Phosphorylation and GlcNAcylation of the RNA polymerase II C-Terminal domain and their effect on recognition by Pin1 and Protein Phosphatase 1

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INTRODUCTION

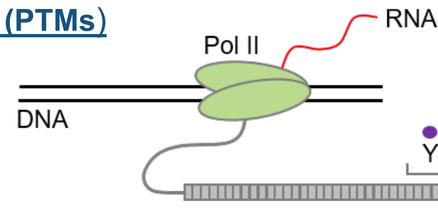
C-terminal domain (CTD) of the RNA polymerase II subunit RBP1 consists of 52 multiple repeats of the consensus ($Y_1S_2P_3T_4S_5P_6S_7$)-unit.[1]

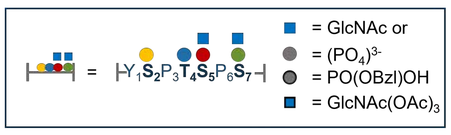
CTD landing hub for proteins to regulate[2]

- Transcription
- Splicing
- Chromatin remodeling

CTD (consensus) undergoes posttranslational modifications (PTMs)

- **Phosphorylation** (Y_1, $S_{2/5/7}$, T_4)
- Glycosylation (GlcNAc) ($S_{5/7}$)
- *Cis*/*trans*-Isomerization ($P_{3/6}$)





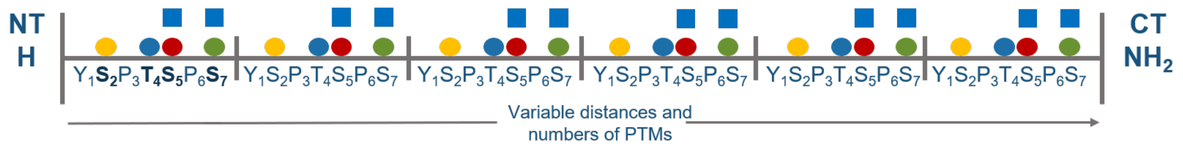
CONCEPT

Unknown if CTD readers and CTD modifiers:

- engage in **multivalent interactions**
- recognize specific **multiphosphorylation** patterns
- are affected by **glycosylation (GlcNAC)**

Idea:

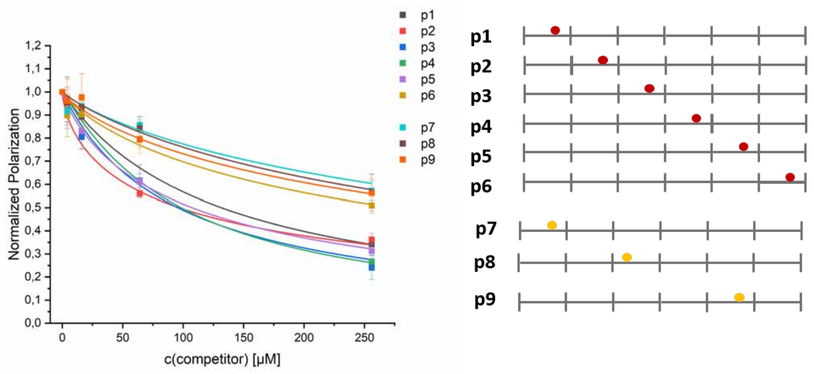
But: No indication how many and which of the 52 repeats carry PTMs



- **Chemical synthesis (SPPS)** of CTD-like peptides with **defined** multiple variations of **phospho-(glyco) sites**
- **Screening** to decipher the multiphoshorylation(-glycosylation)-specific recognition patterns (**FP – Assay**)

