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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.

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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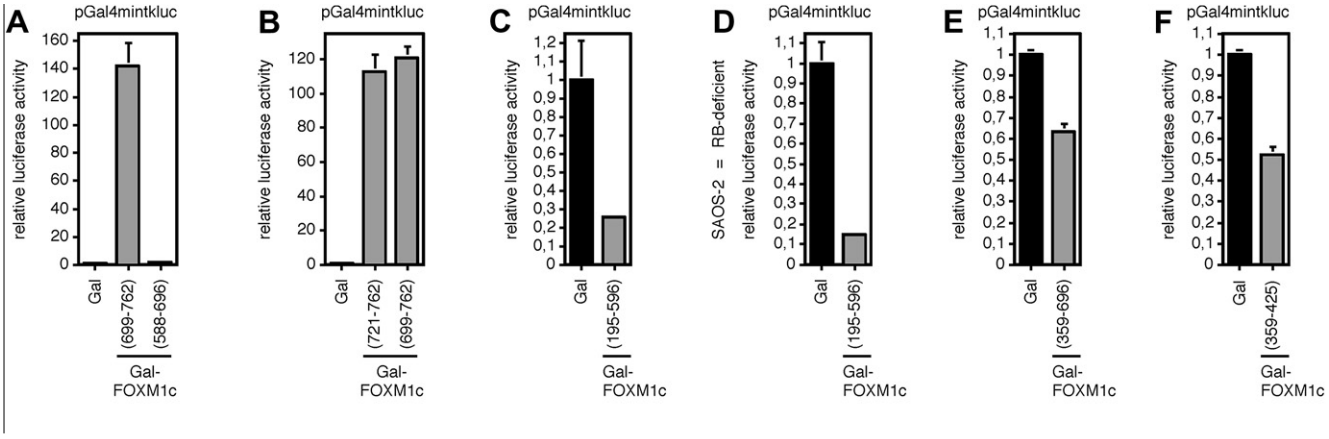

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FOXM1c		cyclin D3/Cdk6		cyclin D1/Cdk4	
762 aa	763 aa	in vitro	in vivo	345-537	529-763
S-4	S-4	#	#		
S-35	S-35		#		
S-330	S-331				
S-450	***S-451***	#		***S-451***	
S-480					
S-488	***S-489***	#		***S-489***	
S-507	***S-508***	#		***S-508***	
T-509	***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***

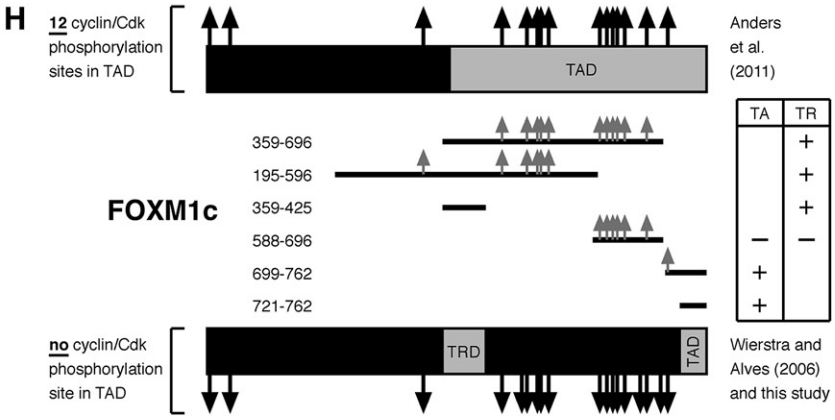

Fig. 1. The TAD of FOXM1c lacks any potential cyclin/Cdk phosphorylation site S/T-P so that a phosphorylation of the FOXM1c-TAD by cyclinD1/Cdk4 is excluded. (A–F) Mapping of the TAD of FOXM1c. RK-13 cells (A–C, E, and F) or RB-deficient SAOS-2 cells (D) were transiently transfected with the reporter 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,

pFOXM1c(189–377; 424–762), pFOXM1c(1–232), pGal-FOXM1c-(195–596), pGal-FOXM1c(359–762), pGal-FOXM1c(359–425), pGal-FOXM1c(359–425; 568–762), pGal-FOXM1c(721–762), pGal-FOXM1c(699–762), pGal-FOXM1c(588–762), pGal-FOXM1c-(588–696), pGST-FOXM1c(1–477) [3], pGST-FOXM1c(359–565) [4], pGal-FOXM1c(359–425; 568–621; 671–762) [9] and pGal-FOXM1c(588–621; 671–762) [8] were described previously.

