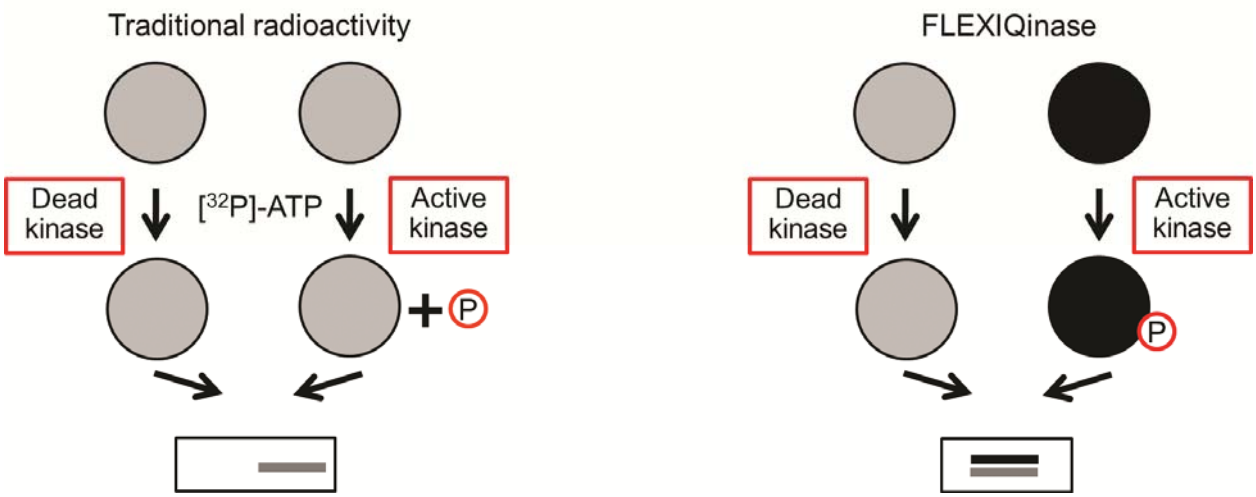
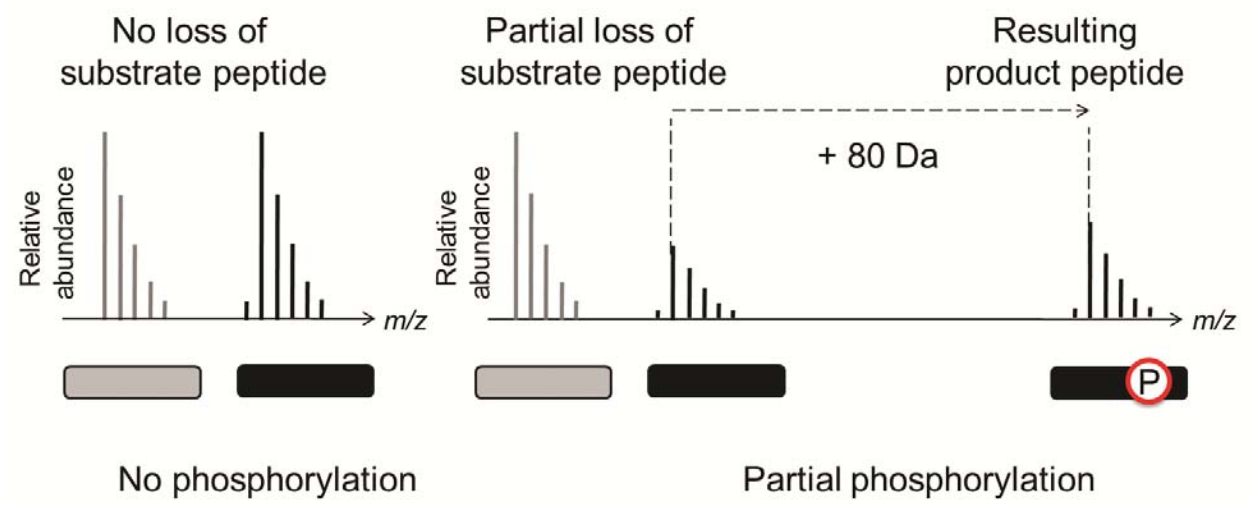
A