RESULTS

1. Positional scan of monophoshorylated CTD-peptides (FP-Assay)

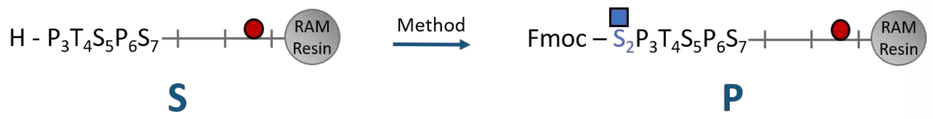


	IC_{50} [µM]
p1	**131 ± 22**
p2	97 ± 9
p3	**96 ± 9**
p4	99 ±3
p5	107 ± 2
p6	**273 ± 25**
p7	416 ± 186
p8	**360 ± 44**
p9	342 ± 38

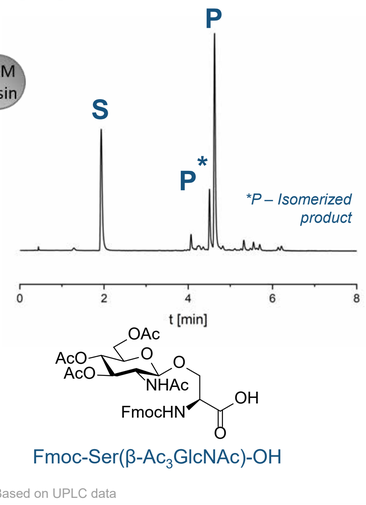
→ **Pin1 prefers pS_5** over pS_2

→ Pin1 has **lower affinity** for pS_5 near **C-terminus**

3. Optimization of Synthesis of phospho-glyco peptides

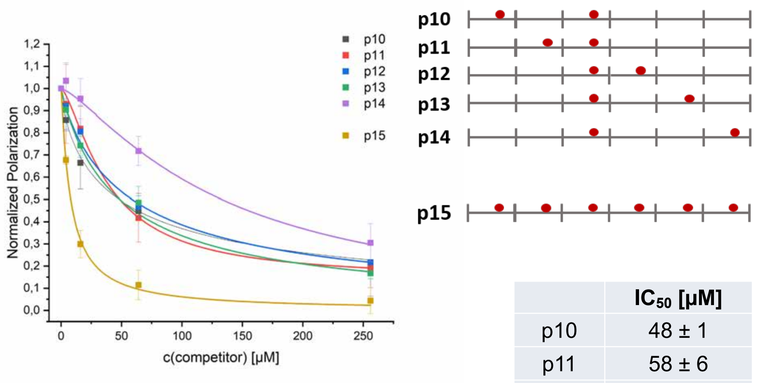


	Method I	Method II	Method III	Method IV	Method V
Fmoc-Ser(β-Ac_3GlcNAc)-OH	2,5 eq.	2,5 eq.	10 eq.	10 eq.	10 eq.
Activator System	HCTU/HOBt (2,5/2,5 eq.)	HATU/HOAt (2,5/2,5 eq.)	HATU/HOAt (10/10 eq.)	HATU/HOAt (10/10 eq.)	HATU/HOAt (10/10 eq.)
Base	DIEA (5 eq.)	DIEA (5 eq.)	NMM (10 eq.)	TMP (10 eq.)	TMP (10 eq.)
Time/Temp.	12 h/rt	12 h/rt	12 h/rt	12 h/rt	**1 h**/40 °C
Isomerization	18:82	10:90	7:93	7:93	7:93
Yield* [%]	63	55	85	95	**100**



*Based on UPLC data

2a. Spatial screening for homobivalency-enhanced binding (FP-Assay)

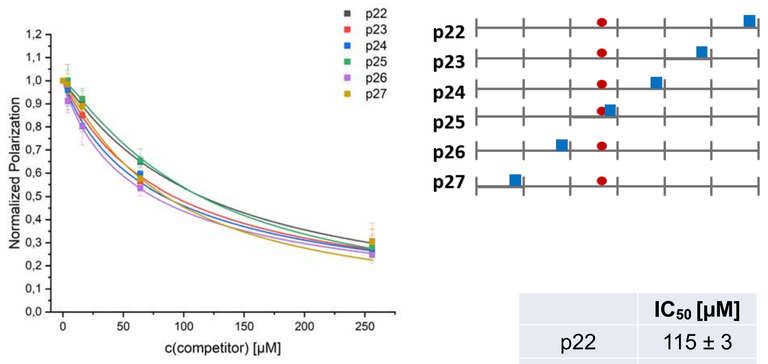


	IC_{50} [µM]
p10	48 ± 1
p11	58 ± 6
p12	59 ± 3
p13	49 ± 3
p14	137 ± 11
p15	8 ± 0,3

