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FOXM1c is activated by cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1, but repressed by GSK-3α ☆

Inken Wierstra [a,*], Jürgen Alves [b,1]

[a] Institute of Molecular Biology, Medical School Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
[b] Institute of Biophysical Chemistry, Medical School Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

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Abstract

Two different inhibitory domains, N-terminus and central domain, keep FOXM1c almost inactive despite its strong transactivation domain. Here, we demonstrate that cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1 activate FOXM1c. Cyclin E/Cdk2 does not target its transactivation domain or its DNA-binding domain. Instead, its activating effect strictly depends on the presence of either the central domain or the N-terminus of FOXM1c and thus is completely lost if both inhibitory domains are deleted. Cyclin E/Cdk2 activates FOXM1c by releasing its transactivation domain from the repression by these two inhibitory domains. However, it does not directly increase the transactivation potential of the TAD. The DNA-binding is not affected by cyclin E/Cdk2, neither directly nor indirectly. These two activating effects of cyclin E/Cdk2 via central domain and N-terminus are additive. Cyclin A/Cdk2 and cyclin A/Cdk1 show similar characteristics. GSK-3α, another proliferation-associated kinase, represses FOXM1c.

Keywords: FOXM1c; Cyclin E/Cdk2; Cyclin A/Cdk2; Cyclin A/Cdk1; GSK-3α; Transcription factor

Different cyclin/Cdk complexes, which act consecutively and are subject to tight regulation by multiple mechanisms, control the cell cycle [1–4]: cyclin D1(D2, D3)/Cdk4(6) and cyclin E/Cdk2 together control the G1/S-transition and thus become active in early or late G1-phase, respectively. In S-phase, cyclin A/Cdk2 becomes active, as does cyclin A/Cdk1 in G2-phase. Cyclin B/Cdk1 controls the G2/M-transition and thus becomes active at the end of G2-phase. At the restriction point in late G1-phase, the cell cycle is finally induced and then progresses independently of growth factors [1–6]. RB controls the restriction point and for passage through the restriction point its phosphorylation by cyclin D1(D2,D3)/Cdk4(6) and cyclin E/Cdk2 is required [1–5,7–12].

The forkhead/winged helix transcription factor FOXM1, whose expression correlates strictly with proliferation [13–19], represents a typical proliferation-associated gene: it promotes proliferation by stimulating G1/S- as well as G2/M-transition [14–18,20–25]. Accordingly, it controls the expression of genes that regulate S-phase entry and of those that regulate M-phase entry [14–19,21–25]. Both proliferation and antiproliferation signals regulate its function as transcriptional activator [13,16,18,19,21,24,26–28]. Furthermore, an implication of FOXM1 in tumorigenesis is assumed [21–23,25,27,29].

The splice variant FOXM1c (MPP2) can be a very potent transactivator because it possesses a very strong transactivation domain (TAD) [30]. However, the TAD is repressed by two different inhibitory domains (IDs) [30].

☆ *Abbreviations*: aa, amino acid; Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; DBD, DNA-binding domain; dn, dominant-negative; FOX, forkhead box; FKH, forkhead domain; GSK-3, glycogen synthase kinase-3; ID, inhibitory domain; NLS, nuclear localization signal; NRD, negative-regulatory domain; PI3K, Phosphatidylinositol 3-kinase; SODA, solid phase DNA-binding assay; TAD, transactivation domain; TRD, transrepression domain.

* Corresponding author. Fax: +49 511 883536.
E-mail addresses: iwiwiwi@web.de (I. Wierstra), alves@bpc.mh-hannover.de (J. Alves).
[1] Fax: +49 511 532 2909.

