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Cyclin D1/Cdk4 increases the transcriptional activity of FOXM1c without phosphorylating FOXM1c

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ABSTRACT

Anders et al. (2011) [11] reported that cyclinD1/Cdk4 and cyclinD3/Cdk6 enhance the transcriptional activity of FOXM1c by phosphorylating its TAD. They defined 12 Cdk consensus sites as essential for the activation of FOXM1c by cyclinD1/Cdk4 and cyclinD3/Cdk6 and stated that the 12 Cdk-sites are positioned within the TAD of FOXM1c. In contrast, this study demonstrates that all potential cyclin/Cdk phosphorylation sites S/T-P of FOXM1c are located outside its TAD so that the TAD of FOXM1c contains no potential cyclin/Cdk site, which excludes a phosphorylation of the FOXM1c-TAD by cyclinD1/Cdk4 and cyclinD3/Cdk6. This study shows that the activation of FOXM1c by cyclinD1/Cdk4 is lost without removal of any cyclin/Cdk site and gained without addition of any cyclin/Cdk site because it depends on a FOXM1c domain with no potential cyclin/Cdk site, namely on the interaction domain for the tumor suppressor RB, which binds to and represses FOXM1c. CyclinD1/Cdk4 activates FOXM1c because cyclinD1/Cdk4 releases FOXM1c from its repression by RB through removal of RB from FOXM1c. For this purpose, cyclinD1/Cdk4 phosphorylates only RB, but not FOXM1c, so that cyclinD1/Cdk4 increases the transcriptional activity of FOXM1c without phosphorylating FOXM1c and activates FOXM1c independently of cyclin/Cdk phosphorylation sites in FOXM1c. In summary, this study changes the model of Anders et al. (2011) [11] completely because it disproves their central conclusion that cyclinD1/Cdk4 and cyclinD3/Cdk6 enhance the transcriptional activity of FOXM1c by phosphorylating its TAD at the 12 Cdk-sites.

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1. Introduction

The activating transcription factor FOXM1c possesses a forkhead domain as DBD [1–5] and a very strong acidic TAD (Fig. S-1) [3]. However, wild-type FOXM1c is inactive because the TAD is inhibited by three IDs, namely by the NRD-N, the TRD and the NRD-C (Fig. S-1) [3–10].

The NRD-N inhibits the TAD directly because the direct binding of the NRD-N to the TAD blocks the TAD completely [3,4,6,9]. The TRD inhibits the TAD indirectly by actively transrepressing against the transactivation by the TAD [3,8]. The NRD-C inhibits the TAD indirectly through recruitment of the corepressor RB because the tumor suppressor RB binds directly to the NRD-C of FOXM1c and thereby represses indirectly the FOXM1c-TAD without interacting with the FOXM1c-TAD [6,9]. Since the TRD and the NRD-C coincide in the central domain of FOXM1c the central domain functions as a dual ID for the TAD (Fig. S-1).

Inactive wild-type FOXM1c can be converted into a very strong transactivator if the TAD is released from its inhibition by the three IDs through activating signals, which switch-off the IDs [6,7,9,10].

FOXM1c is strongly activated by cyclinD1/Cdk4 [6,7,9,11] and cyclinD3/Cdk6 [11].

Anders et al. [11] reported that cyclinD1/Cdk4 and cyclinD3/Cdk6 *in vitro* phosphorylate the same two FOXM1c fragments and they mapped the cyclinD3/Cdk6 phosphorylation sites in FOXM1c *in vitro* and *in vivo* (Fig. 1G). CyclinD1/Cdk4 and cyclinD3/Cdk6 stabilize the FOXM1c protein by preventing its ubiquitin-dependent proteasomal degradation [11]. Additionally, cyclinD1/Cdk4 [6,7,9,11] and cyclinD3/Cdk6 [11] increase the transcriptional activity of FOXM1c independently of an effect on its protein stability.

These two effects can easily be discrimated because cyclinD1/Cdk4 and cyclinD3/Cdk6 augment the stability of endogenous FOXM1c and exogenous FOXM1 expressed from a weak promoter whereas their effects on the stability of exogenous FOXM1c expressed from a very strong promoter are negligible [11]. In the present study, an effect of cyclinD1/Cdk4 on the FOXM1c protein stability is excluded because the exogenous FOXM1c analyzed

Abbreviations: aa, amino acid; Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; CMV, cytomegalovirus; DBD, DNA binding domain; dn, dominant-negative; FOXM1c, Forkhead box M1, splice variant c; GST, glutathione-*S*-transferase; HA, hemagglutinin; HSV, herpes simplex virus; ID, inhibitory domain; INK4A, inhibitor of Cdk4 A; p16, CDKN2A, Cdk inhibitor 2A; NLS, nuclear localization signal; NRD, negative-regulatory domain; RB, retinoblastoma protein, RB1, retinoblastoma 1; SV40, simian virus 40; TK, thymidine kinase; TAD, transactivation domain; TRD, transrepression domain.

