Protein Phosphorylation: Regulation of Protein Binding and Pathway Cross-talk

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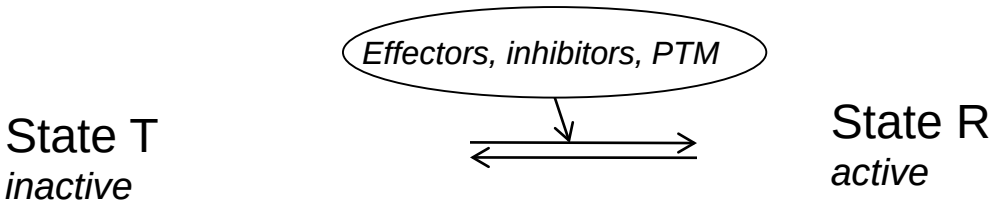
Mechanisms of regulation of protein activity

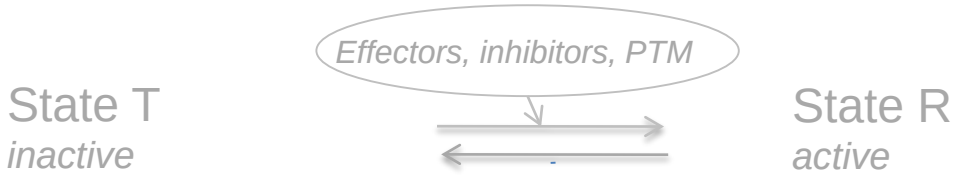
- Availability/abundance
  - Gene expression, translation
  - Translocation of proteins or substrates
  - Turnover
- Proteolytic activation
- Inteins

Mechanisms of protein regulation



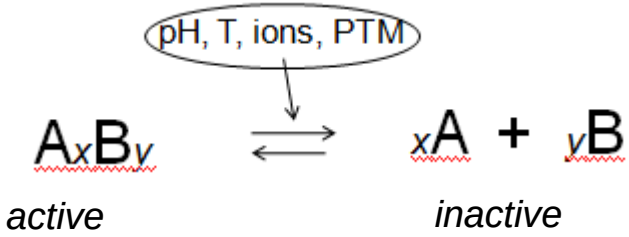
- Binding another protein or small molecule
- Reversible covalent post-translational modifications
- Allosteric activation and inhibition

Mechanisms of protein regulation



- Binding of another protein or small molecule
- Reversible covalent post-translational modifications
- Allosteric activation and inhibition

Transitions between different oligomeric states



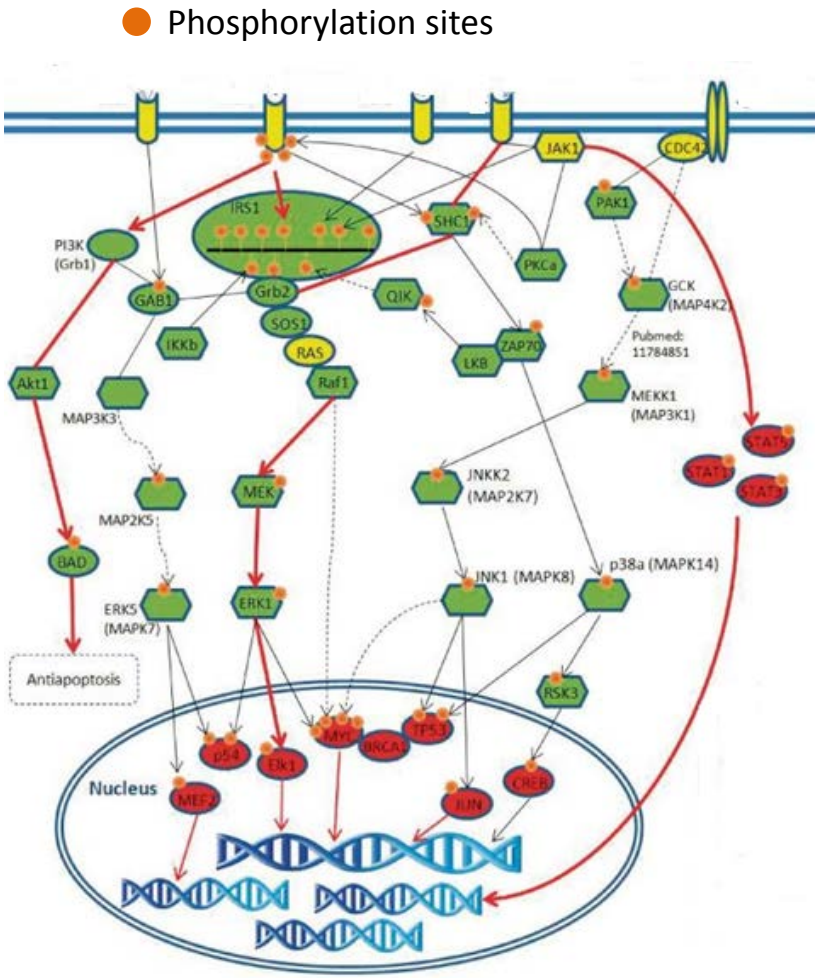
$$K = e^{-\Delta G^0_{diss} / RT}$$

$$K_d = \frac{[A]^x \times [B]^y}{[A_x B_y]}$$

Phosphorylation regulates many signaling processes

Phosphorylation can:

- change structural properties, stability, activity, dynamics;
- regulate protein-protein binding;
- Structural changes produced by phosphorylation are largest among other PTMs.



Adapted from Lee et al, NAR 2011

Methods and databases

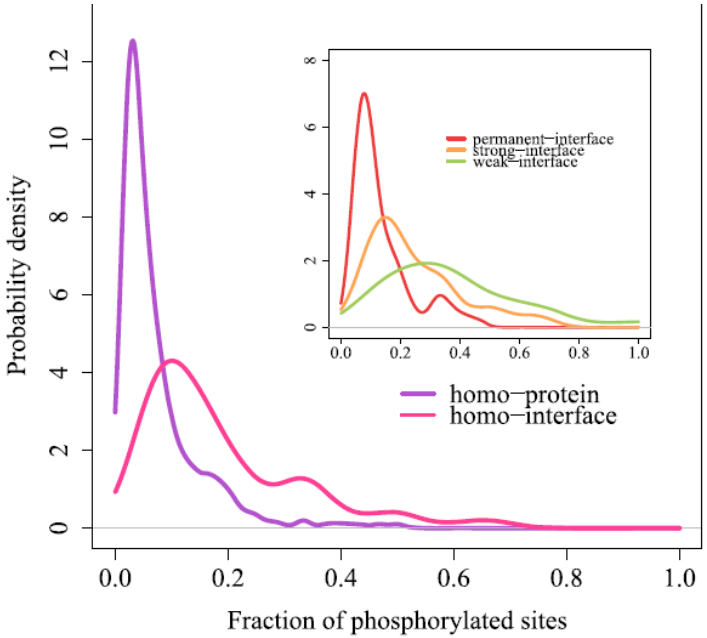
Bioinformatics	Sequence alignment, structure superposition, annotation of intrinsically disordered regions (**Disopred** (Ward et al, 2004), **PONDR-FIT** (Xue et al, *2010)*
Structural Modeling	Homology modeling of protein structures, in silico phosphorylation: **NEST/Jackal** (Petrey et al, 2003), **Modeller** (Eswar et al, 2006), **FoldX** (Guerois et al, 2002)
Energy calculations	Molecular Mechanics Poisson-Boltzmann approach (**Charmm** (MacKerrel et al, 1998))
Dynamics	All-atom Molecular Dynamics simulations (**NAMD** (Phillips et al, 2005))
Phosphorylation and pathway databases	**PhosphoSitePlus** (Hornbeck et al, 2014) **KEGG** (Kanehisa et al, 2012), **Reactome** (Croft et al, 2011), **PID** (Schaefer et al, 2009)

