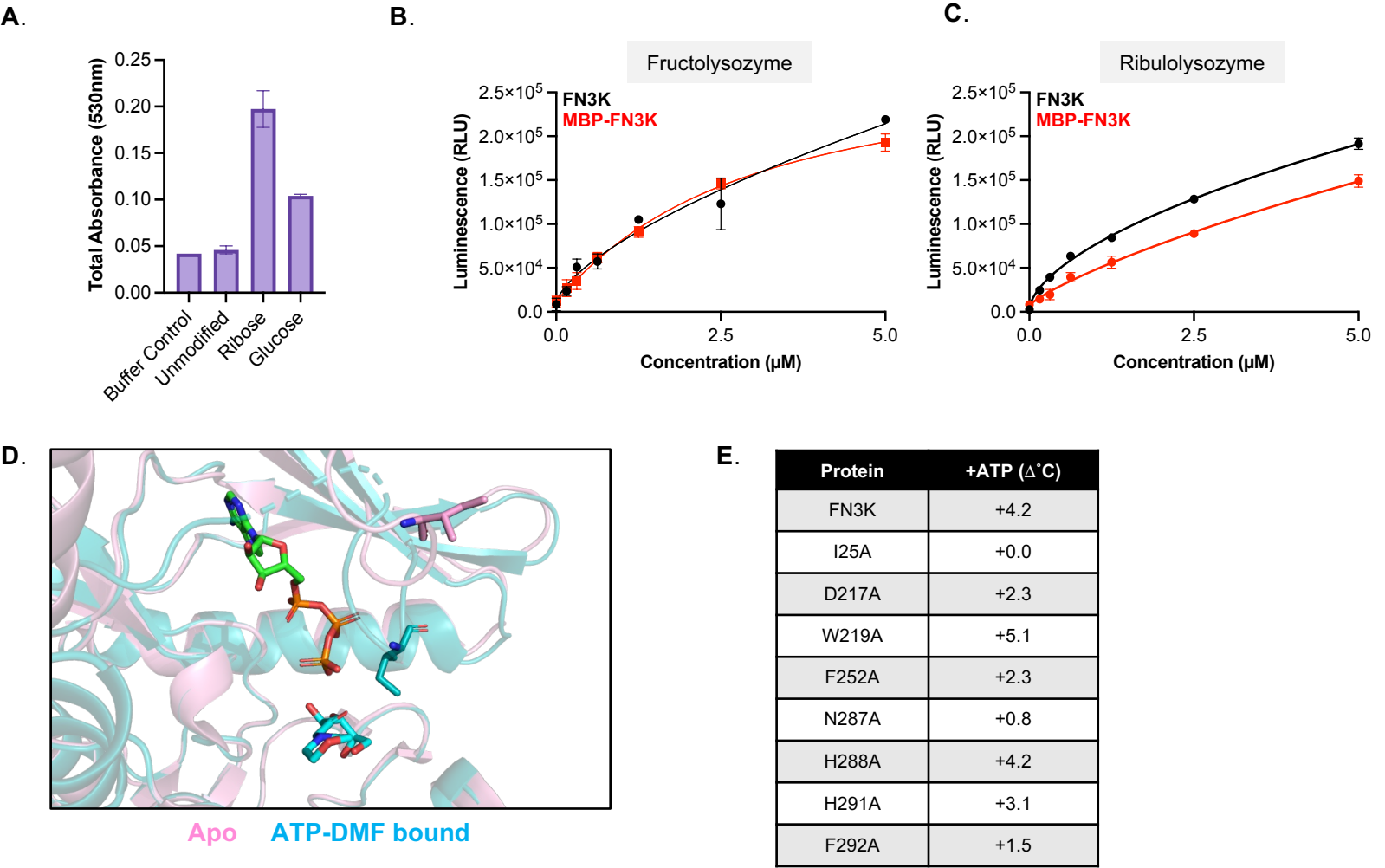


**Figure S1.** Kinase activity of substrate binding mutations in HsFN3K



**Figure S2.** Ancestral reconstruction of fructosamine kinase family generates stable proteins with high homogeneity.

**A.**

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

-----MEQLLRAELRTATLRAFGGPGAGCISEGRAYDTDAGPVFVKVNRRTQARQMF  
-----MEELLRLRELGCSSVRATGHSGGGCISQGRSYDDQGRVVFVKVNPKEARRMF  
MAAMSEDPMEALLKRELGTAVLKATGHSGGGCISQGQSYDTRGRVVFVKINHKAEARRMF  
MAAMSEDPMEALLKRELGTAVLKATGHSGGGCISQGQSYDTRGRVVFVKINHKAEARRMF  
MAAMSEDPMEALLKRELGTAVLKATGHSGGGCISQGQSYDTRGRVVFVKINHKAEARRMF  
MAAMSEDPMEALLKRELGTAVLKATGHSGGGCISQGQSYDTRGRVVFVKINHKAEARRMF

52  
52  
60  
60  
60  
60

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

EGEVASLEALRSTGLVRVPRPMKVIDLPGGGAFFVMEHLKMKSLSSQASKLGEQMA DLHL  
EGEMASLTAILKNTNTVKVPKPIKVLDAPGGGSVLVMEHMDMRHLSSHAAKLGAQLADLHL  
EGEMASLEAILKNTNTVKVPKPIKVLDLPGGGAVFVMEHLDMRGLSKHS AKLGEQLADLHL  
EGEMASLEAILKTETVKVPKPIKVLDLPGGGAVL VMEHLDMRGLSRHS AKLGTQLADLHL  
EGEMASLEAILKTETVKVPKPIKVLDLPGGGAVL VMEHLDMRGLSRHS AKLGEQLADLHL  
EGEMASLEAILKTETVKVPKPIKVLDLPGGGAVFVMEHLDMRGLSKHS AKLGEQLADLHL

112  
112  
120  
120  
120  
120

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

YNQKLRREKLKEEENTVGRRGGEAEFPQYVDKFGFHTVTCCGFIPQVNEWQDDWPTFFARHR  