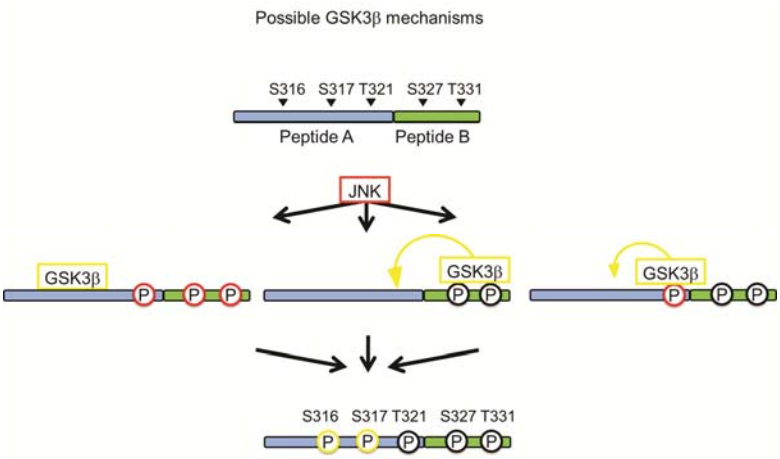


B

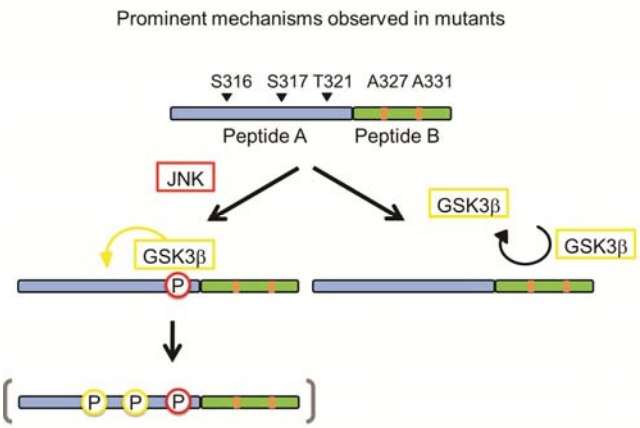


Supplementary Figure 1. The 1-kinase assay. (a) Comparison between the traditional and the FLEXIQinase workflow. Grey and black circles indicate light and heavy labeled proteins, respectively. (b) Scheme of possible substrate and product peptide spectral profiles. Grey and black bars indicate light and heavy peptides, respectively.