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The N-terminus functions as specific negative-regulatory domain (NRD), named NRD-N, which inhibits the TAD completely by directly binding to it [30]. The central domain acts by two different mechanisms as ID for the TAD: first, it is a strong RB-independent transrepression domain (TRD) [30]. Second, it functions as RB-recruiting NRD, named NRD-C [31]. Accordingly, FOXM1c is repressed by the tumor suppressor RB [31]. On the other hand FOXM1c is strongly activated by the G1-phase proliferation signal cyclin D1/Cdk4 [31]. Cyclin D1/Cdk4 releases FOXM1c from this repression by RB and from the repression by its own inhibitory N-terminus thereby strongly activating FOXM1c [31].

In this study we demonstrate that FOXM1c is activated by cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1, too. The activating effect of cyclin E/Cdk2 strictly depends on the presence of either the central domain or the NRD-N so that it is completely lost if both are deleted. Cyclin E/Cdk2 reduces the repression of the TAD by these two IDs. However, it does not directly increase its transactivation potential. The DNA-binding is not affected by cyclin E/Cdk2, neither directly nor indirectly. The two activating effects of cyclin E/Cdk2 on FOXM1c via the central domain and via the NRD-N are additive. Cyclin A/Cdk2 and cyclin A/Cdk1 show similar characteristics. In addition, FOXM1c is repressed by GSK-3α, another proliferation-associated kinase.

Materials and methods

Plasmids. pGal0 (C. Dang), pGal4mintkluc (R. Janknecht), and expression plasmids for cyclin A, cyclin D1, cyclin E, Cdk1, Cdk2, Cdk4, Cdk2dn (dominant-negative), Cdk4dn, $p21^{CIP1/WAF1}$ (R. Bernards), $p27^{KIP1}$ (J. Koh, R. Bernards), β-catenin (pcDNAβ415, J. Behrens), GSK-3α, and GSK-3β (J.R. Woodgett) were kind gifts. pEQ176P2 [32], $p(MBS)_3$-mintk-luc, pFOXM1c [27], pFOXM1c(189–762), pFOXM1c(1–347; 574–762), [28], pFOXM1c(189–348; 573–762)NLS, pFOXM1c(189–425; 568–762), pGal(GAL4–DNA-binding domain)-FOXM1c, pGal-FOXM1c(359–762), pGal-FOXM1c(359–425; 568–762), pGal-FOXM1c (588–762), and pGal-FOXM1c(721–762) [30] have been described previously. The protein expression of all constructs was verified [30].

Cell culture, transient transfections, luciferase reporter gene assays, and whole cell lysates. Cultivation of RK-13 and COS7 cells, transient transfections using the calcium phosphate co-precipitation method [33], luciferase reporter gene assays [34], and preparation of whole cell lysates were carried out as described before [31].

Solid phase DNA-binding assays (SODAs). SODAs were performed according to Larsson et al. [35] with modifications as described previously [30] using the oligonucleotide hfh-11 (5′-TCGAGTACGTTGTTAT TTGTTTTTTTCGTCGA-3′; [36]) and the antibody α-FOXM1c(C-20) (sc-502) from Santa Cruz (Santa Cruz, CA, USA).

Results

Cyclin E/Cdk2 activates FOXM1c via the central domain and via the N-terminus

The functional domains of FOXM1c [30] are summarized in Fig. 2B (top): the forkhead/winged helix transcription factor FOXM1c possesses a very strong C-terminal TAD (amino acid (aa) 721–762), which is repressed by two different IDs. The N-terminus (aa 1–188) functions as specific NRD-N, which inhibits the TAD completely by directly binding to it. The central domain (aa 349–572) and its part aa 349–425 function as ID for the TAD by two different mechanisms, namely as RB-independent TRD (aa 359–425) [30] and as RB-recruiting NRD-C (aa 349/359–425) [31].

FOXM1c as well as its counterpart Gal-FOXM1c were strongly activated by cyclin E/Cdk2 (Fig. 1). FOXM1c proteins bind to the reporter construct $p(MBS)_3$-mintk-luc via their own forkhead domain, so that their transcriptional effects are the sum of the DNA-binding properties and the transactivation/transrepression potential of FOXM1c. In contrast, Gal-FOXM1c fusion proteins, which bind to the reporter construct pGal4mintkluc via the heterologous GAL4–DNA-binding domain (DBD), represent only the transactivation/transrepression potential of FOXM1c. Consequently, the activation of both FOXM1c and Gal-FOXM1c demonstrates that cyclin E/Cdk2 increases the transactivation potential of FOXM1c (Fig. 1).

