Supplemental Figure legends. - Trajtenberg *et al.*

Fig. S1. Multiple sequence alignment of 57 'NarQ-like' and 'DesK-like' proteins sharing ABD-DHp architectures, following Pfam definitions. Homologous sequences were identified and aligned as described in 'Experimental Procedures'. Identical positions are displayed in bold white over a red background; columns featuring conservative substitutions are red boxed. Sequences are labelled with their corresponding Pfam IDs and species codes, separated by an underscore.

Fig. S2. Solvent accessible surface of the nucleotide-binding pocket and neighboring region in HKs. The surface is colored with a red-blue ramp, according to negative-positive mapped electrostatic potential. Comparison of members of three different HK families reveals that HPK7 has a shallower ATP-binding cleft, mainly due to a much shorter ATP-lid loop, which does not contain extra α-helices. **(A)** DesKABD; **(B)** PhoQ; **(C)** CheA. Note the basic patch surrounding the ATP phosphates, less pronounced in DesKABD.

Fig. S3. Structural comparison between independently refined DesK ATP binding domains, pinpointing statistically flexible regions. (A) Error scaled difference distance matrix for crystal structures 3EHG (this report) and 2.1Å resolution 3EHH (ATP-binding domain only). Spanning residues 244-367 are indicated on the axes. Spatial shifts smaller than 2.5σ are shown in grey. Higher than 2.5σ shifts are depicted with a color ramp : red stands for expansion and blue for contraction. α-helices (blank boxes) and β-strands (black) are indicated on the *x*-axis as reference. **(B)** Superposition of the two model compared in *(A)*, using the C^{α} atoms that were found to be conformationally invariant at the 2.5σ level (in grey). The purple regions are significantly shifted (including expansion or contraction). Bound ATP is shown in stick representation. **(C)** Error scaled difference distance matrix for crystal structures 3EHG (this report) and 2.65Å resolution 3GIE (ATP-binding domain only). Spanning residues 244-367 are indicated on the axes. Spatial shifts smaller than 1.5σ are shown in grey. Higher than 1.5σ shifts are depicted with a color ramp : red stands for expansion and blue for contraction. α-helices (blank boxes) and β-strands (black) are indicated on the *x*-axis as reference. **(D)** Superposition of the two models compared in *(C)*, using the C^{α} atoms that were found to be conformationally invariant at the 1.5σ level (in grey). The purple regions are significantly variable. Bound ATP is shown in stick representation.

Fig. S4. Reproducibility of the docking protocol. (A-D) Data of four independent simulation runs were used to calculate the rmsd of the resulting ABD models, with respect to the best scoring structure of each run. Note that two populations of structures are reproducibly obtained. **(E)** Superposition of two models, each representative of these two clusters.

Fig. S5. Effect of using different DHp domain structures, corresponding to different functional states of DesKC, on the docking simulations to the ABD. The left column displays two cartoons with transparent solvent accessible surface, viewed at 90°, for each conformational state; on the right column, a close-up of each of these docked models allows for visualization of His188 and ATP, highlighting the resulting distances and arrangements. Complementary details are listed in Table S2. **(A)** DHp in the wild-type kinase state (pdb 3GIE). In the close-up view, the distance between His188 and γP ATP is indicated (3Å). **(B)** Same as *(A)*, but using the DHp domain of $DesKC_{H188V}$ (pdb 3EHH), corresponding to the phosphatase state. Val188 has been substituted by a His for the sake of this analysis. Clearly, the resulting distance between ATP and His188 is too large (8.8Å) to be compatible with the phosphorylation reaction. **(C)** Best docking structure with the DHp domain in the phosphotransferase state (pdb 3GIG). Note that the interacting surface is considerably smaller compared to the kinase state (correlated to a significantly lower Haddock score, see Table S2).

Fig. S6. Disulphide crosslinking assay for control mutants. SDS-PAGE of the crosslinking reaction performed with the enginnered mutant proteins **(A)** Q193C-G334C; and, **(B)** S196C-G334C. No detectable bands are present in *(A)* corresponding to the expected increment in molecular weight due to covalent dimerization. Cysteine at position 196 allowed for the appearance of a faint band with the

expected dimeric molecular mass (marked with an arrow), sensible to DTT treatment. However, note the extremely reduced kinetics and yield with respect to the disulphide bonding induced by a Cys at the original target position 192 (see Fig. 4B).