→ No bivalency-enhanced binding

→ No multivalency-enhanced binding of Pin1

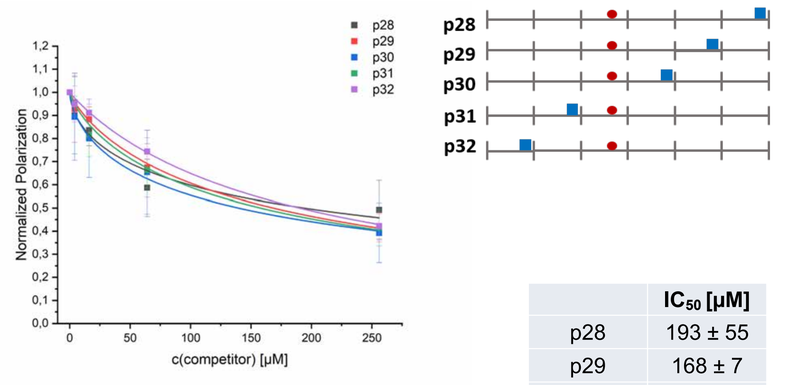
4a. GlcNAcylated (S_7) phospho-peptides (FP-Assay)



	IC_{50} [µM]
p22	115 ± 3
p23	113 ± 20
p24	**91 ± 8**
p25	**83 ± 5**
p26	**114 ± 5**
p27	74 ± 2

→ GlcNAcylation at S_7 does not influence recognition of pS_5 by Pin1

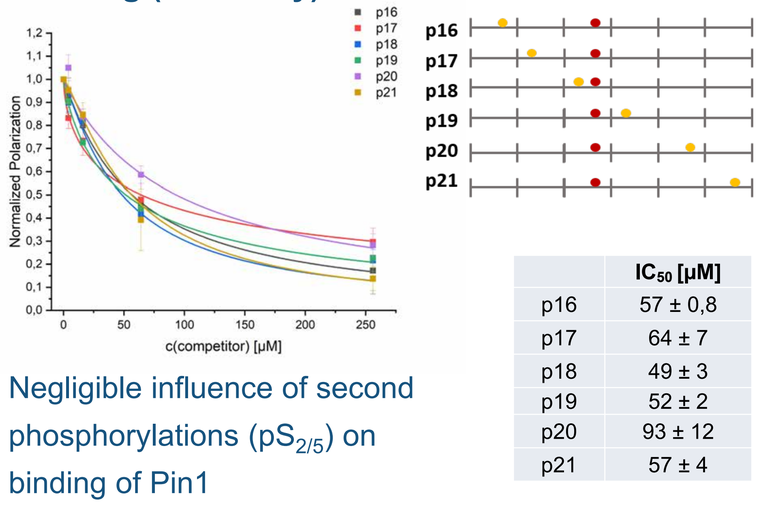
4b. GlcNAcylated (S_5) phospho-peptides (FP-Assay)



	IC_{50} [µM]
p28	193 ± 55
p29	168 ± 7
p30	**139 ± 10**
p31	**158 ± 16**
p32	190 ± 9

