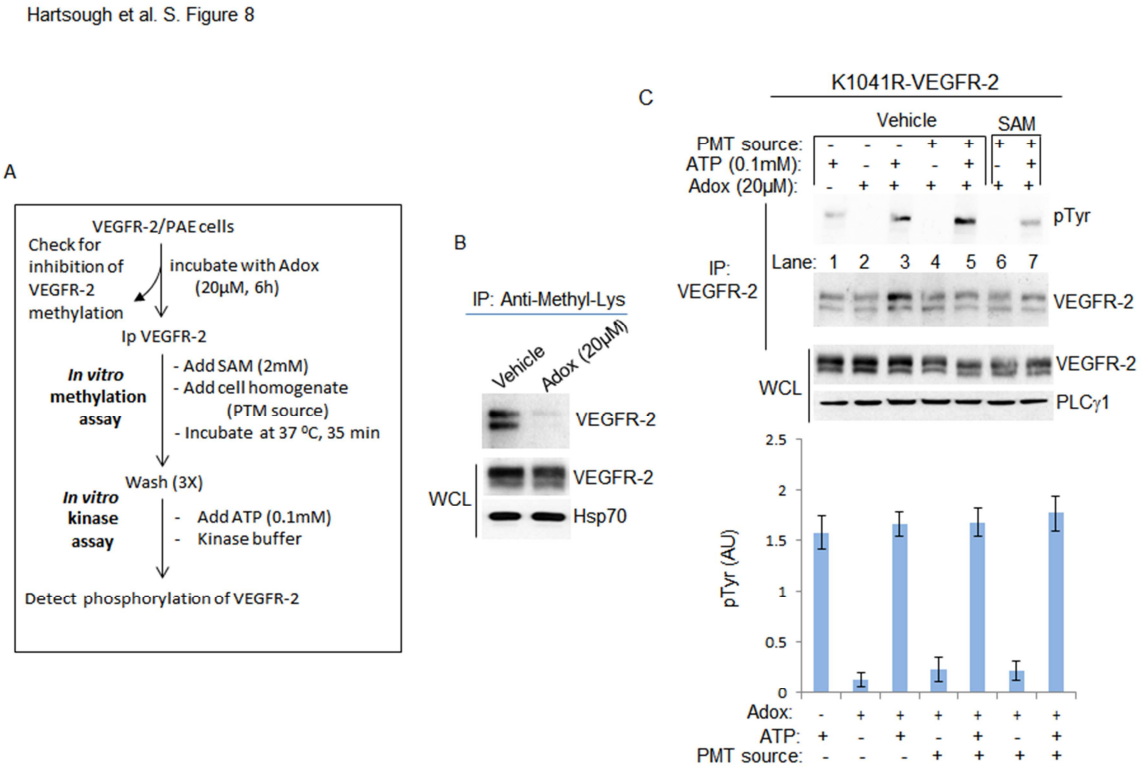


Figure S8. Remethylation of the K1041R VEGFR-2 mutant does not increase its tyrosine phosphorylation:
(A) Schematic of in vitro re-methylation and kinase assay of VEGFR-2 is shown.
(B) PAE cells expressing VEGFR-2 were treated with vehicle or Adox. Methyl-lys immunoprecipitates were blotted for VEGFR-2. Whole cell lysates were blotted for VEGFR-2 and HSP70 as a loading control. The blots are representative of three independent experiments.
(C) PAE cells expressing the K1041R VEGFR-2 mutant were treated with Adox to generate hypo-methylated VEGFR-2. The hypo-methylated K1041-VEGFR-2 was immunoprecipitated and subjected to in vitro methylation and kinase assays. The same membrane was re-blotted for VEGFR-2. Whole cell lysates were blotted for VEGFR-2 and PLCγ1 as a loading control. Result is presented as induction of tyrosine phosphorylation of the K1041R VEGFR-2 mutant normalized to total VEGFR-2 protein. Median of three independent experiments is shown. Error bars= range.