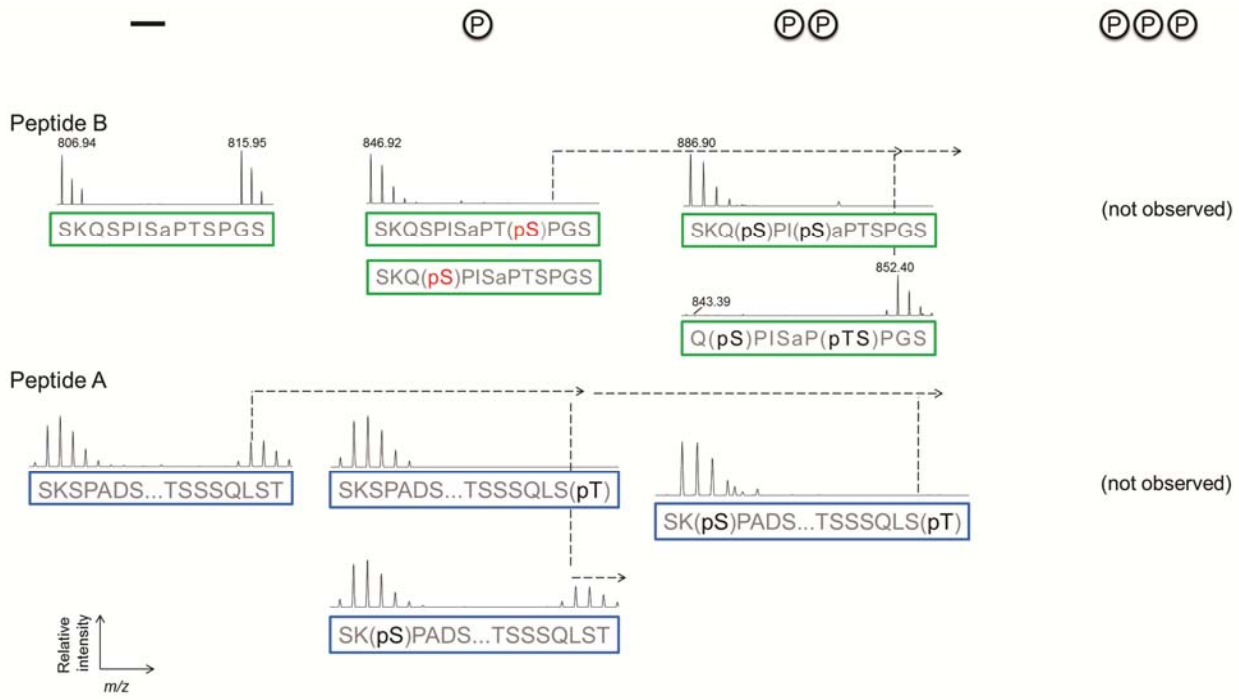
A



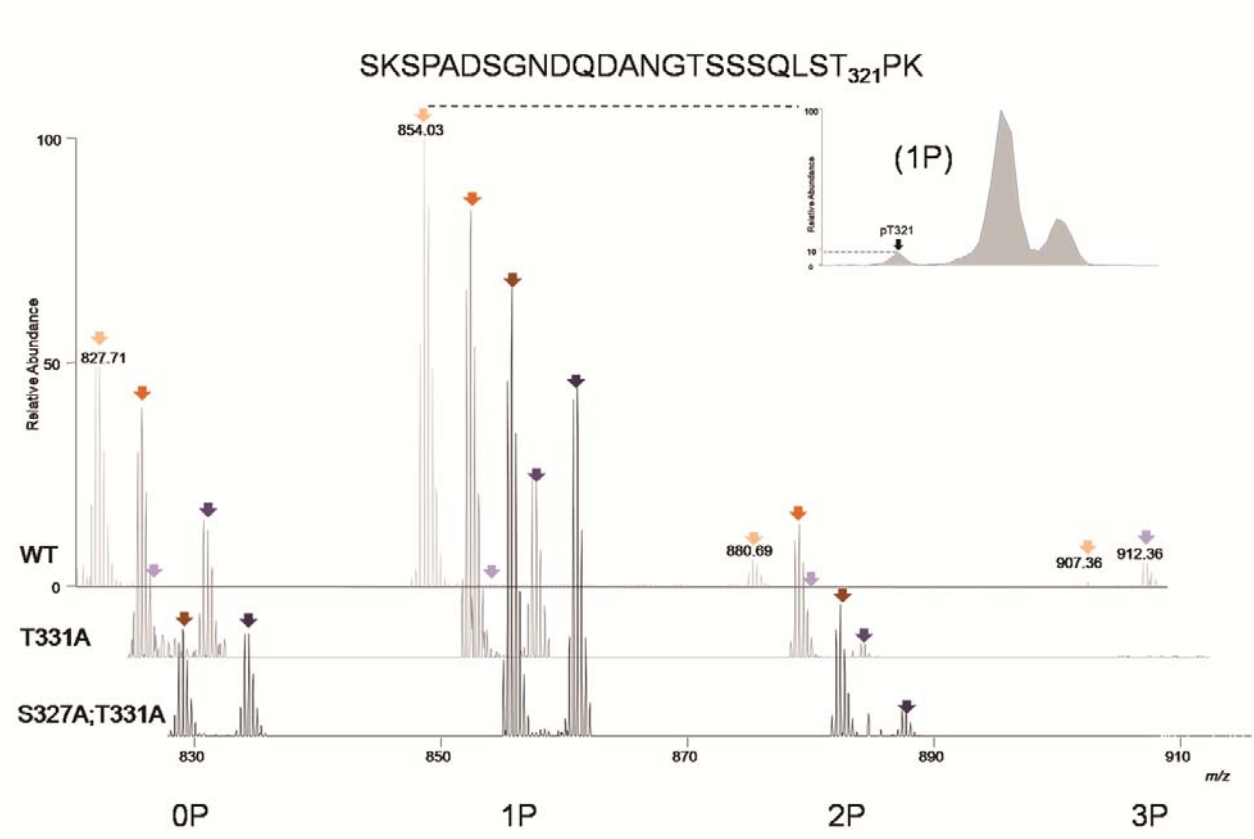
B



Supplementary Figure 2. Interpretation of the JNK-GSK3β FLEXIQinase assays. (a) The possible JNK-dependent GSK3 β mechanisms as interpreted from the WT DCX FLEXIQinase assay (Fig. 3b). (b) The prominent mechanism that are revealed as interpreted from the S327A;T331A DCX FLEXIQinase assay (Fig. 3c). JNK-dependent sites are red and black indicates either JNK or GSK3β-dependent phosphorylation. The grey parenthesis indicates a phospho-peptide that is likely to be present, but not observed with MS.



Supplementary Figure 3. Peak chase profiles from JNK/GSKβ-treated single mutant DCX (T331A). The phosphorylation status is indicated on top where a dash indicates an unmodified peptide, and the number of P's indicate the number of phosphorylation sites on the peptides below. Representative light-to-heavy peak profiles are shown for the phosphorylation sites indicated below them. Green and blue boxes include all possible phospho-acceptor sites for peptides B and A, respectively. Dashed lines connect heavy substrate peaks with their expected heavy product peaks, until an end product is observed. (not observed) is noted if the peak chase profile indicates a product should be present but not observed with MS. JNK-dependent sites are red and black indicates either JNK or GSK3b-dependent phosphorylation.