Because of the inhibitory N-terminus wildtype FOXM1c and Gal-FOXM1c were (almost) inactive in the absence of exogenous cyclin E/Cdk2. However, in its presence both transactivated strongly (or at least significantly) demonstrating that cyclin E/Cdk2 reduces the inhibition of the TAD by the NRD-N (Fig. 1).

Cyclin E/Cdk2 functions in concert with cyclin D1/Cdk4 to control the G1/S-transition [1–5]. Both are required to inactivate RB, which controls the restriction point in late G1-phase, because its prior partial

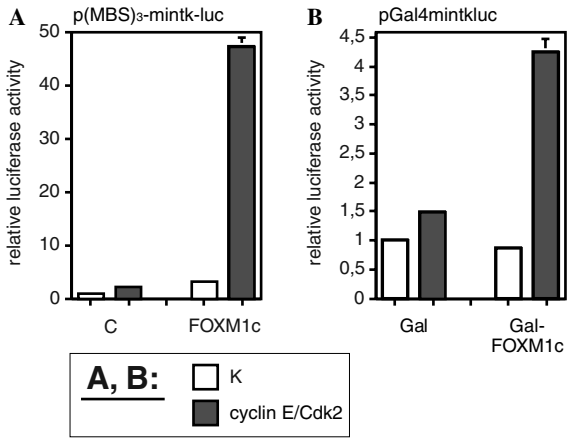

Fig. 1. FOXM1c is activated by cyclin E/Cdk2. (A) RK-13 cells were transiently transfected with pFOXM1c or as control (C) the empty vector and with the reporter construct $p(MBS)_3$-mintk-luc. Either expression plasmids for cyclin E and Cdk2 or as control (K) the empty vector were cotransfected. The relative luciferase activity of $p(MBS)_3$-mintk-luc in combination of the control (C) with the control (K) was set as 1. (B) RK-13 cells were transiently transfected with expression plasmids for Gal or Gal-FOXM1c and with the reporter construct pGal4mintkluc. Either expression plasmids for cyclin E and Cdk 2 or as control (K) the empty vector were cotransfected. The relative luciferase activity of pGal4mintk-luc in the combination of Gal with the control (K) was set as 1.

phosphorylation by cyclin D1/Cdk4 is the prerequisite for its subsequent full phosphorylation by cyclin E/Cdk2 [1–5,7–12]. Therefore the activation of FOXM1c and Gal-FOXM1c by cyclin E/Cdk2 was compared to their activation by cyclin D1/Cdk4 and both cyclin-dependent kinases (Cdks) together. Supplementary Fig. 1A and B demonstrates that both FOXM1c and Gal-FOXM1c are predominantly activated by cyclin D1/Cdk4 [31] whereas their activation by cyclin E/Cdk2 is considerably smaller. Thereby cyclin E/Cdk2 might or might not enhance further their already very strong activation by cyclin D1/Cdk4 (Supplementary Fig. 1A and C).

To characterize in detail the activation of FOXM1c by cyclin E/Cdk2, a variety of deletion mutants of FOXM1c and Gal-FOXM1c were tested for activation by cyclin E/Cdk2 (Figs. 2 and 3; data not shown) and cyclin D1/Cdk4 + cyclin E/Cdk2 (Supplementary Fig. 2; data not shown). Their expression and in case of the former ones DNA-binding and nuclear localization had been shown before [30].

The two N-terminal deletion mutants FOXM1c(189–762) and Gal-FOXM1c(359–762), whose TADs were not inhibited by the NRD-N and which thus transactivated strongly, were still significantly activated by cyclin E/Cdk2 (Figs. 2A and 3A). Consequently, cyclin E/Cdk2 increases the transactivation potential of FOXM1c also independently of the inhibitory N-terminus (Figs. 2B and 3B).