DNKKLGEMRLKEAGTVGRGGQEERPFVARFGFDVVTCCGYLPQVNDWQEDWVVFFARQR  
HNQKLGEKLKKEAGTVGKGAGQSEFPQYVDKFGFHTVTCCGYIPQVNEWQDDWPTFFARHR  
HNQKLGEKLKKEAGTVGKGAGQSEVQVVDQFGFHTVTCCGYLPQVNDWQDDWPTFFARQR  
HNQKLGEKLKKEAGTVGKGAGQSEVQYVDKFGFHTVTCCGYLPQVNDWQDDWPTFFARQR  
HNQKLGEKLKKEAGTVGKGAGQSEVQYVDKFGFHTVTCCGYLPQVNEWQDDWPTFFARQR

172  
172  
180  
180  
180  
180

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

LQAQLDLIEKDYADREARELWSRLQVKIPDLFCGLEIIVPALLHGD LWSGNVAEDDV-GPI  
IQPQMDMVEKESGDREALQLWSALQLKIPDLFRDLEIIPALLHGD LWGNGVAEDSS-GPV  
LQAQLDLIEKDYGDREARELWSQLQLKIPDLFC DVEIIVPALLHGD LWGNGVAEDDSGEPI  
IQPQMDMIEKRSGDREARELWSQLQLKIPDLFC DMEIIVPALLHGD LWGNGVAEDDSGEPI  
IQPQMDMIEKRYGDREARELWSQLQLKIPDLFC DMEIIVPALLHGD LWGNGVAEDDSGEPI  
LQPQLDMIEKRYGDREARELWSQLQLKIPDLFC DVEIIVPALLHGD LWGNGVAEDDSGEPI

231  
231  
240  
240  
240  
240

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

IYDPASFYGHSEFELAIALMFGGFPRSFYTAYHRKIPKAPGFDQRLLLYQLFNYL NHWNH  
IFDPASFYGHSEYELAIAGMFGGFSSSFYSAYHGKIPKAPGF EKRLQLYQLFHYL NHWNH  
IFDPASFYGHSEFELAIAGMFGGFSSSFYSAYHSKIPKAPGF EKRLKLYQLFNYL NHWNH  
IFDPASFYGHSEYELAIAGMFGGFSSSFYSAYHSKIPKAPGF EKRLKLYQLFHYL NHWNH  
IFDPASFYGHSEYELAIAGMFGGFSSSFYSAYHSKIPKAPGF EKRLKLYQLFHYL NHWNH  
IFDPASFYGHSEYELAIAGMFGGFSSSFYSAYHSKIPKAPGF EKRLKLYQLFHYL NHWNH