Existing restriction enzyme sites in pGal-FOXM1c(359–762) were used to create the deletion mutant pGal-FOXM1c(359–696). The protein expression of Gal-FOXM1c(359–696) was verified in Fig. S-2B.

pGal0 (C. Dang) and pGal4mintkluc (R. Janknecht, A. Nordheim) as well as the expression plasmids for HA-RB(379–928) (W.G. Kaelin), p16 (J. Koh), cyclinD1, Cdk4, dnCdk4 and dnCdk2 (R. Bernards) were generous gifts. pGEX-3X was from Pharmacia.

2.2. Antibodies

α-FOXM1c(C-20) (sc-502), α-RB(C-15) (sc-50) and α-GAL4-DBD(RK5C1) (sc-510) were purchased from Santa Cruz.

2.3. Cell culture, transient transfections, luciferase reporter gene assays and total cell lysates

Cell culture, transient transfections, luciferase reporter gene assays and total cell lysates were performed as described before [6].

2.4. GST-fusion proteins, GST-pull-down assays and Western blotting

Preparation of GST-fusion proteins, GST-pull-down assays and Western blotting were carried out as described previously [6].

3. Results and discussion

3.1. 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 and cyclinD3/Cdk6 is excluded

Anders et al. state that cyclinD1/Cdk4 and cyclinD3/Cdk6 enhance the transcriptional activity of FOXM1c by phosphorylating its TAD at the 12 Cdk-sites. Accordingly, they show three FOXM1c maps with the 12 Cdk-sites inside the TAD, which reaches from approx. aa 370 until the last aa 763 (Fig. 1H, top panel). However, they did not map the FOXM1c-TAD experimentally.

The mapping of the TAD requires the analysis of Gal-FOXM1c-fusion proteins, which bind to GAL4-binding sites in the reporter construct with their heterologous yeast GAL4-DBD independently of the forkhead-DBD of FOXM1c so that they represent only the transactivation (or transrepression) potential of FOXM1c, but not its DNA-binding activity.

The analysis of Gal-FOXM1c-fusion proteins (Fig. 1H) allowed the precise mapping of the TAD (aa 721–762) (Fig. 1B), the TRD (aa 359–425) (Fig. 1F) and a segment without any transactivation or transrepression potential (aa 588–696) (Fig. 1A). Gal-FOXM1c(721–762) comprises the complete TAD (Fig. 1H) because it transactivated as strongly as Gal-FOXM1c(699–762) (Fig. 1B) although Gal-FOXM1c(699–762) was by far better expressed than Gal-FOXM1c(721–762) (Fig. S-2A). The transrepression by Gal-FOXM1c(195–596) in both RK-13 cells (Fig. 1C) and RB-deficient SAOS-2 cells (Fig. 1D) revealed that the TRD functions independently of RB.

Consequently, all 18 potential cyclin/Cdk phosphorylation sites S/T-P of FOXM1c are located outside the TAD so that the TAD of FOXM1c possesses no potential cyclin/Cdk site (Fig. 1H, bottom panel), which excludes a phosphorylation of the FOXM1c-TAD by cyclinD1/Cdk4 and cyclinD3/Cdk6. This result disproves the statements of Anders et al. that cyclinD1/Cdk4 and cyclinD3/Cdk6 phosphorylate FOXM1c in its TAD and that the FOXM1c-TAD contains the 12 Cdk-sites (Fig. 1H, top panel).

In reality, the 12 Cdk-sites of Anders et al. are positioned outside the true FOXM1c-TAD (aa 721–762) (Fig. 1H, bottom panel), but Anders et al. sketched them within their wrong TAD because they enlarged the TAD arbitrarily up to approx. aa 370 (Fig. 1H, top panel) so that it included the TRD and the segment without any transactivation potential (Fig. 1H), which both are definitely no TAD (Fig. 1A and C–F). Actually, 11 of the 12 Cdk-sites are part of transrepressing FOXM1c fragments (aa 359–696 and aa 195–596) (Fig. 1C–E and H).

In summary, the TAD of FOXM1c lacks any potential cyclin/Cdk site (Fig. 1H, bottom panel) so that it cannot be phosphorylated by cyclinD1/Cdk4 and cyclinD3/Cdk6. This finding changes the model of Anders et al. completely because it disproves their central conclusion that cyclinD1/Cdk4 and cyclinD3/Cdk6 enhance the transcriptional activity of FOXM1c by phosphorylating its TAD.

3.2. CyclinD1/Cdk4 does not directly target the TAD of FOXM1c, but increases the transactivation by the FOXM1c-TAD only indirectly

FOXM1c proteins bind with their forkhead-DBD to FOXM1c-binding sites in the reporter construct so that they represent both the transactivation potential and the DNA-binding activity of FOXM1c.