Effect of phosphorylation on protein-protein binding

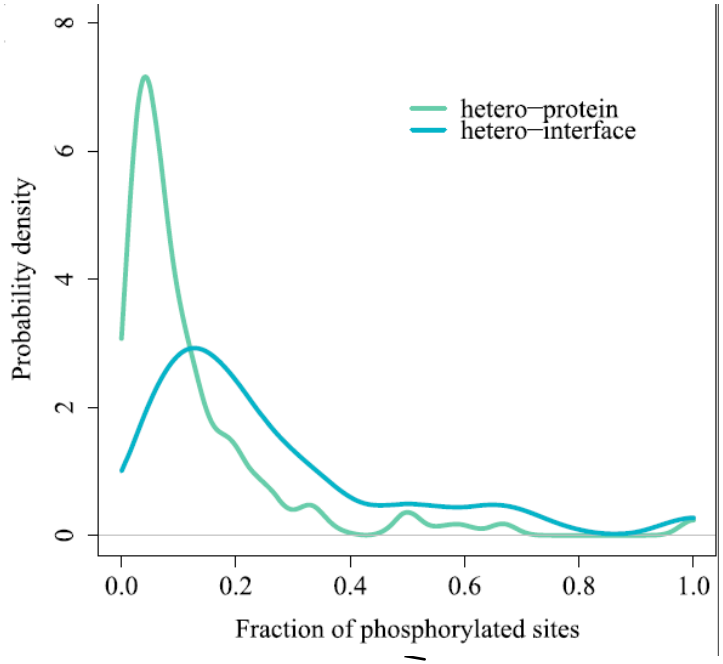
- Coupling between phosphorylation and protein binding. Can and how often phosphorylation regulates PPIs in human complexes?
- Effect of phosphorylation on binding affinity.
- Structural consequences of phosphorylation.
- Evolution of phosphorylation sites.

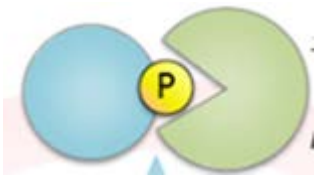
Phosphorylation at interfaces

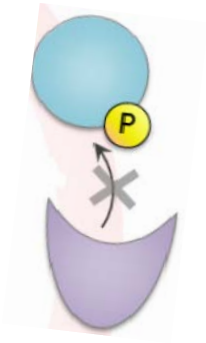
Homooligomers

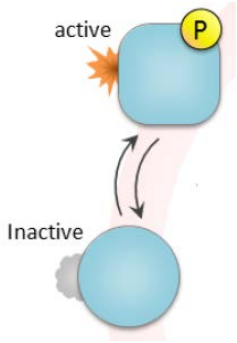


Heterooligomers









For hetero- and weak transient homooligomers phosphosites are associated with binding interfaces (p-value<<0.001)

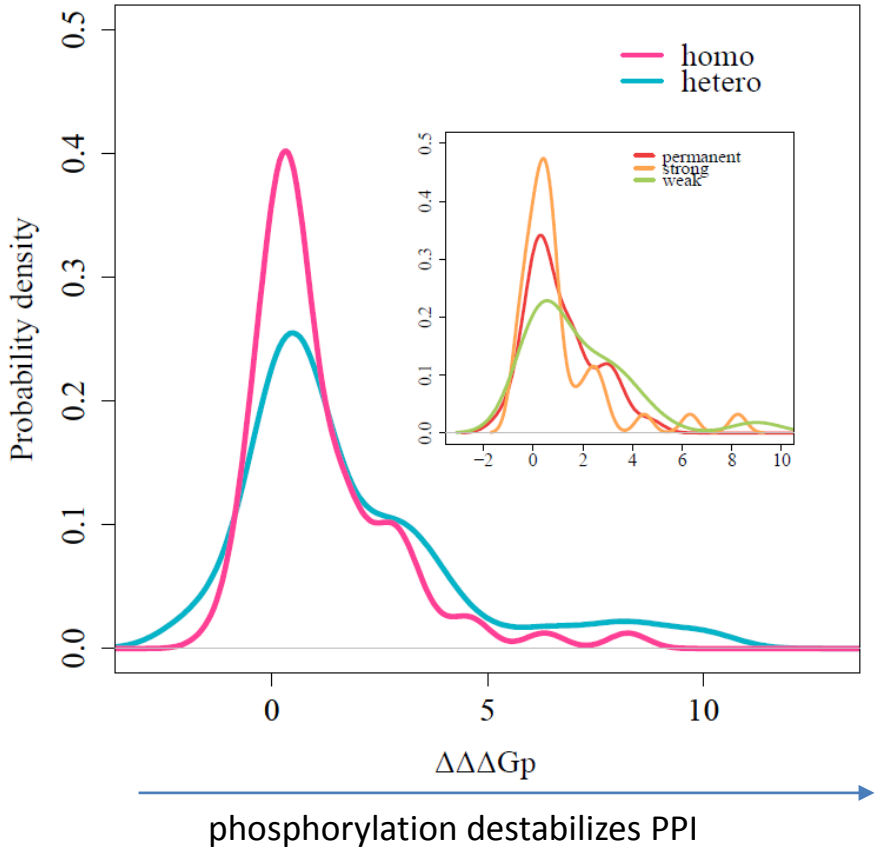
Nishi et al, *Structure*, 2011

Structural environment of phosphorylated sites

	Hetero-oligomer	Homo-oligomers		
		Weak	Strong	Permanent
Protomer ASA	2.2e-16	0.06	0.32	0.14
ΔASA	1.7e-09	0.61	0.36	0.05
No. of hydrogen bonds per site	5.3e-04	0.04	0.27	0.20
No. of residue-residue contacts per site	1.1e-03	0.50	0.42	0.45
Binding hot spots, $\Delta\Delta\Delta G^{ala}$	2.7e-04	0.18	0.67	0.39
$\Delta\Delta\Delta G^{p}$	1.3e-12	2.8e-05	1.1e-04	1.6e-05
Evolutionary conservation of site	0.016	0.55	0.11	0.65

- Phosphosites form ***more hydrogen bonds*** than non-phosphosites on interfaces for hetero- and weak homo-oligomers.
- Phosphosites are located at ***binding hot spots*** for heterooligomers.
- Phosphosites on interfaces ***are more conserved*** than non-phosphosites on interfaces for heterologomers.

