Supporting Information

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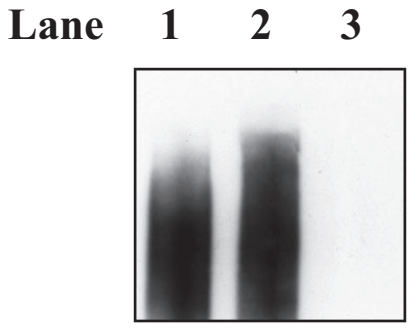

Fig. S1. Phosphorylation of the serine residue in the linker region preceding the initiating methionine of Pellino 1 is not required for activation by IRAK1. Cleavage of the GST-tag from Pellino 1 with PreScission Protease leaves a pentapeptide sequence GPLGS, preceding the initiating methionine residue of Pellino 1, which is phosphorylated at the serine residue by IRAK1 in vitro (Fig 2*B*). The mutation of the GPLGS serine residue to Ala (Lane 2) does not affect the activation of Pellino 1 by IRAK1 upon incubation with MgATP as compared to wild type Pellino 1 (Lane 1). Lane 3; same as Lane 2 except that IRAK1 was omitted from the phosphorylation reaction. The E2 conjugating enzyme used to assay Pellino 1 was Ubc13-Uev1a. Further details are described under *Experimental Procedures*.