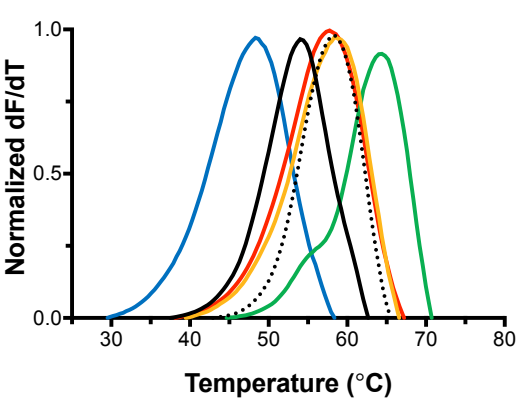
291  
291  
300  
300  
300  
300

HsFN3K  
HsKT3K  
ancFN3K  
ancKT3K2  
ancKT3K1  
ancFN3KRP

FGREYRSPSLGTMRRLLK---- 309  
FGSGYRGSSLNIMRNLVK---- 309  
FGTGYRSSSLNIMRKLLKCLKA 322  
FGTGYRGSSLNIMRNLVKCLKA 322  
FGTGYRSSSLNIMRNLLKCLKA 322  
FGTGYRSSSLNIMRNLLKCLKA 322

309  
309  
322  
322  
322  
322

**B.**



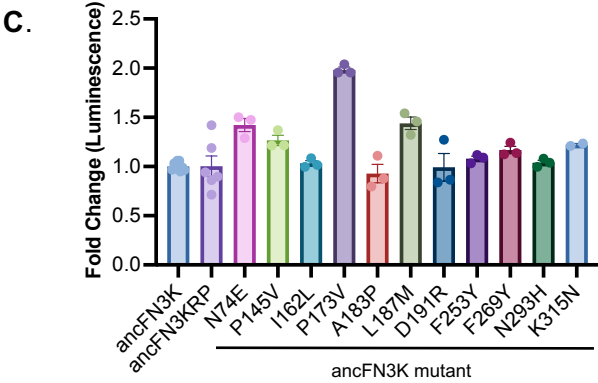
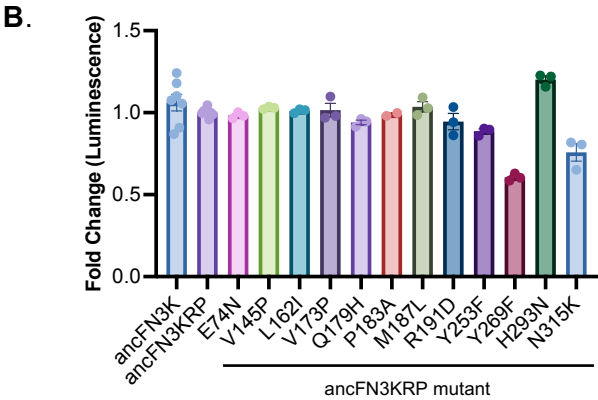
**C.**

|                      |        |
|----------------------|--------|
| HsFN3K               | 54.4°C |
| HsKT3K               | 58.4°C |
| ancFN3KRP            | 57.7°C |
| ancKT3K <sup>1</sup> | 58.7°C |
| ancKT3K <sup>2</sup> | 64.8°C |
| ancFN3K              | 47.9°C |

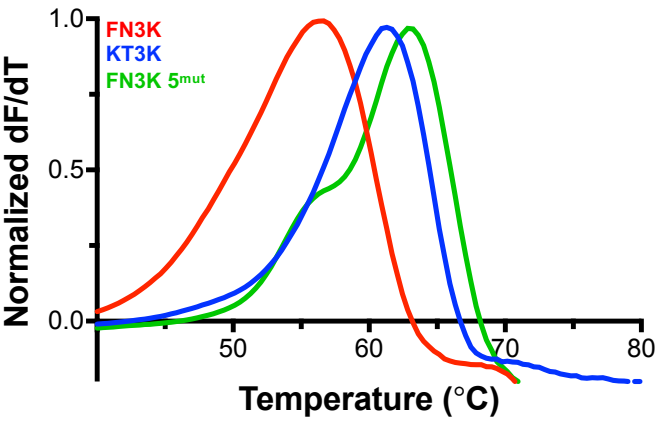
Figure S3. Switch mutations in ancFN3Ks.