Since cyclinD1/Cdk4 had no effect on Gal-FOXM1c(721–762) and Gal-FOXM1c(588–762) it does not directly affect the TAD of FOXM1c (Fig. 2D and E). Nonetheless, cyclinD1/Cdk4 enhanced the transactivation potential of Gal-FOXM1c(359–762) so that it ultimately increases the transactivation by the FOXM1c-TAD, but only indirectly, namely dependently on the central domain (aa 359–587) (Fig. 2D and E).

In contrast, cyclinD1/Cdk4 does not affect the DNA-binding by the FOXM1c-DBD, neither directly nor indirectly [7]. Accordingly, cyclinD1/Cdk4 had no effect on FOXM1c(189–348; 573–762)NLS, but enhanced the transcriptional activity of FOXM1c(189–762), verifying that cyclinD1/Cdk4 does not directly affect the TAD or the DBD of FOXM1c and that it indirectly increases the transactivation by the FOXM1c-TAD, namely dependently on the central domain (aa 349–572) (Fig. 2C and E).

Hence, cyclinD1/Cdk4 does not target the TAD of FOXM1c directly. This finding disproves the statement of Anders et al. that cyclinD1/Cdk4 enhances the transcriptional activity of FOXM1c by phosphorylating its TAD.

3.3. CyclinD1/Cdk4 does not affect the transrepression by the TRD of FOXM1c

The central domain of FOXM1c functions as RB-independent TRD (Fig. 1C, D, F and H) so that cyclinD1/Cdk4 could increase the transactivation by the FOXM1c-TAD indirectly and dependently on the central domain by reducing the transrepression by the TRD (Fig. 2A). However, cyclinD1/Cdk4 does not reduce the transrepression by the FOXM1c-TRD because the transrepression by Gal-FOXM1c(195–596) was unaffected by cyclinD1/Cdk4 (Fig. 2A and B). Thus, cyclinD1/Cdk4 does not target the TRD of FOXM1c.

3.4. The activation of FOXM1c by cyclinD1/Cdk4 is independent of cyclin/Cdk phosphorylation sites in FOXM1c

Irrespective of the position of the 12 Cdk-sites within FOXM1c, cyclinD1/Cdk4 may enhance the transcriptional activity of FOXM1c by phosphorylating the 12 Cdk-sites so that the 12 Cdk-sites may