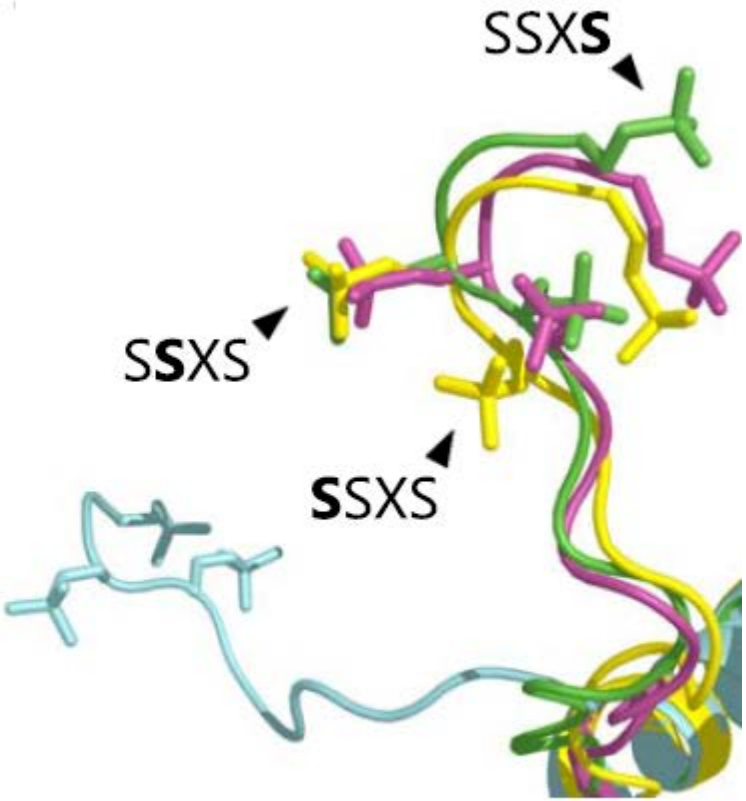
Change in binding affinity upon (de)phosphorylation



$\Delta\Delta\Delta G_p$ = change in binding affinity upon phosphorylation = $\Delta\Delta G_{WT} - \Delta\Delta G_{phospho}$

- *in silico* (de)phosphorylation – FoldX (*Guerois et al, JMB 2002*);
- For majority of complexes attaching of phosphate does not alter the binding considerably (< 2kcal/mol)
- For ~30% of complexes phosphate attachment destabilized the complex by > 2kcal/mol

R-Smad: phosphorylation promotes trimer formation



Comparison of phosphorylated model of Smad1 with actual phosphorylated structure of Smad2 (1khx):

Structure - yellow

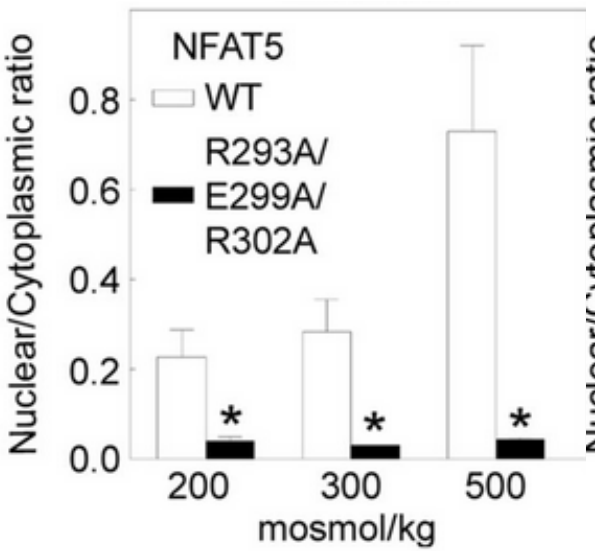
Model – green/cyan/blue

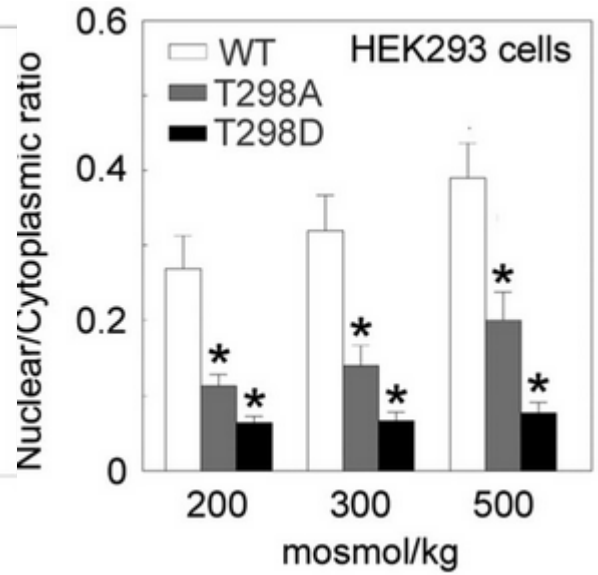
Effect of *in silico* (de)phosphorylation on R-Smad complex formation

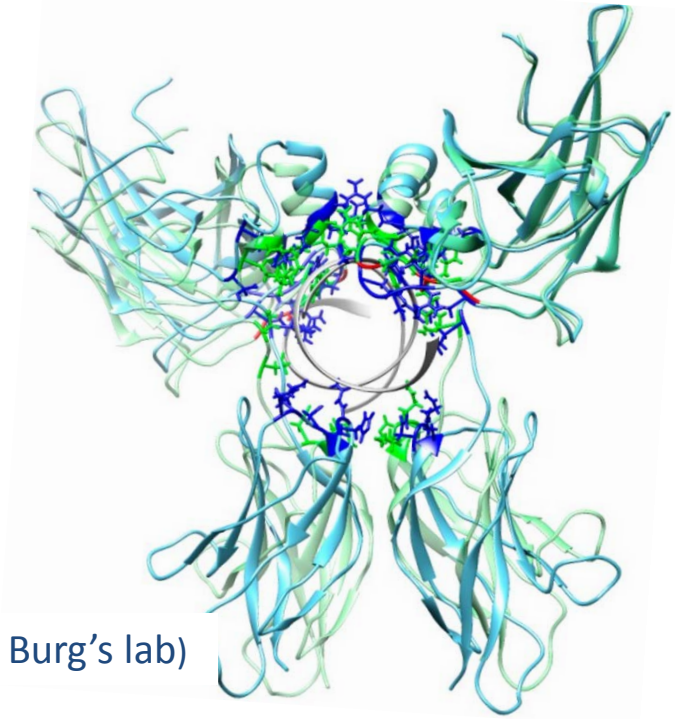
(de)phosphorylation - ΔΔΔG, kcal/mol, values in red show stabilizing effect of phosphorylation