→ Minor but significant weakening of Pin1 binding due to GlcNAcylation at S_5

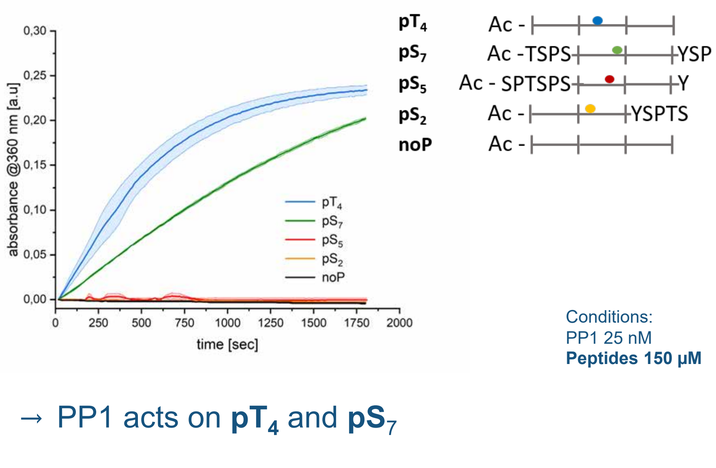
2b. Spatial screening for heterobivalency-enhanced binding (FP-Assay)



	IC_{50} [µM]
p16	57 ± 0,8
p17	64 ± 7
p18	49 ± 3
p19	52 ± 2
p20	93 ± 12
p21	57 ± 4

→ Negligible influence of second phosphorylations ($pS_{2/5}$) on binding of Pin1

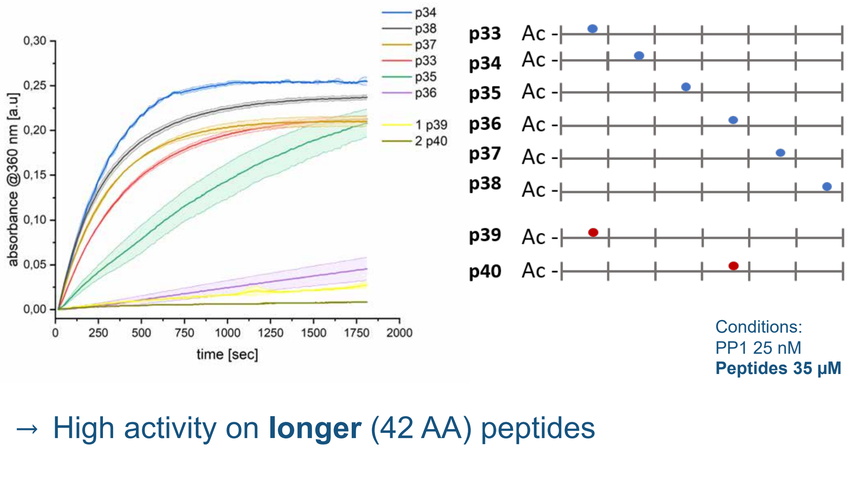
5a. Phosphatase (PP1) activity:



Conditions:
PP1 25 nM
Peptides 150 µM

→ PP1 acts on **pT_4** and **pS_7**

5b. Phosphatase (PP1) activity: positional scan



Conditions:
PP1 25 nM
Peptides 35 µM

→ High activity on **longer** (42 AA) peptides

CONCLUSION & OUTLOOK

The CTD modifier Pin1 prefers pS_5 over pS_2. Contrary to previous report[3], bivalency-enhanced binding was not observed. Current data suggests remarkable tolerance of Pin1 for *O*-GlcNAcylation at gS_7, though binding to gS_5 is slightly reduced. This modification may hinder future phosphorylation at this site. Future work will examine the effect of *O*-GlcNAcylation at T_4 (reported to induce turn-like structures[4]), at gS_7 in biphosphorylated (pS_5) peptides and the influence of multi-*O*-GlcNAcylation. Furthermore, the influence of a third phospho site (pS_2) on the recognition of Pin1 will be explored.
The phosphatase PP1 has a preference for removing phosphorylation at pT_4 and pS_7 residues and showed higher activity on longer peptides. For the phosphatase PP1 the effect of *O*-GlcNAcylation on the activity of CTD-like peptides with pT_4 residues is not known and needs also to be tested. Other phosphatases like Ssu7 that dephosphorylates pS_5 residues and interact together with Pin1 will be used to decipher which phosphorylation patterns are important for the recognition of Pin1.

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