Supplementary Figure 4. The relative MS1 intensities of the light (JNK-treated) and heavy peaks (JNK/GSK3β-treated) of the unmodified and phospho-peptides of DCX peptide A. Light and heavy peaks are labeled with orange and purple arrows, respectively, with increasing color darkness with increasing mutations. The corresponding WT and mutants are labeled at the left of each *m/*z axis. The light variant of the singly phosphorylated WT peptide is analyzed in detail (inset displays extracted ion chromatogram). The total intensity of this single peak (at m/z = 854.03) is the combined signal of three predominant singly phospho-peptides (1P). The chromatographic peak corresponding to the pT321 peptide is 10 % of the higher downstream peak.

Phosphorylation sites on WT-DCX	Specificity
MNGLPSPTH(pS)AHCSFYR	JNK
MNGLPSP(pT)HSAHCSFYR	JNK
p(TRT)LQALSNEKK.A	JNK
TSANMKAPQ(pS)LASSNSAQAR	JNK
TSANMKAPQSLAS(pS)NSAQAR	JNK
APQSLASp(SNS)AQAR	JNK
APQ(pS)LASSNSAQAR	JNK
GNPSATAGPKA(pS)PTpPQK	JNK
GNPSATAGPKASP(pT)PQK	JNK
GNPSATAGPKAp(SPT)PQK	JNK
TSAK(pS)PGPMRR	JNK
SK(pS)PADSGNDQDANGTSSSQLSTPK	JNK
SKSPADSGNDQDANGTSSSQLS(pT)PK	JNK/GSK3β
p(SKS)PADSGNDQDANGTSSSQLS(pT)PK	JNK/GSK3β
(pS)K(pS)PADSGNDQDANGTSSSQLSTPK	JNK
p(SKS)PAD(pS)GNDQDANGTSSSQLSTPK	JNK
SK(pS)PADSGNDQDANGp(TS)SSQLS(pT)PK	GSK3β>>JNK
SKQ(pS)PISTP(pT)SPGSLRK	JNK/GSK3β
SKQ(pS)PIS(pT)PT(pS)PGSLRK	JNK/GSK3β
QSPIS(pT)PTSPGSLRK	JNK/GSK3β
QSPISTPT(pS)PGSLRK	JNK/GSK3β

Supplementary Table 1

JNK and GSK3β sites identified in this study. GSKβ>>JNK, this phospho-combination is observed only post GSKβ treatment