Protein	Site	Phospho -site	Dimer AB	Dimer BC	Dimer AC	Average ΔΔΔG, kcal/mol
Smad2 (1khx)	SSXS	S->pS	0.59	0.59	0.59	0.59
	SSXS	pS->S	0.88	0.88	0.88	0.88
	SSXS	pS->S	1.53	1.53	2.86	1.97
Smad1 (1khu)	SSXS	S->pS	-2.11	1.58	-0.22	-0.25
	SSXS	S->pS	-0.9	1.49	-1.74	-0.38
	SSXS	S->pS	-1.45	-1.87	-1.08	-1.47

Mutations in DNA-binding loop of NFAT5 produce unique outcomes on binding and dynamics







Izumi et al, *Am J Physiol Cell Physiol.* 2012 (experimental data from M. Burg's lab)

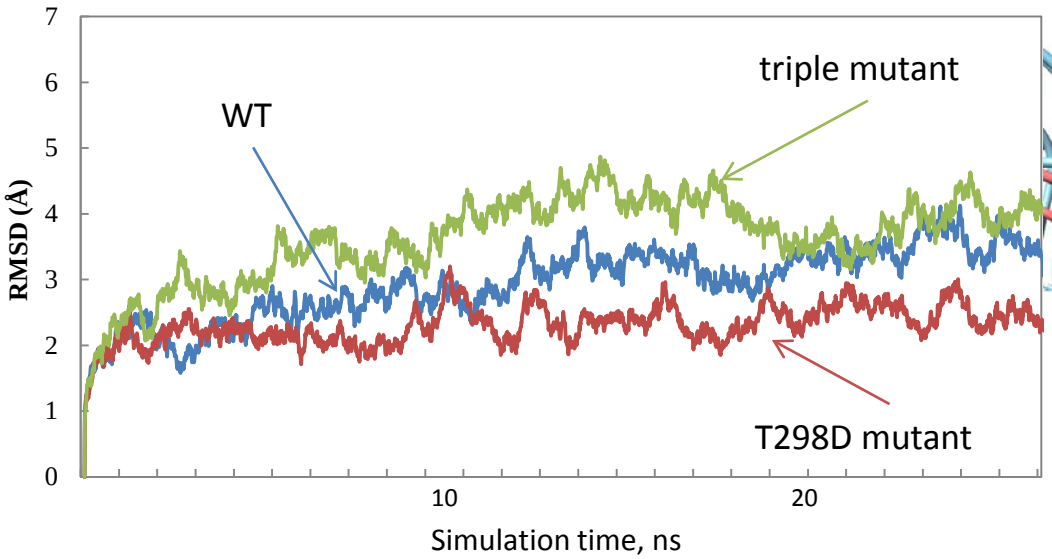
Binding energy, kcal/mol

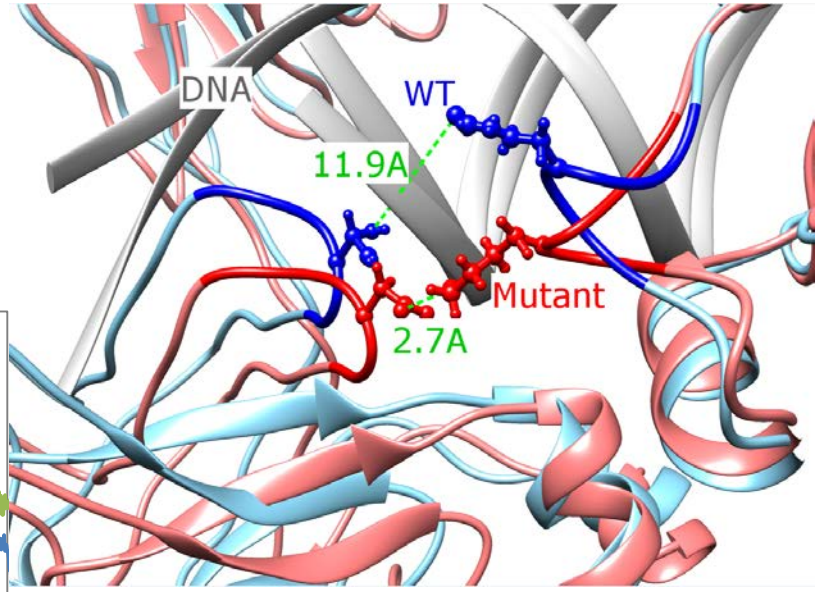
	Complex - DNA	Chain C - DNA	Chain D - DNA	Chain C – Chain D
Wild type	10.00 (0.62)	3.61 (0.39)	2.89 (0.39)	2.73 (0.23)
T298D	9.24 (0.54)	3.79 (0.23)	2.76 (0.23)	2.92 (0.23)
R293, E299, R302	6.47 (0.62)	3.13 (0.23)	2.36 (0.23)	2.78 (0.31)

Li et al, *J of Phys Chem*, 2013

Phosphomimetic mutation T298D constraints movements of two chains

Changes in conformation , Molecular Dynamics simulations (NAMD program, explicit solvent)





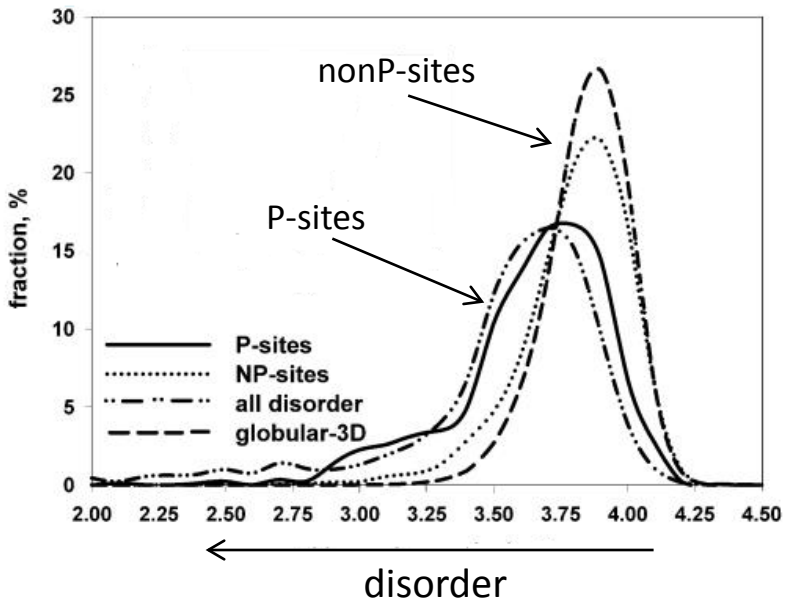
Effect of T298D on structure, formation of an extra salt bridge between two chains in a dimer