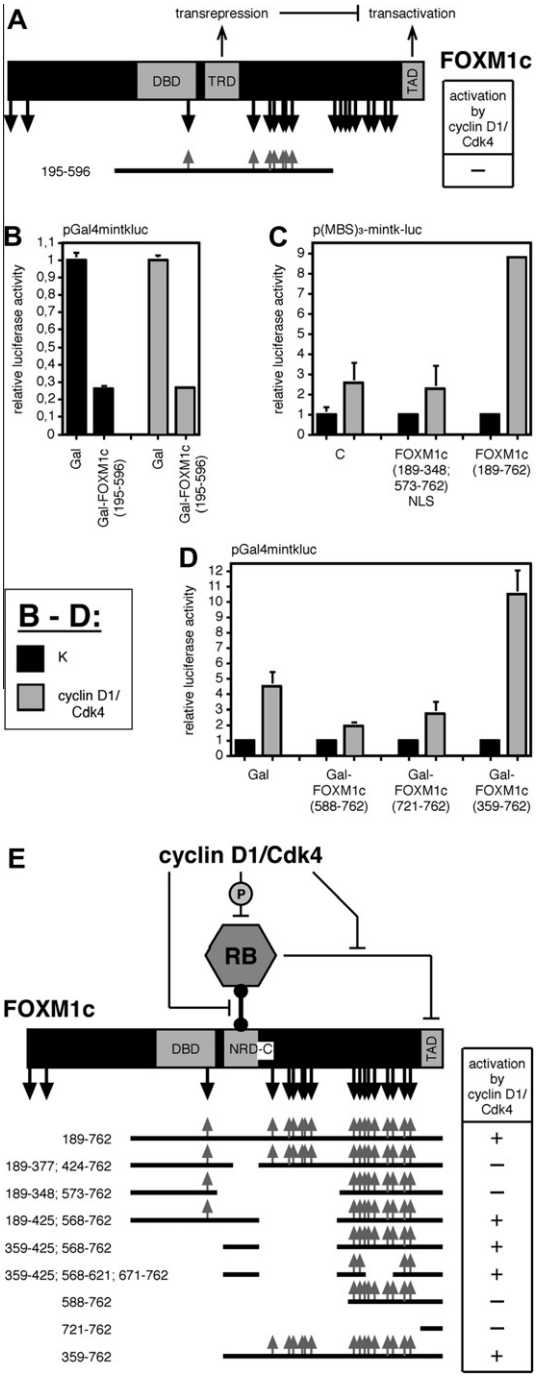

Fig. 2. (A and B) CyclinD1/Cdk4 does not affect the transrepression by the TRD of FOXM1c. (C–E) CyclinD1/Cdk4 does not directly target the TAD of FOXM1c, but increases the transactivation by the FOXM1c-TAD only indirectly. (A and E) For a description see legend to Fig. 1H. It is indicated whether the FOXM1c proteins are activated by cyclinD1/Cdk4 (+) or not (−). (E) The thick dot-ended line marks the direct interaction between RB and the NRD-C of FOXM1c. The encircled P indicates the phosphorylation of RB by cyclinD1/Cdk4. The fold transactivation by each FOXM1c protein in the absence of exogenous cyclinD1/Cdk4 is listed in Fig. S-5. (B and D) RK-13 cells were transiently transfected with pGal4mintkluc and with expression plasmids for Gal or the indicated Gal-FOXM1c-fusion proteins. (C) RK-13 cells were transiently transfected with the reporter construct $p(MBS)_3$-mintk-luc (three FOXM1c-binding sites upstream of the minimal *TK* promoter of HSV) and with pFOXM1c(189–762), pFOXM1c(189–348; 573–762)NLS or the empty vector (control C). (B–D) Either expression plasmids for cyclinD1 and Cdk4 or the empty vector (control K) were cotransfected. (B) The relative luciferase activity of pGal4mintkluc in the control Gal was set to 1. (C) The relative luciferase activity of $p(MBS)_3$-mintk-luc in the control K was set to 1. (D) The relative luciferase activity of pGal4mintkluc in the control K was set to 1.

mediate the activation of FOXM1c by cyclinD1/Cdk4. Therefore, the importance of the 12 Cdk-sites of Anders et al. for the activation of FOXM1c by cyclinD1/Cdk4 was tested.

Surprisingly, in contrast to FOXM1c(189–762), FOXM1c(189–377; 424–762) was not activated by cyclinD1/Cdk4 although FOXM1c(189–377; 424–762) retained all sixteen potential cyclin/Cdk sites of FOXM1c(189–762) and the 12 Cdk-sites (Figs. 2C and E and 3A and B). This astonishing failure of cyclinD1/Cdk4 to activate FOXM1c(189–377; 424–762) (Fig. 3A and B) demonstrates that the 12 Cdk-sites do not mediate the activation of FOXM1c by cyclinD1/Cdk4 (Fig. 2E).

Accordingly, FOXM1c(189–348; 573–762)NLS and Gal-FOXM1c(588–762) were not activated by cyclinD1/Cdk4 despite their possession of seven of the 12 Cdk-sites (Figs. 2C–E and 3B, and C), confirming that these seven sites do not mediate the activation of FOXM1c by cyclinD1/Cdk4.

Vice versa, cyclinD1/Cdk4 still activated Gal-FOXM1c(359–425; 568–621; 671–762) (Fig. 3C) although it lacked 11 (or nine) potential cyclin/Cdk sites and nine (or eight) of the 12 Cdk-sites (Fig. 2E). Therefore, these missing nine (or eight) of the 12 Cdk-sites do not mediate the activation of FOXM1c by cyclinD1/Cdk4.

Consistently, FOXM1c(189–425; 568–762) and Gal-FOXM1c(359–425; 568–762), which lacked five of the 12 Cdk-sites, were still activated by cyclinD1/Cdk4 (Figs. 2E and 3A–C), verifying that these missing five sites do not mediate the activation of FOXM1c by cyclinD1/Cdk4.

The deletion of aa 622–670 removes five or three potential cyclin/Cdk sites: First, it deletes the three sites S-626, S-637 and T-661. Second, although T_{619}-P and S_{671}-P are barely retained this deletion may eliminate the two sites T-619 and S-671 because it changes their flanking sequences, which are important for substrate recognition by cyclinD1/Cdk4 [14].

In summary, the 12 Cdk-sites of Anders et al. do not mediate the activation of FOXM1c by cyclinD1/Cdk4 because cyclinD1/Cdk4 failed to activate FOXM1c(189–377; 424–762) (Fig. 3A and B), which contained the 12 Cdk-sites (Fig. 2E). Since FOXM1c(189–377; 424–762) possessed all the potential cyclin/Cdk sites of FOXM1c(189–762), which was strongly activated by cyclinD1/Cdk4, the failure of cyclinD1/Cdk4 to activate FOXM1c(189–377; 424–762) demonstrates that cyclinD1/Cdk4 activates FOXM1c independently of cyclin/Cdk phosphorylation sites in FOXM1c (Figs. 2C and E and 3A and B). These findings change the model of Anders et al. completely because they disprove their central conclusion that cyclinD1/Cdk4 enhances the transcriptional activity of FOXM1c by phosphorylating FOXM1c at the 12 Cdk-sites.