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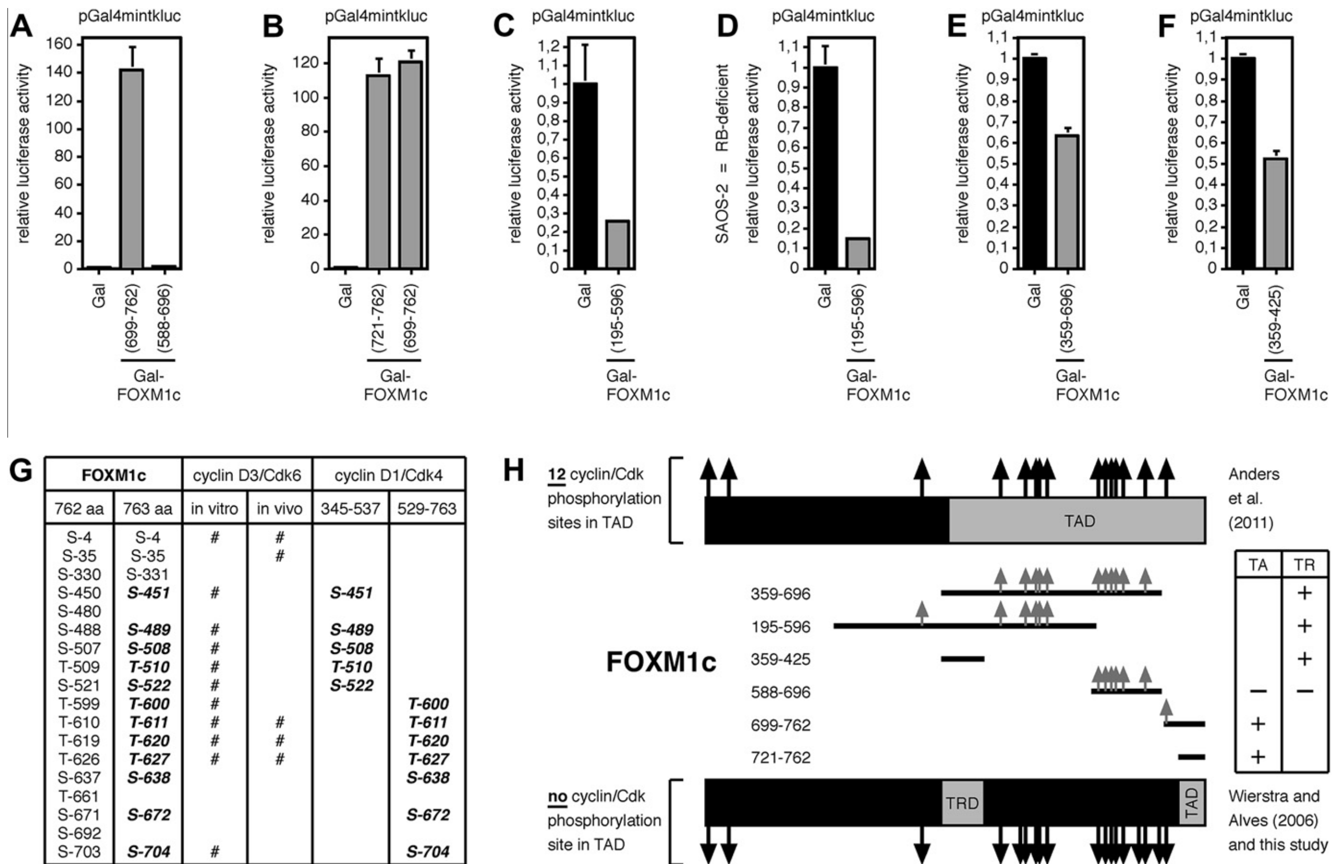

FOXM1c		cyclin D3/Cdk6		cyclin D1/Cdk4	
762 aa	763 aa	in vitro	in vivo	345-537	529-763
S-4	S-4	#	#		
S-35	S-35		#		
S-330	S-331				
S-450	***S-451***	#		***S-451***	
S-480					
S-488	***S-489***	#		***S-489***	
S-507	***S-508***	#		***S-508***	
T-509	***T-510***	#		***T-510***	
S-521	***S-522***	#		***S-522***	
T-599	***T-600***	#			***T-600***
T-610	***T-611***	#	#		***T-611***
T-619	***T-620***	#	#		***T-620***
T-626	***T-627***	#	#		***T-627***
S-637	***S-638***				***S-638***
T-661					
S-671	***S-672***				***S-672***
S-692					
S-703	***S-704***	#			***S-704***