Li et al, *J of Phys Chem*, 2013

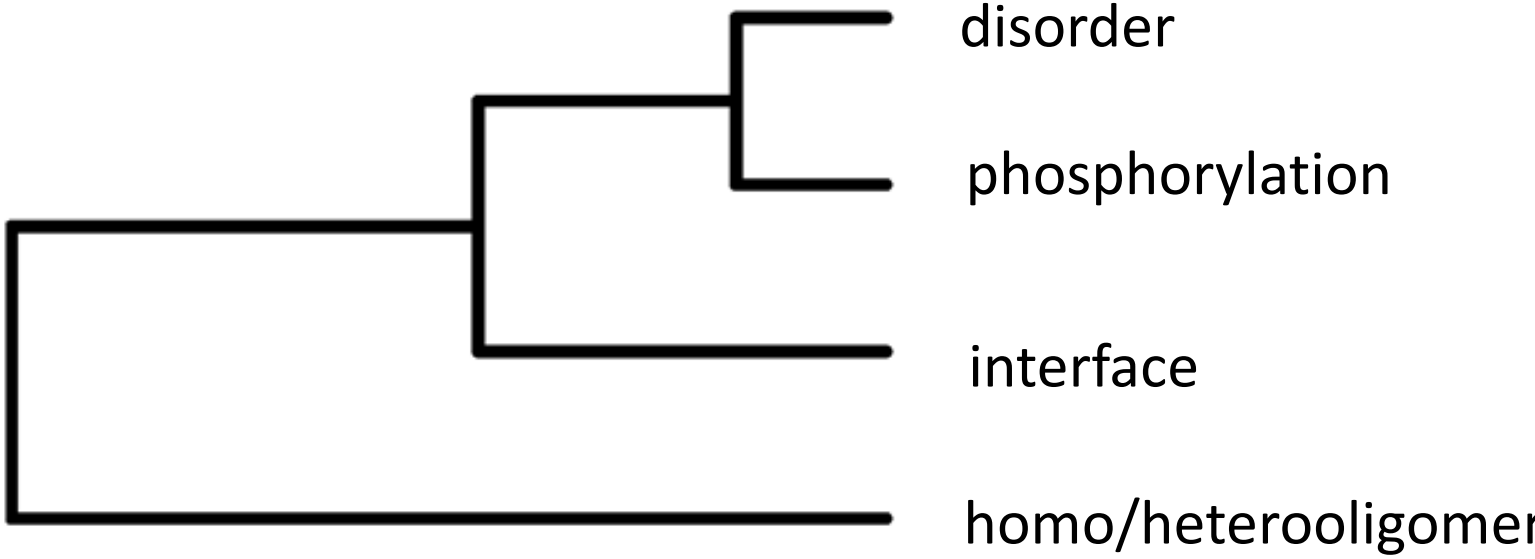
Null hypothesis: mechanism of coupling between (de)phosphorylation and protein binding involves disorder–order or order–disorder transitions

P-sites are associated with disorder

Iakoucheva et al, NAR 2004



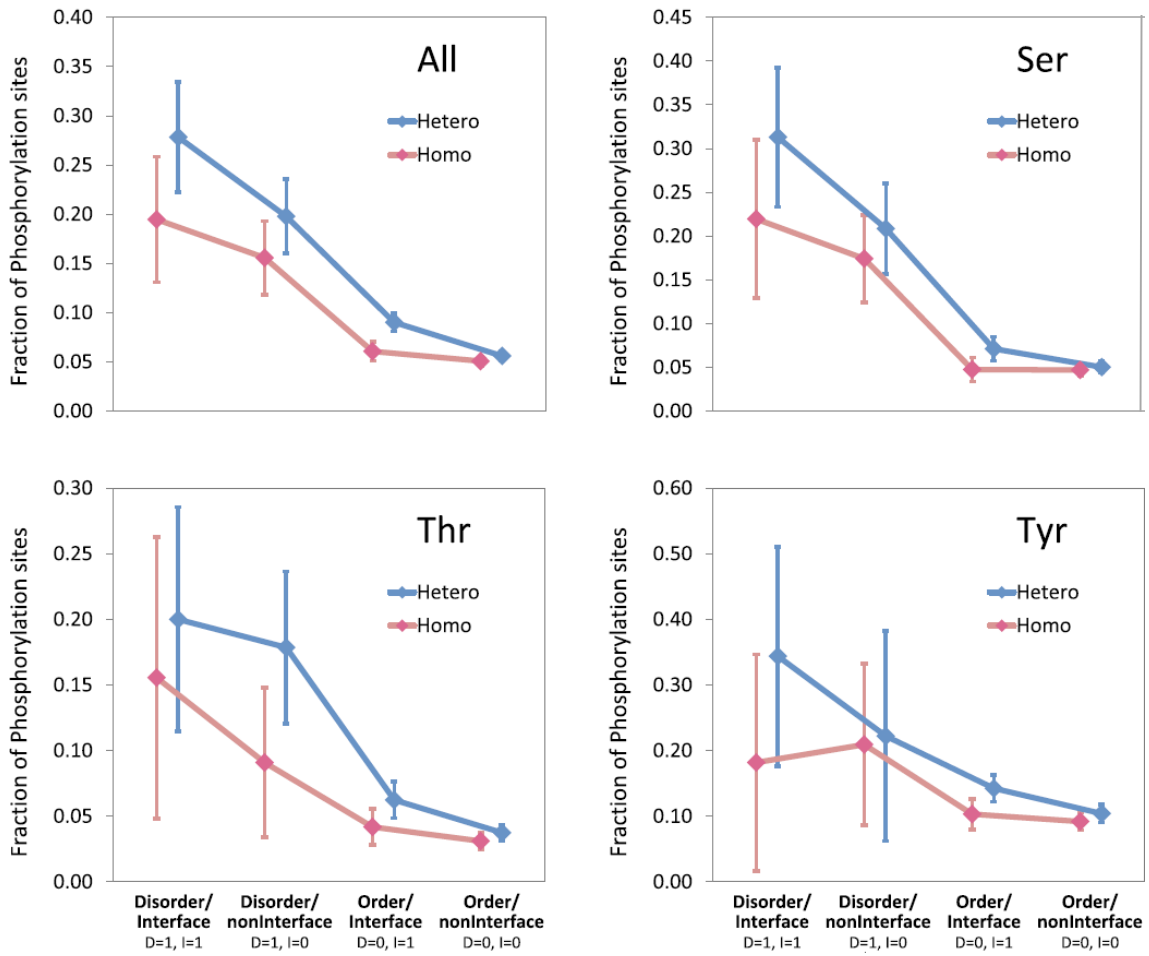
The association between features for protein sites:
disordered/structural, Interface/surface/core, phosphorylated/non-phosphorylated