These results for the activation of FOXM1c by cyclinD1/Cdk4 were confirmed by corresponding findings for the repression of FOXM1c by dnCdk4 (Figs. S-3A and B and S-4A and B). Moreover, FOXM1c was repressed by the CKI $p16^{INK4A}$ (Figs. S-3C and S-4C), an inhibitor of cyclinD1/Cdk4 [14].

3.5. The activation of FOXM1c by cyclinD1/Cdk4 is no direct effect on FOXM1c and is not caused by the phosphorylation of FOXM1c, but instead depends on RB and is mediated by the phosphorylation of RB

CyclinD1/Cdk4 strongly activated Gal-FOXM1c(359–762) and FOXM1c(189–762) in RK-13 cells whereas both FOXM1c proteins were unaffected by cyclinD1/Cdk4 in RB-deficient SAOS-2 cells (Fig. 3D–G). This failure of cyclinD1/Cdk4 to activate FOXM1c in RB-deficient SAOS-2 cells demonstrates that the activation of FOXM1c by cyclinD1/Cdk4 is no direct effect on FOXM1c and is not caused by the phosphorylation of FOXM1c, but instead depends on RB and is mediated by the phosphorylation of RB.

Hence, the inability of cyclinD1/Cdk4 to activate FOXM1c in SAOS-2 cells (Fig. 3D and F) reveals that cyclinD1/Cdk4 increases