A.

| Site | ancFN3KRP | ancFN3K | KT3K | FN3K | Site |
|------|-----------|---------|------|------|------|
| 74   | E         | N       | N    | G    | 66   |
| 145  | V         | P       | R    | P    | 137  |
| 162  | L         | I       | L    | I    | 154  |
| 173  | V         | P       | V    | P    | 165  |
| 179  | Q         | H       | Q    | H    | 171  |
| 183  | P         | A       | P    | A    | 175  |
| 187  | M         | L       | M    | L    | 179  |
| 191  | R         | D       | E    | D    | 183  |
| 253  | Y         | F       | Y    | F    | 244  |
| 269  | Y         | F       | Y    | F    | 260  |
| 293  | H         | N       | H    | N    | 284  |
| 315  | N         | K       | N    | R    | 306  |

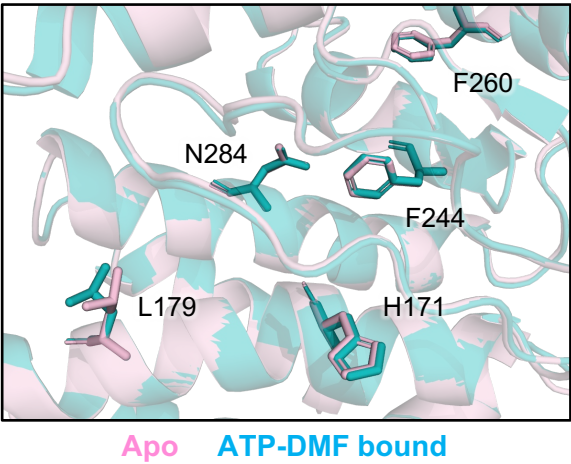


**Figure S4.** Mutations in FN3K make them more KT3K-like.



**Figure S5.** Intramolecular contact analysis of HsFN3K and HsKT3K

**A.**



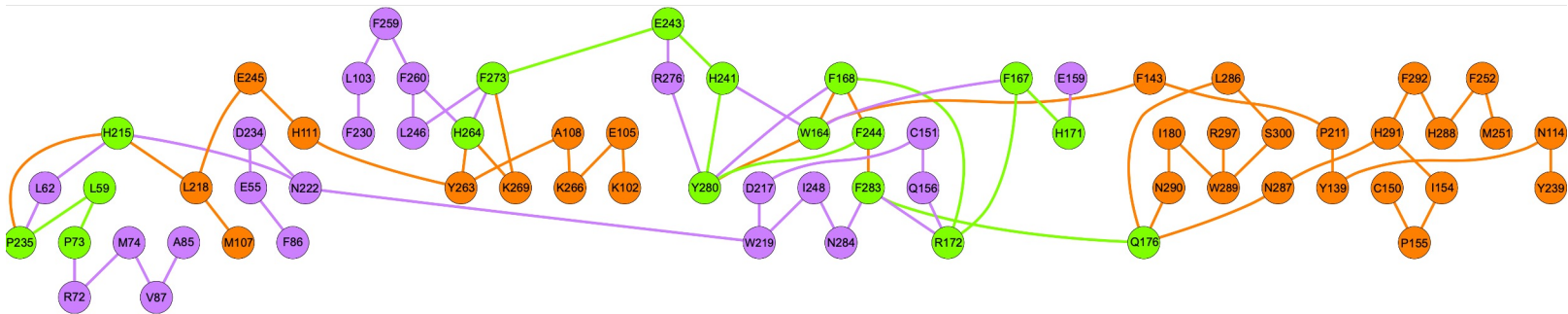
**B.**

| HsFN3K N284 |      |
|-------------|------|
| F283        | 1.33 |
| Y285        | 1.34 |
| H288        | 2.91 |
| Q281        | 2.97 |
| Y280        | 3.08 |
| L282        | 3.19 |
| N287        | 3.30 |
| L286        | 3.33 |
| M251        | 3.49 |
| I248        | 3.64 |
| A247        | 4.06 |
| F244        | 4.29 |
| F252        | 4.79 |
| W289        | 4.99 |
| D217        | 5.16 |
| L279        | 5.73 |
| Y296        | 5.86 |

| HsKT3K H284 |      |
|-------------|------|
| F283        | 1.33 |
| Y285        | 1.34 |
| H288        | 2.83 |
| Q281        | 3.13 |
| Y280        | 3.30 |
| L282        | 3.31 |
| Y244        | 3.32 |
| L286        | 3.41 |
| N287        | 3.43 |
| I248        | 3.53 |
| M251        | 3.79 |
| G216        | 4.03 |
| A247        | 4.14 |
| C151        | 4.71 |
| D217        | 4.75 |
| W289        | 5.01 |
| F252        | 5.20 |
| Y168        | 5.82 |
| Y296        | 5.93 |
| E245        | 5.93 |

Figure S6. Protein Structure Network analysis of HsFN3K and AtFN3K