Fig. 1. The TAD of FOXM1c lacks any potential cyclin/Cdk phosphorylation site S/T-P so that a phosphorylation of the FOXM1c-TAD by cyclinD1/Cdk4 is excluded. (A–F) Mapping of the TAD of FOXM1c. RK-13 cells (A–C, E, and F) or RB-deficient SAOS-2 cells (D) were transiently transfected with the reporter construct pGal4mintkluc (four GAL4-binding sites upstream of the minimal *TK* promoter of HSV) and with expression plasmids for Gal (yeast GAL4-DBD) or the indicated Gal-FOXM1c-fusion proteins (GAL4-DBD fused to FOXM1c fragment). The relative luciferase activity of pGal4mintkluc in the control Gal was set to 1. (G) CyclinD3, D1/Cdk6, 4 phosphorylation sites in FOXM1c. FOXM1c possesses 18 potential cyclin/Cdk phosphorylation sites S/T-P (first column), 15 of which are considered Cdk consensus sites in Anders et al. [11] (second column). Human FOXM1c was cloned with either 762 or 763 aa so that the aa numbering in this study (for 762 aa) differs from that in Anders et al. [11] (for 763 aa). Anders et al. [11] mapped 11 *in vitro* and five *in vivo* cyclinD3/Cdk6 phosphorylation sites (#) in FOXM1c (third and fourth column). CyclinD1/Cdk4 *in vitro* phosphorylated the same two FOXM1c fragments as cyclinD3/Cdk6, namely aa 345–537 and aa 529–763, which included the Cdk consensus sites listed (fifth and sixth column) [11]. Those 12 Cdk consensus sites, which Anders et al. [11] defined as essential for the activation of FOXM1c by cyclinD1/Cdk4 and cyclinD3/Cdk6, are marked (bold and italics). (H) All potential cyclin/Cdk phosphorylation sites S/T-P of FOXM1c are located outside the TAD. FOXM1c is depicted as a black rectangle and its functional domains as grey boxes. The 18 potential cyclin/Cdk phosphorylation sites S/T-P in FOXM1c are shown as arrows. Gal-FOXM1c-fusion proteins are depicted as thick black lines, but their GAL4-DBD is not shown. It is indicated whether the Gal-FOXM1c-fusion proteins transactivate (TA = +) or transrepress (TR = +) or do neither of the two (TA = – and TR = –).

was expressed from the very strong CMV and SV40 early promoters. Additionally, the expression of all FOXM1c proteins analyzed was scrutinized and compared previously (Fig. S-2; data not shown) [3,8,9].

Anders et al. [11] state that cyclinD1/Cdk4 and cyclinD3/Cdk6 enhance the transcriptional activity of FOXM1c by phosphorylating its TAD. They define 12 Cdk consensus sites as essential for the activation of FOXM1c by cyclinD1/Cdk4 and cyclinD3/Cdk6 (Fig. 1G) [11], which are called the 12 Cdk-sites from here onwards. Anders et al. [11] claim that the 12 Cdk-sites are positioned within the TAD of FOXM1c (Fig. 1H, top panel) and that cyclinD1/Cdk4 and cyclinD3/Cdk6 activate FOXM1c through phosphorylation of its TAD at the 12 Cdk-sites.

In total, FOXM1c possesses 18 potential cyclin/Cdk phosphorylation sites S/T-P (Fig. 1G), which are named potential cyclin/Cdk sites in the following. They include the 12 Cdk-sites of Anders et al. [11] (Fig. 1G).

In contrast to Anders et al., Wierstra and Alves [3,6] showed that all 18 potential cyclin/Cdk phosphorylation sites S/T-P of FOXM1c are located outside the TAD so that the TAD of FOXM1c lacks any potential cyclin/Cdk site (Fig. 1H, bottom panel), which excludes a phosphorylation of the FOXM1c-TAD by cyclinD1/Cdk4 and cyclinD3/Cdk6. Since the FOXM1c-TAD contains no potential cyclin/Cdk site (Fig. 1H, bottom panel) [3,6] cyclinD1/Cdk4 and cyclinD3/Cdk6 must activate FOXM1c without a phosphorylation of its TAD. In fact, Wierstra and Alves [6,7,9] demonstrated how cyclinD1/Cdk4 increases the transactivation potential of FOXM1c not only without phosphorylating the FOXM1c-TAD, but even without phosphorylating FOXM1c at all.

Since the results of Wierstra and Alves [3,6,7,9] contradict the statements of Anders et al., the aim of the present study was to perform deciding experiments in order to settle how cyclinD1/Cdk4 increases the transcriptional activity of FOXM1c. The results obtained disprove the statements of Anders et al., but confirm and extend the findings of Wierstra and Alves [3,6,7,9]. In particular, the present study changes the model of Anders et al. completely because it disproves their central conclusion that cyclinD1/Cdk4 enhances the transcriptional activity of FOXM1c by phosphorylating its TAD.

2. Materials and methods

2.1. Plasmids

p(MBS)$_3$-mintk-luc [12], pFOXM1c(189–762) [13], pFOXM1c-(189–425; 568–762), pFOXM1c(189–348; 573–762)NLS,

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