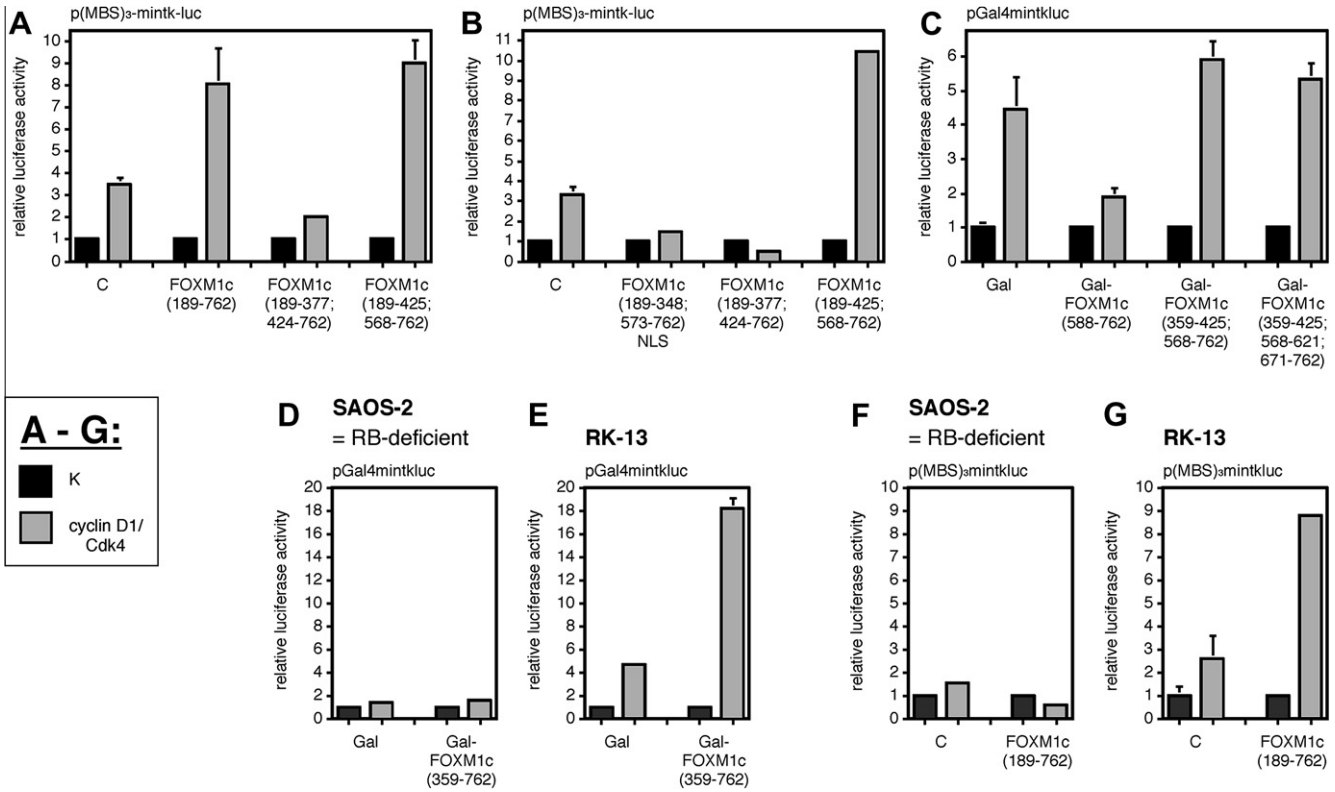

Fig. 3. (A–C) The activation of FOXM1c by cyclinD1/Cdk4 is independent of cyclin/Cdk phosphorylation sites in FOXM1c, but instead depends on a FOXM1c domain without any cyclin/Cdk phosphorylation site, namely on the NRD-C. (D–G) The activation of FOXM1c by cyclinD1/Cdk4 is no direct effect on FOXM1c and is not caused by the phosphorylation of FOXM1c, but instead depends on RB and is mediated by the phosphorylation of RB. (A, B, F, and G) RK-13 cells (A, B, and G) or RB-deficient SAOS-2 cells (F) were transiently transfected with $p(MBS)_3$-mintk-luc and with expression plasmids for the indicated FOXM1c proteins or the empty vector (control C). (C–E) RK-13 cells (C, and E) or RB-deficient SAOS-2 cells (D) were transiently transfected with pGal4mintkluc and with expression plasmids for Gal or the indicated Gal-FOXM1c-fusion proteins. (A–G) Either expression plasmids for cyclinD1 and Cdk4 or the empty vector (control K) were cotransfected. (A, B, F, and G) The relative luciferase activity of $p(MBS)_3$-mintk-luc in the control K was set to 1. (C–E) The relative luciferase activity of pGal4mintkluc in the control K was set to 1.

the transactivation potential of FOXM1c without phosphorylating FOXM1c. This finding 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 FOXM1c at the 12 Cdk-sites.

Since Gal-FOXM1c(359–762) and FOXM1c(189–762) possess the 12 Cdk-sites (Fig. 2E) they would be activated by cyclinD1/Cdk4 in SAOS-2 cells if cyclinD1/Cdk4 would increase the transcriptional activity of FOXM1c through its phosphorylation at the 12 Cdk-sites. Consequently, the failure of cyclinD1/Cdk4 to activate these two FOXM1c proteins in SAOS-2 cells (Fig. 3D and F) disproves the statement of Anders et al. that cyclinD1/Cdk4 enhances the transcriptional activity of FOXM1c through phosphorylation of FOXM1c at the 12 Cdk-sites.

3.6. The activation of FOXM1c by cyclinD1/Cdk4 depends on a FOXM1c domain without any cyclin/Cdk phosphorylation site, namely on the NRD-C

Surprisingly, since cyclinD1/Cdk4 activated FOXM1c(189–762), but not FOXM1c(189–377; 424–762), the activation by cyclinD1/Cdk4 was lost through removal of the segment aa 378–423, which contains no potential cyclin/Cdk site (Figs. 2C and E and 3A and B).

Conversely, since cyclinD1/Cdk4 activated FOXM1c(189–425; 568–762), but not FOXM1c(189–348; 573–762)NLS, the activation by cyclinD1/Cdk4 was gained through addition of the segment aa 349–425, which lacks any potential cyclin/Cdk (Figs. 2C, E and 3A, B). Accordingly, cyclinD1/Cdk4 activated Gal-FOXM1c(359–425; 568–762) and Gal-FOXM1c(359–425; 568–621; 671–762), but not Gal-FOXM1c(588–762), verifying that the activation by cyclinD1/Cdk4 was acquired through addition of the segment aa 359–425 with no potential cyclin/Cdk site (Figs. 2D, E and 3C).

Hence, astonishingly, the activation by cyclinD1/Cdk4 depends on a FOXM1c domain without any potential cyclin/Cdk site, namely on the NRD-C (aa 359–425) (Fig. 2E).

3.7. RB binds to the NRD-C of FOXM1c

The NRD-C is the interaction domain for RB because RB bound to aa 359–477 of FOXM1c as evidenced by the interactions of HA-RB(379–928) with GST-FOXM1c(1–477) and GST-FOXM1c(359–565) in pull-down assays (Fig. 4A). Thus, the activation of FOXM1c by cyclinD1/Cdk4 depends on FOXM1c's interaction domain for RB (Fig. 2E).

Additionally, the activation of FOXM1c by cyclinD1/Cdk4 requires RB because it was lost in RB-deficient SAOS-2 cells (Fig. 3D and F). Consequently, the activation of FOXM1c by cyclinD1/Cdk4 relies on the binding of RB to the NRD-C of FOXM1c (Fig. 2E).