The repression of FOXM1c(189–762) and Gal-FOXM1c(359–762) by a dominant-negative (dn) form of Cdk2 (Cdk2dn) and the Cdk inhibitors (CKIs) $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ confirmed this activation by cyclin E/

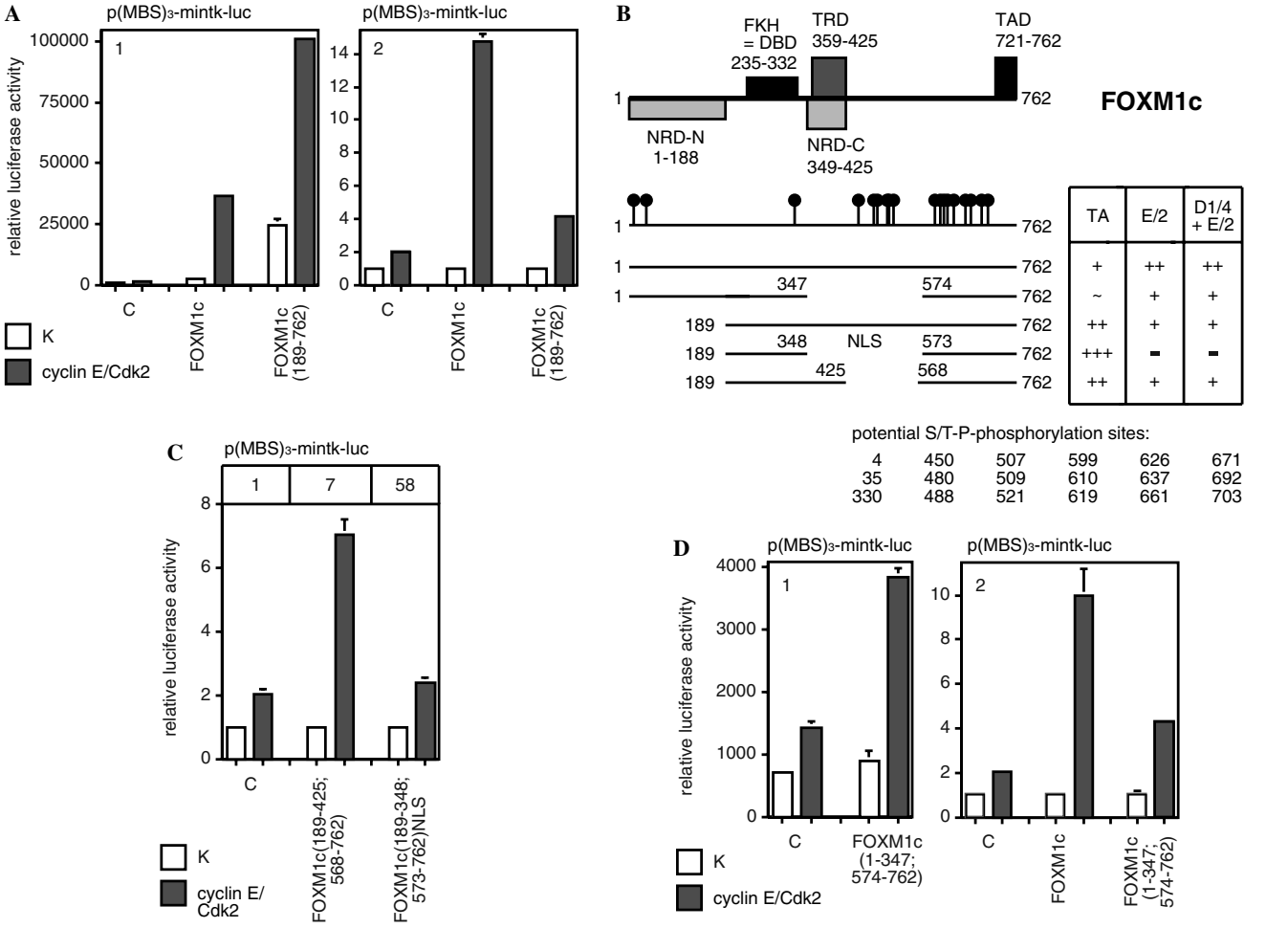

Fig. 2. Cyclin E/Cdk2 activates FOXM1c via the central domain and via the N-terminus. (A, C, and D) RK-13 cells were transiently transfected with p(MBS)$_3$-mintk-luc and expression plasmids for FOXM1c proteins or as control (C) the empty vector. Either expression plasmids for cyclin E and Cdk2 or as control (K) the empty vector were cotransfected. In (C) the relative luciferase activity of p(MBS)$_3$-mintk-luc in the control (K) was set as 1. In (C) the transactivation by the FOXM1c proteins in the control (K), i.e., in the absence of exogenous cyclin E/Cdk2, is indicated by numbers above the columns. (B) Map of functional domains and potential cyclin/Cdk phosphorylation sites S/T-P (needle heads) of the FOXM1c proteins. FOXM1c(189–348; 573–762)NLS contains the heterologous nuclear localization signal (=NLS) of SV40 large T between aa 348 and aa 573. TA (=transactivation): it is shown whether the FOXM1c proteins transactivate very strongly (=+++), strongly (=++), weakly (=+) or (almost) not (= ~). Note that the given transactivation for FOXM1c(189–425; 568–762) is corrected by expression. E/2 and D1/4 + E/2: It is indicated whether cyclin E/Cdk2 (=E/2) and cyclin D1/Cdk4 + cyclin E/Cdk2 (=D1/4 + E/2; see Supplementary Fig. 2) activate the FOXM1c proteins strongly (=++), medium (=+) or not (=−). NRD, negative-regulatory domain; FKH, forkhead domain; DBD, DNA-binding domain; TRD, transrepression domain; TAD, transactivation domain.

