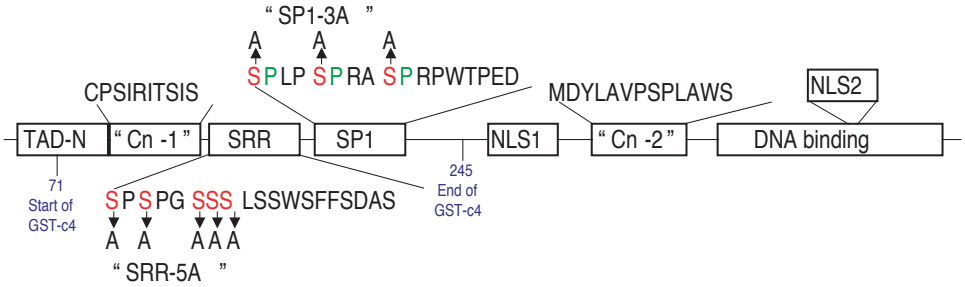
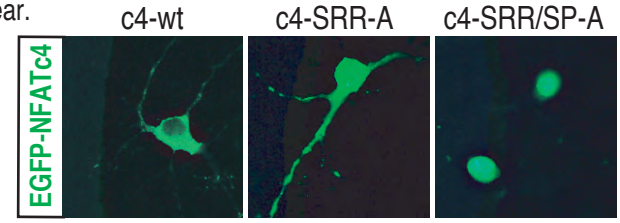
a. Diagram of NFATc4 constructs .

A recombinant fusion protein between GST and residues 71-245 of human NFATc4 was generated and used for in vitro kinase assays. The residues mutated in constructs designated "SRR5A," "SP13A," and "SRR5A/SP13A" are shown in red; the prolines in the SP1 region are shown in green. TAD-N, N-terminal transactivation domain; "Cn-1" and "Cn-2," calcineurin-binding domains; SRR, serine-rich region; SP1, serine-proline rich region; NLS, nuclear localization sequence.



b. Serine-to-alanine mutations of in the SRR- and SP-region of NFATc4 result in Ca^{2+}-independent nuclear localization of EGFP-NFATc4

EGFP -tagged full length NFATc4 constructs were transfected into P0 hippocampal neurons. Mutation of serine 170,172,175,176 and 177 to alanine in c4-SRR-A (schematic in Supp. Fig.3a) results in both cytoplasmic and nuclear localization of EGFP-NFATc4 in hippocampal neurons. Additional mutation of serine 213, 217 and 221 in the SP-region (schematic in Supp.Fig.7a) renders EGFP-NFATc4 constitutively nuclear.



c. DYRK1a targets the third serine in the SP1 region, permitting processive phosphorylation of the second, then the first serine by GSK-3.

Tryptic digests of GST-c4 and GST-c4 phosphorylated in vitro by rDYRK1a were subjected to MALDI-tandem time-of-flight mass spectrometry. A mass peak of 2409.141 representing the sequence ASPRPWTPEDPWSLYGPSPGGR (residues 214-235 of NFATc4) was present in the unphosphorylated sample. A new peak with mass of 2489.094 representing the same peptide plus one phosphate appeared in the phosphorylated sample. As the SRR5A/SP13A mutant was not phosphorylated in vitro by DYRK1a , the mass spectrometric data suggest that S215 of NFATc4 is targeted by DYRK1a. Thus, S-A mutations of the SP1 region of GST-c4 SRR5A were made in all possible combinations. An in vitro kinase assay as in Fig. 2 was performed on each mutant. Some background phosphorylation of each construct was visible. However, a shift in electrophoretic mobility was observed only in the "SSS" and "ASS" mutants, although the "ASS" mutant displayed a smaller mobility shift and less isotope incorporation, suggesting that DYRK1a primes the third serine in the SP1 region for processive phosphorylation of the second and first serines by GSK-3. The shift in electrophoretic mobility even under denaturing conditions appears to correlate with GSK-3 phosphorylation and may indicate a fundamental conformational change consistent with nuclear export of full-length NFATc proteins.