3.8. CyclinD1/Cdk4 releases FOXM1c from its repression by RB and exogenous RB restores the activating effect of cyclinD1/Cdk4 on FOXM1c in SAOS-2 cells

HA-RB(379–928), which was bound to the NRD-C (Fig. 4A), repressed Gal-FOXM1c(359–762) and cyclinD1/Cdk4 relieved this inhibition of Gal-FOXM1c(359–762) by HA-RB(379–928) (Fig. 4B, panel 2). Thus, cyclinD1/Cdk4 activates FOXM1c because

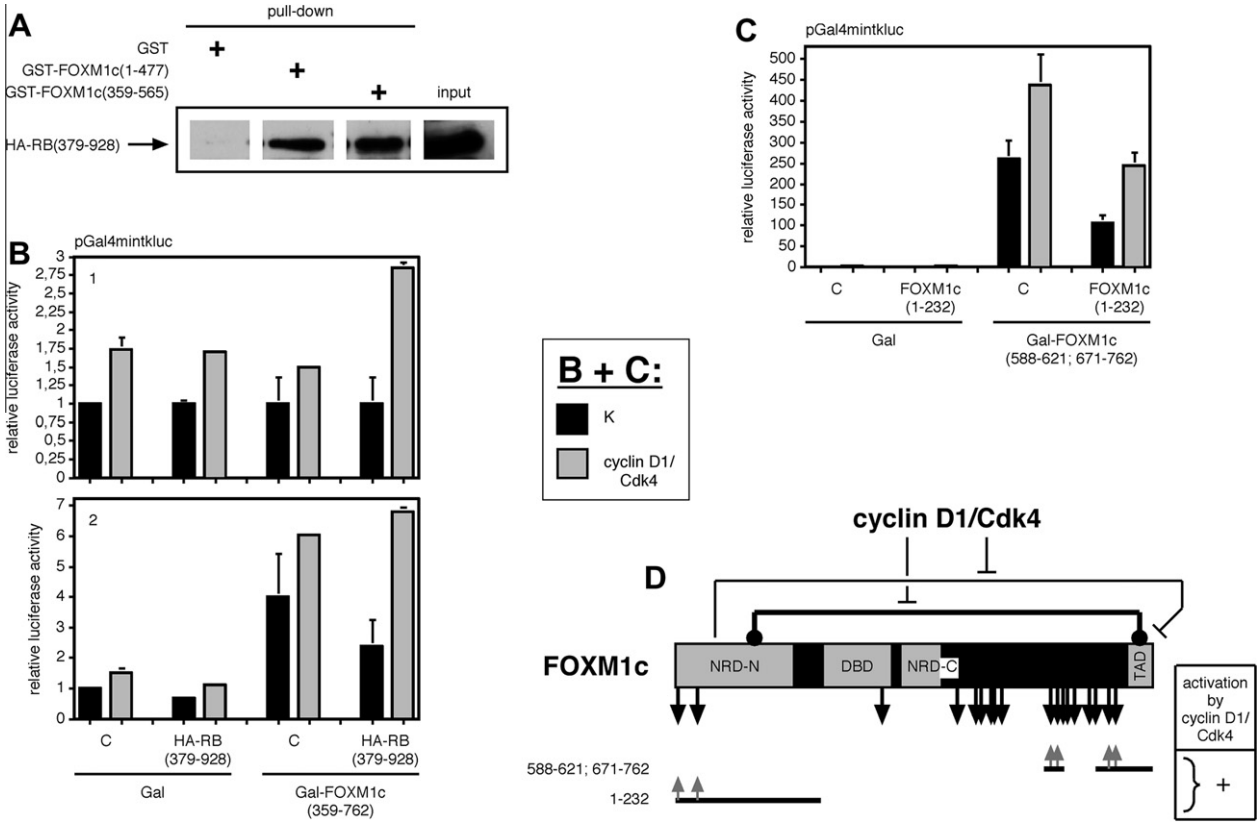

Fig. 4. (A) RB binds to the NRD-C of FOXM1c. GST-pull-down assays were performed with purified GST, GST-FOXM1c(1–477) or GST-FOXM1c(359–565) and total cell lysates of RK-13 cells transiently transfected with expression plasmids for HA-RB(379–928), dnCdk4 and dnCdk2. Bound HA-RB(379–928) was detected in a Western blot using α-RB(C-15) as primary antibody. (B) CyclinD1/Cdk4 releases FOXM1c from its repression by RB and exogenous RB restores the activating effect of cyclinD1/Cdk4 on FOXM1c in SAOS-2 cells. (C and D) Most cyclinD3,D1/Cdk6,4 phosphorylation sites in FOXM1c are dispensable for the cyclinD1/Cdk4-mediated release of the FOXM1c-TAD from its inhibition by the NRD-N. (B and C) RK-13 cells were transiently transfected with pGal4mintkluc and with expression plasmids for Gal or the indicated Gal-FOXM1c-fusion proteins. Either expression plasmids for cyclinD1 and Cdk4 or the empty vector (control K) were cotransfected. (B) Either the expression plasmid for HA-RB(379–928) or the empty vector (control C) were cotransfected. In panel 1, the relative luciferase activity of pGal4mintkluc in the control K was set to 1. In panel 2, the relative luciferase activity of pGal4mintkluc in the combination of control Gal with control K with control C was set to 1. (C) Either pFOXM1c(1–232) or the empty vector (control C) were cotransfected. The relative luciferase activity of pGal4mintkluc in the combination of control Gal with control K with control C was set to 1. (D) For a description see legend to Fig. 1H. The thick dot-ended line marks the interaction between the TAD and the NRD-N of FOXM1c.