A. AtFN3K HsFN3K Shared



|        |                                                                       |     |
|--------|-----------------------------------------------------------------------|-----|
| AtFN3K | MAVASLSICFSARPHLLLRNFSRPKFVAMAAMSEDPiREWILTEGKATQITKIGSVGGG           | 60  |
| HsFN3K | -----MEQLLRAELRTATLRAFGGPGAG                                          | 23  |
|        | :. : : * : : : : * . * . *                                            |     |
| AtFN3K | CINLASHYQTDAGSFFVKTNRS-IGPAMFEGEALGLEAMYETRTIRVNPCHKAGELPTGG          | 119 |
| HsFN3K | CISEGRAYDTDAGPVFVKVNRRTQARQMFEGEVASLEALRSTGLVRVPRPMKVIDLPGGG          | 83  |
|        | ** . . * : *** . *** . * . * * * . * : * : * . * . * * *              |     |
| AtFN3K | SYIIMEFIDFGGSRGNQAE LGRKLAEMHKAG-----KTSKGF                           | 156 |
| HsFN3K | AAFVMEHLKMKSLSSQASKLGEQMDLHLYNQKLREKLKEEENTVGRRGEGAEPQYVDKF           | 143 |
|        | : : * * : : : . . : : * * : : * : * . : . *                           |     |
| AtFN3K | GFEVDNTIGSTPQINTWSSDWIEFYGEKRLGYQLKLARDQYGD SAIYQKGHTLIQNMAPL         | 216 |
| HsFN3K | GFHTVTC CGFIPQVNEWQDDWPTFFARHRLQAQLDLIEKDYADREARELWSRLQVKIPDL         | 203 |
|        | ** . . * * * : * . * * * : : : * * * * . : * . * : * : *              |     |
| AtFN3K | FENVVIEPCL LHGDLWSGNIAYDKNNEPVILDPACYYGHNEADFGMSW-CAGFGESFYNA         | 275 |
| HsFN3K | FCGLEIVPALLHGD LWSGNVAED-DVGPIIYDPASFYGHSEFELAIALMFGGFPRSFFTA         | 262 |
|        | * . : * * . * * * * * * : * : * : * * . : * * * : : : . * * . * * : * |     |
| AtFN3K | YFKVMPKQAGYEKRRDLYLLYHYNLFGSGYRSSAMSIIDDYLRMLKA                       | 326 |
| HsFN3K | YHRKIPKAPGFDQRLLYQLFN YLNHWNHFGREYRSPSLGTMRRLLK----                   | 309 |
|        | * . : * * * : : * * * : : * * * * * : * * * * : : : * :               |     |

**Figure S7.** Neofunctionalization of fructosamine repair by FN3Ks begins with a destabilization event.