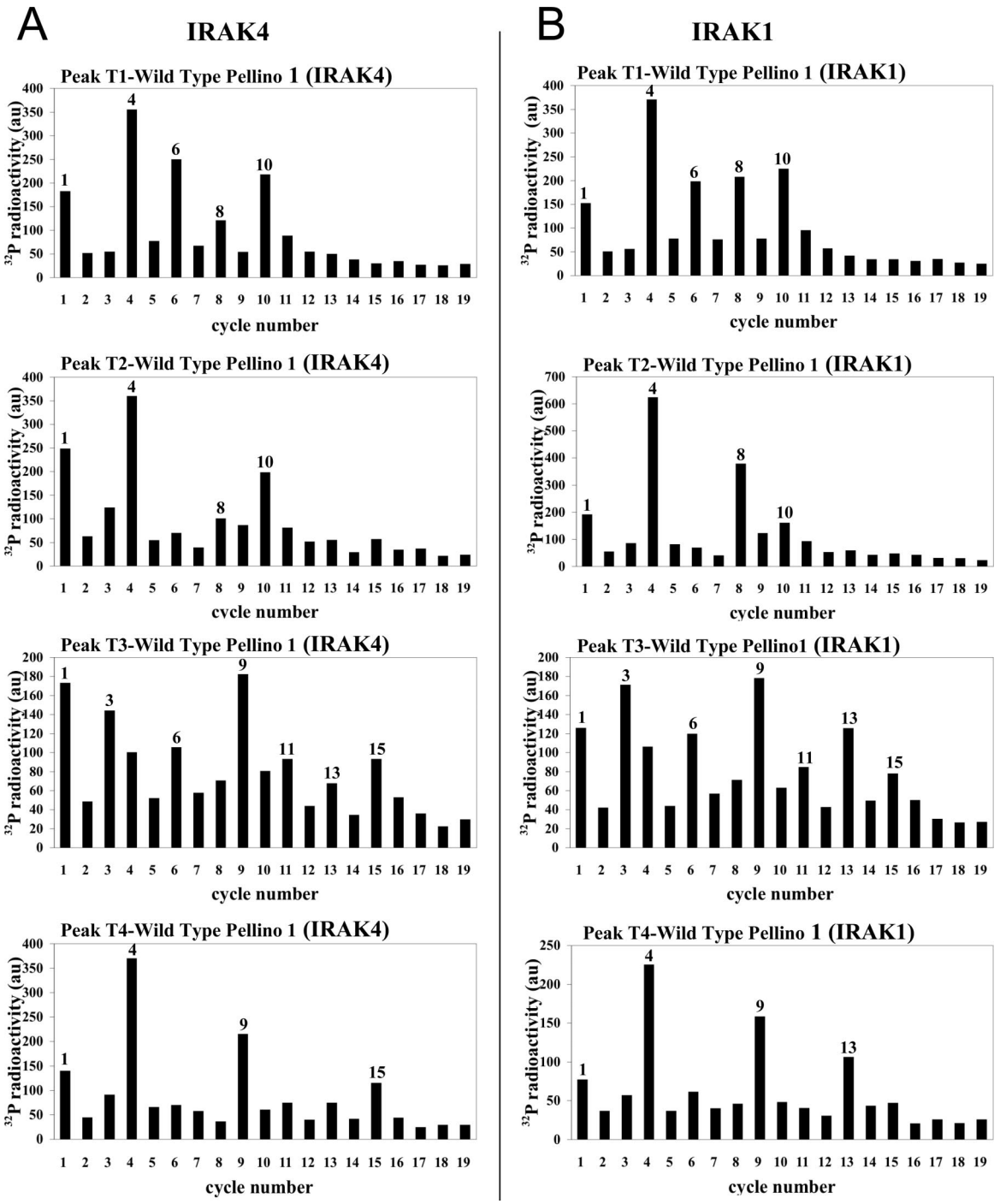

Fig. S2. Solid phase sequencing of Peaks T1 to T4 from Figure 4. (*A*) The left column shows solid phase sequence analysis of Peaks T1–T4 from Fig 4*A*, which were derived by tryptic digestion of wild-type Pellino 1 that had been phosphorylated by IRAK4. Mass spectrometry analysis showed that Peaks T1 and T2 were, respectively, the triply and doubly phosphorylated versions of the peptide DQHSISYTLSR corresponding to residues 73–83. The cycles at which ^{32}P-radioactivity were released was consistent with phosphorylation at Ser76, Ser78, Thr80, and Ser82. Peak T3 contained two forms of the peptide AISNKDQHSISYTLSR with three and four covalently bound phosphates. The solid phase sequence analysis was consistent with phosphorylation of AISNKDQHSISYTLSR at any three or four of the following residues: Ser70, Ser76, Ser78, Thr80, and Ser82. Peak T4 contained the peptide AISNKDQHSISYTLSR with two covalently bound phosphates and solid phase sequencing was consistent with phosphorylation of this peptide at Ser76 and Ser82. Peak 4 also contained the peptide DQHSISYTLSR with one phosphate attached and the results were consistent with phosphorylation of this peptide at Ser76. (*B*) The right column shows solid phase sequence analysis of Peaks T1–T4 from Fig 4*C*, which were derived by tryptic digestion of wild-type Pellino 1 that had been phosphorylated by IRAK1. The results were very similar to *A*, except that IRAK1 showed a greater preference for the phosphorylation of Thr80 over Ser82 (e.g., see solid phase sequencing of T4 in *B* and *A*, where the thirteenth cycle of Edman degradation corresponds to Thr80 and the fifteenth to Ser82.