cyclinD1/Cdk4 releases FOXM1c from its repression by RB (Fig. 4B). Accordingly, exogenous HA-RB(379–928) restored the activating effect of cyclinD1/Cdk4 on Gal-FOXM1c(359–762) in RB-deficient SAOS-2 cells, where Gal-FOXM1c(359–762) was not activated by cyclinD1/Cdk4 in the absence of exogenous RB (Fig. 4B, panel 1). Therefore, the activation of FOXM1c by cyclinD1/Cdk4 is actually the cyclinD1/Cdk4-mediated release of FOXM1c from its inhibition by RB, but no direct effect of cyclinD1/Cdk4 on FOXM1c (Fig. 4B).

CyclinD1/Cdk4 releases FOXM1c from its repression by RB because cyclinD1/Cdk4 removes RB from the NRD-C by phosphorylating only RB, but not FOXM1c (Fig. 2E) [6]. This mechanism explains why cyclinD1/Cdk4 increases the transactivation potential of FOXM1c without phosphorylating FOXM1c and why cyclinD1/Cdk4 activates FOXM1c independently of cyclin/Cdk phosphorylation sites in FOXM1c.

In summary, the surprising observation that the activation of FOXM1c by cyclinD1/Cdk4 is lost without removal of any cyclin/Cdk phosphorylation site and gained without addition of any cyclin/Cdk phosphorylation site (Fig. 2E) is explained by the finding that the activation of FOXM1c by cyclinD1/Cdk4 is not caused by the phosphorylation of FOXM1c, but instead by the phosphorylation of RB [6]. These results change the model of Anders et al. completely because they disprove their central conclusion that cyclinD1/Cdk4 enhances the transcriptional activity of FOXM1c by phosphorylating FOXM1c at the 12 Cdk-sites.

3.9. Most cyclinD3,D1/Cdk6,4 phosphorylation sites in FOXM1c are dispensable for the cyclinD1/Cdk4-mediated release of the FOXM1c-TAD from its inhibition by the NRD-N

In addition to the RB-dependent and NRD-C-dependent effect, cyclinD1/Cdk4 has a second RB-independent and NRD-N-dependent effect on FOXM1c [6,7,9]: The NRD-N inhibits the TAD completely by directly binding to the TAD (Fig. S-1) [3,4,6,9]. CyclinD1/Cdk4 activates FOXM1c because cyclinD1/Cdk4 releases the FOXM1c-TAD from its inhibition by the NRD-N through disruption of the interaction between the NRD-N and the TAD (Fig. 4D) [6]. For this purpose, cyclinD1/Cdk4 may phosphorylate the NRD-N, but not the FOXM1c-TAD, because the NRD-N possesses two potential cyclin/Cdk sites whereas the FOXM1c-TAD contains no potential cyclin/Cdk site (Fig. 4D) [6].

Because of the direct interaction between the NRD-N and the TAD, the NRD-N inhibits the TAD not only within one FOXM1c molecule, but also if the NRD-N and the FOXM1c-TAD are distributed over two separate proteins so that the NRD-N *in trans* (FOXM1c(1–232)) repressed the transactivation by the FOXM1c-TAD *in cis* (Gal-FOXM1c(588–621; 671–762)) (Fig. 4C). CyclinD1/Cdk4 abrogated this inhibition of the FOXM1c-TAD *in cis* by the NRD-N *in trans*, demonstrating that cyclinD1/Cdk4 disrupted the interaction between FOXM1c(1–232) and Gal-FOXM1c(588–621; 671–762) (Fig. 4C and D).

Gal-FOXM1c(588–621; 671–762) contains only four (or six) potential cyclin/Cdk sites and only three (or five) of the 12 Cdk-sites (Fig. 4D). Consequently, the missing nine (or seven) of the 12 Cdk-sites are dispensable for the cyclinD1/Cdk4-mediated release of the FOXM1c-TAD from its inhibition by the NRD-N and for the cyclinD1/Cdk4-mediated disruption of the interaction between the NRD-N and the TAD (Fig. 4C and D). This dispensability of the vast majority of the 12 Cdk-sites for the NRD-N-dependent activation of FOXM1c by cyclinD1/Cdk4 contradicts the statement of Anders et al. that the 12 Cdk-sites mediate the activation of FOXM1c by cyclinD1/Cdk4.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.037.

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