Supplemental Figure Legends

Figure S1. Kinase activity of substrate binding mutations in HsFN3K. A. NBT colorimetric assay of buffer control, unmodified lysozyme, ribose-glycated lysozyme, and glucose-glycated lysozyme. **B-C.** Kinase assay of MBP-tagged and untagged FN3K on fructolysozyme (B) and ribulolysozyme (C). Each data point represents means of triplicates; error bars indicate standard error. **D.** HsFN3K I25 position in apo (PDB: 9CX8, pink) and ATP-DMF bound (PDB: 9CXM, blue). **E.** Change in melting temperatures of FN3K mutants in the presence of ATP.

Figure S2. Ancestral reconstruction of fructosamine kinase family generates stable proteins with high homogeneity. A. Multiple sequence alignment of HsFN3K, HsKT3K, ancFN3KRP, ancKT3K, ancKT3K, and ancFN3K. Conserved amino acid residues across all proteins are highlighted in gray. Amino acid residues found only in KT3K-like proteins are highlighted in yellow. Substrate binding residues from Figure 1are indicated by black arrows. **B.** Normalized DSF melt curves of HsFN3K (black solid), HsKT3K (dotted), ancFN3KRP (red), ancKT3K^{1} (yellow), ancKT3K^{2} (green), and ancFN3K (blue). **C.** Melting temperatures determined in (B).

Figure S3. Switch mutations in ancFN3Ks. A. The 12 amino acid residues that differ between ancFN3KRP and ancFN3KRP and their corresponding residues in HsFN3K and HsKT3K. **B-C.** Kinase activity of ancFN3KRP mutants (B) and ancFN3K mutants (C) towards ribulolysozyme. Each bar graph represents the activity at 5 μM of enzyme. Each data point represents means of triplicates; error bars indicate standard error.

Figure S4. Mutations in FN3K make them more KT3K-like. A. DSF melt curve of HsFN3K, HsKT3K, and HsFN3K 5^{mut}.

Figure S5. Intramolecular contact analysis of HsFN3K and HsKT3K. A. HsFN3K apo (pink, PDB: 9CX8) versus ATP-DMF bound (blue, PDB: 9CXM) structures. H171, L179, F244, F260, and N284 are represented as sticks. **B.** Intramolecular contact analysis of HsFN3K N284 (B) and AlphaFold predicted HsKT3K H284 (C). Purple represents established substrate-binding residues. Pink represents residues highlighted in this study.

Figure S6. Protein Structure Network analysis of HsFN3K and AtFN3K. A. Difference PSN of AtFN3K (PDB: 6OID, purple) and HsFN3K (PDB: 9CX8, orange). Shared residues in each network are green. Residues are labeled as HsFN3K residues. **B.** Multiple sequence alignment of HsFN3K and AtFN3K. Yellow box highlights alpha-helix where F244 resides.

Figure S7. Neofunctionalization of fructosamine repair by FN3Ks begins with a destabilization event. A. Kinase assay of ancFN3KRP, ancFN3KRP 5^{mut}, ancFN3K,

and HsFN3K against DMF. Each data point represents means of triplicates; error bars indicate standard error. **B**. DSF melting curves and corresponding melting temperatures for ancFN3KRP, ancFN3KRP 5^{mut}, ancFN3K, and HsFN3K.