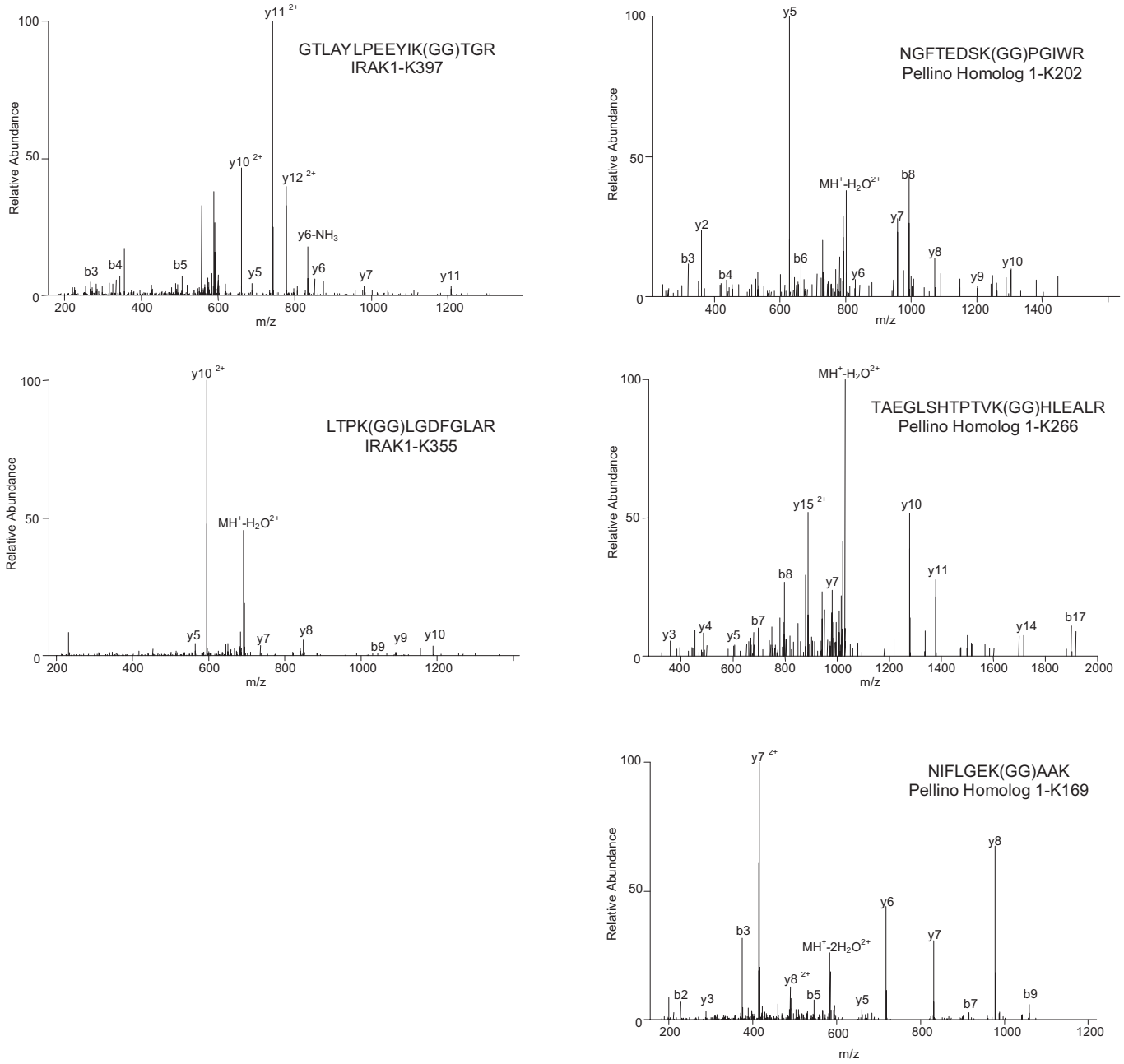

Fig. S3. MS/MS spectra of identified in vitro ubiquitination sites on IRAK1 and Pellino 1 by data-dependent acquisition on a LTQ Orbitrap mass spectrometer. A ubiquitination reaction was set up as described in *Experimental Procedures* using UbcH4 as the E2 conjugating enzyme. After incubation for 1 h, the reaction was terminated with SDS and subjected to SDS/PAGE. The polyubiquitinated species were excised, digested with trypsin, and analysed by MS/MS. The lysine residues on IRAK1 and Pellino 1 to which the first ubiquitin was attached were identified from a mascot (MatrixScience) database search, followed by manual annotation of the MS/MS spectra. The sites of ubiquitination are indicated by the presence of an isopeptide linkage between the C-terminal Gly-Gly sequence of ubiquitin and the ε-amino group of the lysine side chain [denoted as K(GG)].

Other Supporting Information Files

Table S1