Nishi et al, *Mol. Biosys*, 2013

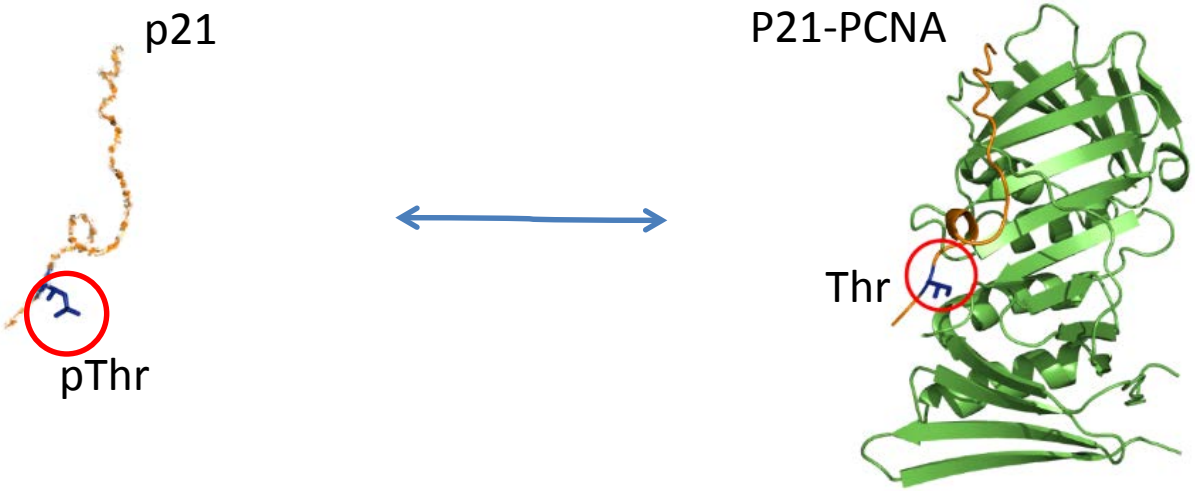
Dependence of phosphorylation state on different factors

Disordered Interface > Disordered Surface > Ordered Interface > Ordered Surface; p-value=0.005

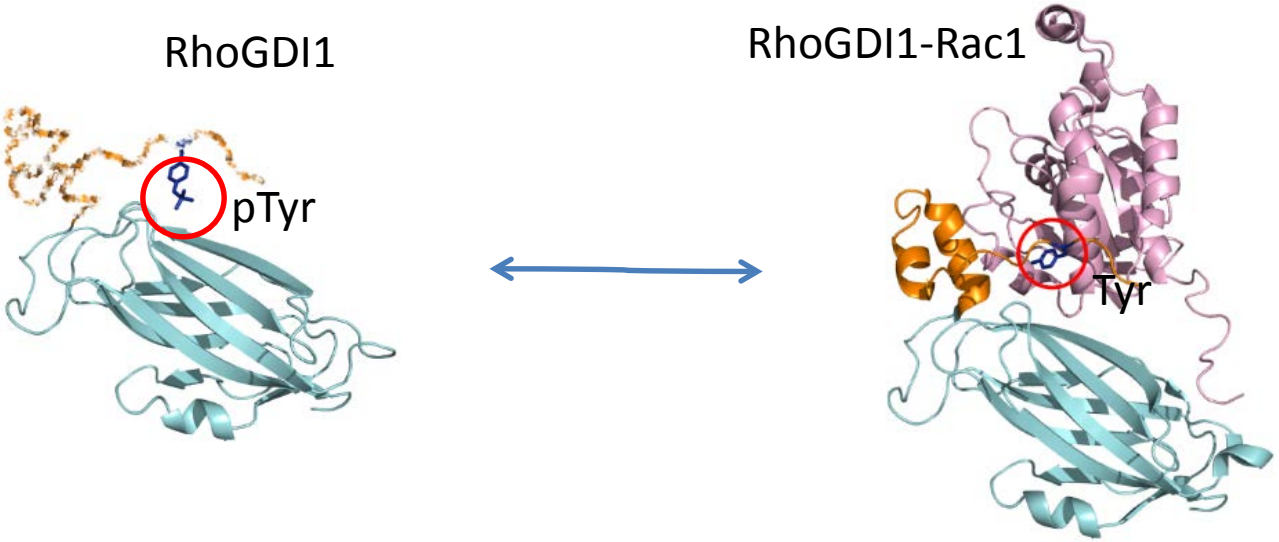


While 25% of disordered interfacial Ser/Thr/Tyr sites are phosphorylated, only 8% of ordered interface Ser/Thr/Tyr sites are phosphorylated

Regulation of disorder↔order transitions upon binding by phosphorylation



Phosphorylation may inhibit the association with proliferating cell nuclear antigen (PCNA)



Phosphorylation promotes dissociation of Rac1 from RhoGDI1

General principles of mediation of functional and pathway crosstalk through phosphorylation

Linking individual phosphorylation events with their functions and pathways

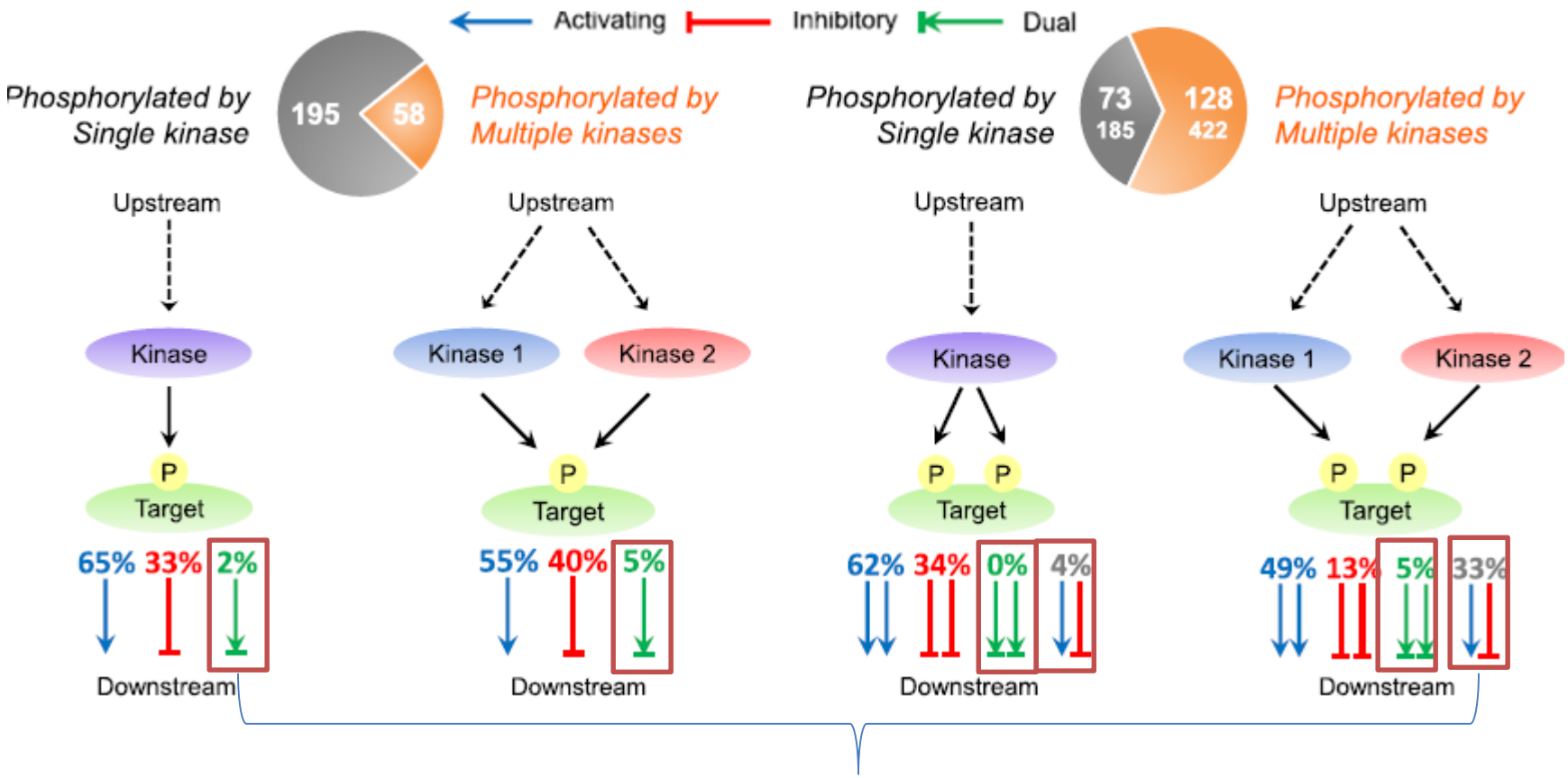
- *pathway crosstalk* - components and their functional states of one pathway may affect the function of another pathway. Pathway crosstalk may be sustained by single proteins through molecular switches provided by phosphorylation.