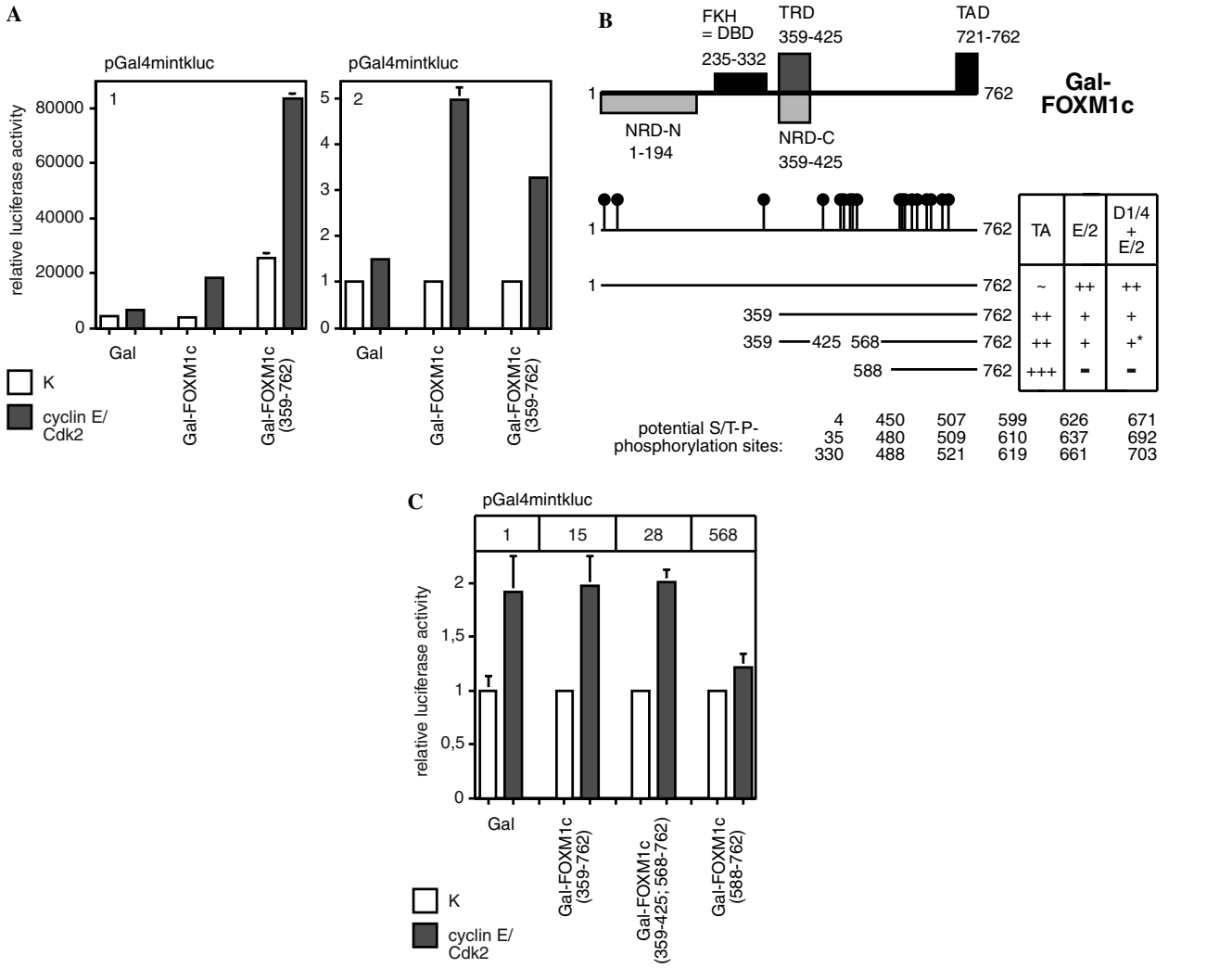

Fig. 3. Cyclin E/Cdk2 does not directly increase the transactivation by the TAD. (A, C) RK-13 cells were transiently transfected with pGal4mintkluc and expression plasmids for Gal or Gal-FOXM1c fusion proteins. Either expression plasmids for cyclin E and Cdk2 or as control (K) the empty vector were cotransfected. The relative luciferase activity of pGal4mintkluc in the control (K) was set as 1. In (C) the transactivation by the Gal-FOXM1c fusion proteins in the control (K), i.e., in the absence of exogenous cyclin E/Cdk2, is indicated by numbers above the columns. (B) Map of functional domains and potential cyclin/Cdk phosphorylation sites S/T-P (needle heads) of the Gal-FOXM1c fusion proteins. The Gal-FOXM1c fusion proteins have the GAL4-DBD (not shown) N-terminal to the FOXM1c sequence (aa indicated in the figure). TA (=transactivation): it is shown whether the Gal-FOXM1c fusion proteins transactivate very strongly (=+++), strongly (=++) or not (=∼). E/2 and D1/4 + E/2: It is indicated whether cyclin E/Cdk2 (=E/2) and cyclin D1/Cdk4 + cyclin E/Cdk2 (=D1/4 + E/2; see Supplementary Fig. 2) activate the Gal-FOXM1c fusion proteins strongly (=++), medium (=+) or not (=−). *Data not shown.

Cdk2 (Supplementary Fig. 1E, F, and H; data not shown). This is consistent with the repression of wildtype FOXM1 by Cdk2dn, $p21^{CIP1/WAF1}$ [18], and $p27^{KIP1}$ [21].

Cyclin E/Cdk2 does not directly increase the transactivation by the TAD

First, the activating effect of cyclin E/Cdk2 on FOXM1c, which is independent of the NRD-N, was analyzed further with mutants lacking the N-terminus (Figs. 2B and 3B).