- Data on single phosphorylation events with *in vivo* kinases, pathways, on how phosphorylation may be associated with activation or inhibition of a target protein

Pathway regulation by single or multiple phosphorylation

Single phosphorylation

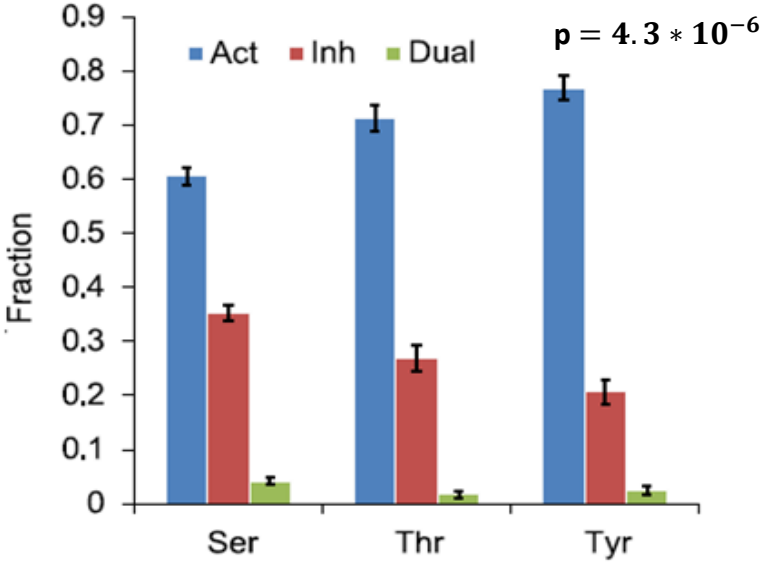
Multiple phosphorylation



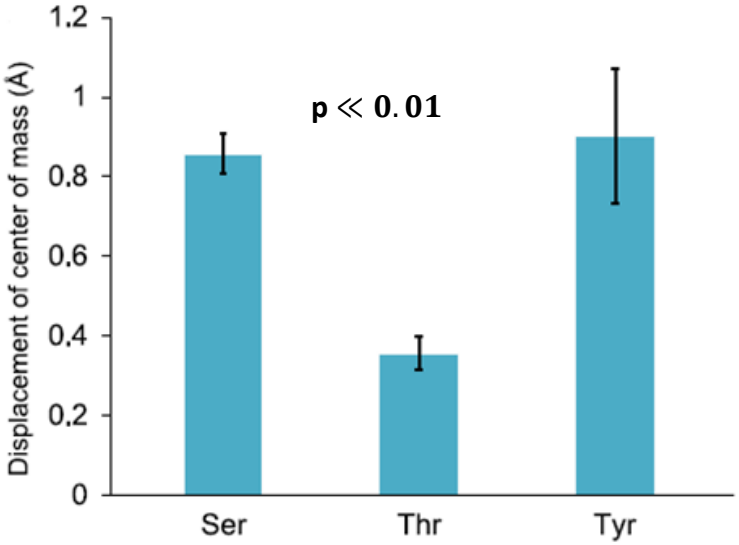
P-sites which might provide the crosstalk

Regulatory properties of phosphorylation sites

Functional effect of phosphorylation depends on residue type



Phosphorylation of Tyr and Ser lead to larger structural changes compared to phosphorylation of Thr

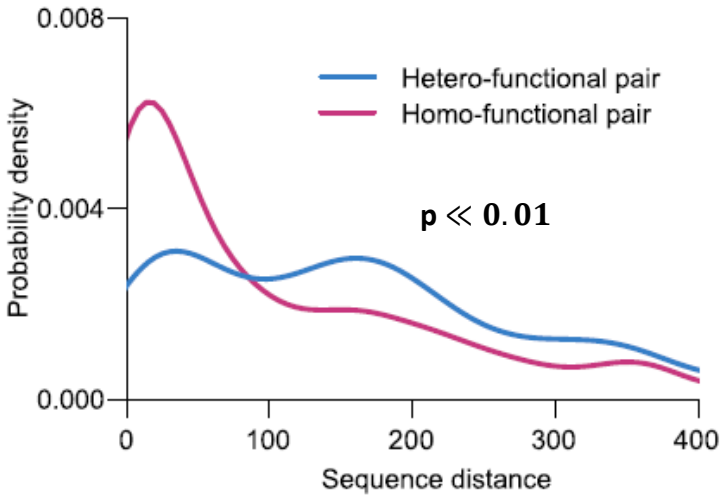


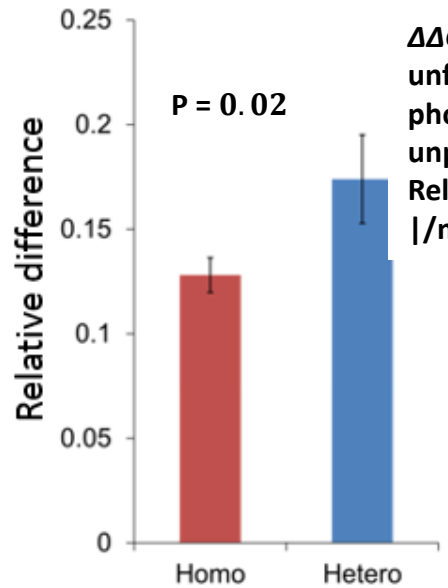
Act – activation of downstream signaling
Inh – inhibition
Dual – activation and inhibition

Nishi et al, under revisions

Crosstalk provided by multiple phosphorylation

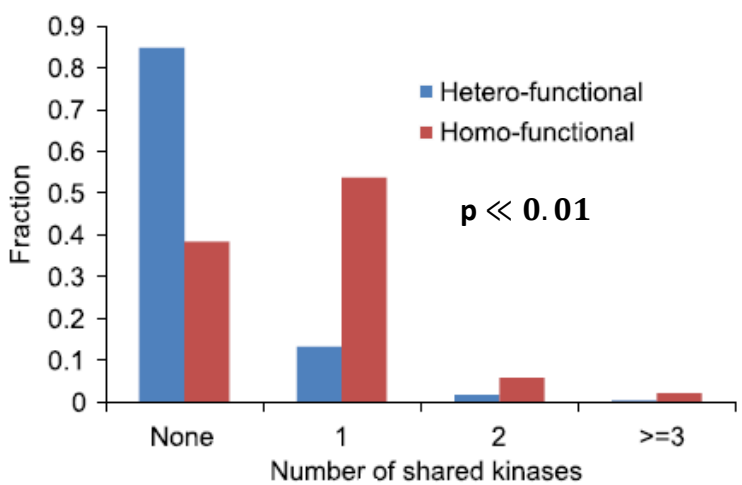
Homo-functional phosphosite pair – sites with the same functional consequences on protein

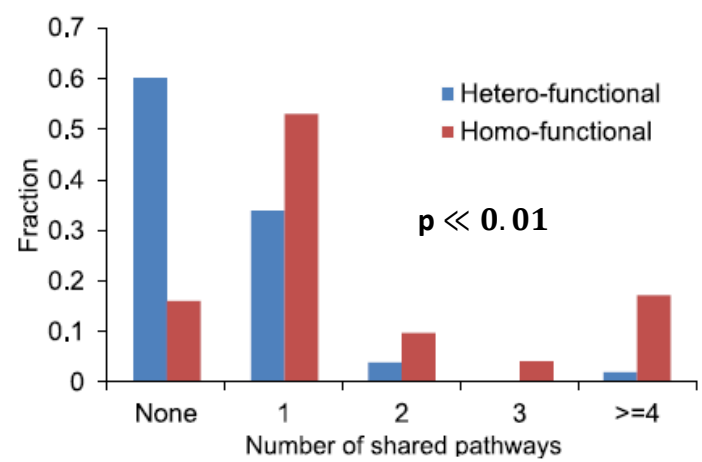


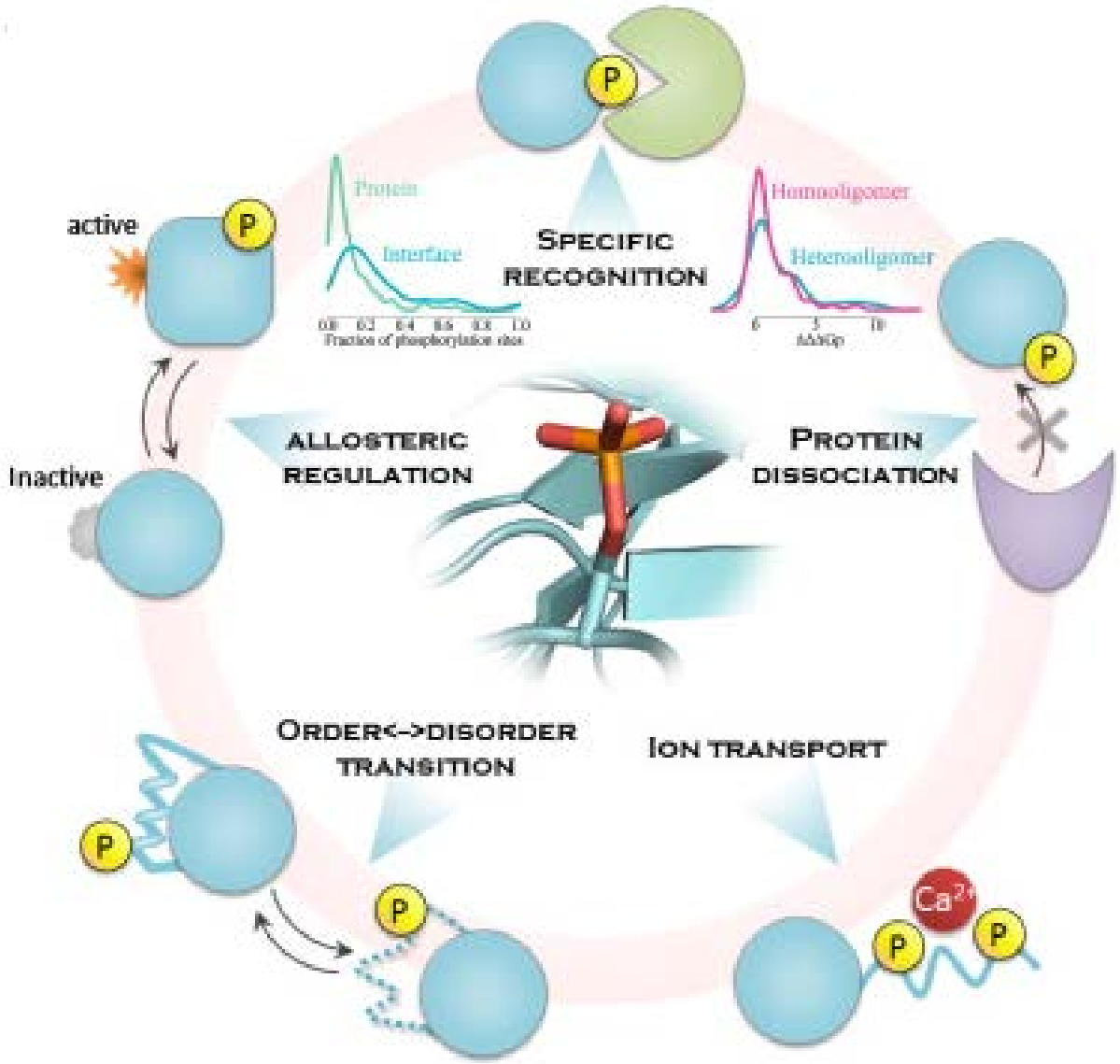


***ΔΔG = G^P - ΔG^U. ΔG^P* and *ΔG^U* - unfolding free energies of phosphorylated and unphosphorylated states. Relative difference b = |ΔΔG$_1$- ΔΔG$_2$ |/max(|ΔΔG$_1$|,|ΔΔG$_2$|)**

Hetero-functional pairs are separated in sequence and space, phosphorylated by different kinases and regulate different pathways







active
Inactive
Protein
Interface
Fraction of phosphorylation sites
Homooligomer
Heterooligomer
SPECIFIC RECOGNITION
ALLOSTERIC REGULATION
PROTEIN DISSOCIATION
ORDER<->DISORDER TRANSITION
ION TRANSPORT
P
Ca2+

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