Cyclin E/Cdk2 activated significantly FOXM1c(189–762) and FOXM1c(189–425; 568–762), which both possessed the inhibitory central domain, whereas it did not activate the maximal transactivator FOXM1c(189–348; 573–762)NLS, which lacked both IDs for the TAD (Fig. 2A and C). Consequently, cyclin E/Cdk2 does not directly increase the transactivation by the TAD or the DNA-binding by the DBD, but this activating effect of cyclin E/Cdk2 on FOXM1c depended specifically on aa 349–425 demonstrating that cyclin E/Cdk2 reduces the repression of the TAD by the central domain (Fig. 2B). Accordingly, the activating effect of cyclin E/Cdk2 on FOXM1c(189–425; 568–762) and FOXM1c(189–762) was equally strong (data not shown).

To show that this activating effect is independent of the DBD (aa 235–332), similar experiments were per-

formed with analogous Gal-FOXM1c fusion proteins (Fig. 3B). Again, cyclin E/Cdk2 activated Gal-FOXM1c(359–762) and Gal-FOXM1c(359–425; 568–762), whose TADs were repressed by the central ID, whereas it did not activate the maximal transactivator Gal-FOXM1c(588–762) demonstrating that this activating effect of cyclin E/Cdk2 depended specifically on aa 359–425 of FOXM1c (Fig. 3A–C). Therefore cyclin E/Cdk2 does not directly increase the transactivation by the TAD, but reduces the repression of the TAD by the central domain and thereby increases indirectly the transactivation by the TAD.

The repression by Cdk2dn (or Cdk2dn + Cdk4dn) confirmed these results because it showed the same specificity as the activation by cyclin E/Cdk2 (Supplementary Fig. 1D, E, and G).

Cyclin E/Cdk2 exerts two independent additive activating effects on FOXM1c

Next, the activating effect of cyclin E/Cdk2 via the NRD-N was analyzed (Fig. 2D). The use of FOXM1c(1–347; 574–762) excluded any involvement of the central domain (Fig. 2B). In the absence of exogenous cyclin E/Cdk2 FOXM1c(1–347; 574–762) was inactive because of the NRD-N. However, its significant transactivation in the presence of exogenous cyclin E/Cdk2 demonstrated that cyclin E/Cdk2 reduces the inhibition of the TAD by the NRD-N independent of the central domain (Fig. 2B and D).

Consequently, cyclin E/Cdk2 exerted two independent activating effects on FOXM1c: the first one depended specifically on aa 349/359–425, i.e., on the central ID for the TAD (Figs. 2B and C, and 3B and C), the second one on the NRD-N (Fig. 2B and D). Figs. 2A and 3A show that cyclin E/Cdk2 activated full-length FOXM1c and its GAL4–DBD fusion product considerably more than the respective N-terminal deletion mutants with only the central ID. Similarly, full-length FOXM1c was considerably more activated by cyclin E/Cdk2 than FOXM1c(1–347; 574–762), which contained the NRD-N but lacked the central ID (Fig. 2D). Therefore, these two independent activating effects of cyclin E/Cdk2 on FOXM1c are additive (Figs. 2B and 3B). Cyclin E/Cdk2 reduces the repression of the TAD by the two IDs, but does not directly increase the transactivation potential of the TAD, so that its two activating effects on FOXM1c depend specifically on the presence of either the central domain or the NRD-N (Figs. 2A–D and 3A–C).

Neither cyclin E/Cdk2 nor cyclin D1/Cdk4 nor both together increase the DNA-binding by the DBD

To examine whether cyclin E/Cdk2 increases indirectly also the DNA-binding by the DBD solid phase DNA-binding assays (SODAs) were performed with whole cell lysates from COS7 cells transiently transfected with expression plasmids for FOXM1c(189–762), cyclins and Cdks (Fig. 4). No significant expression-independent effect of exogenous cyclin E/Cdk2 or exogenous cyclin D1/Cdk4 or their combination on the DNA-binding by FOXM1c(189–762) was observed. Thus neither cyclin E/Cdk2 nor cyclin D1/Cdk4 nor both together increase the DNA-binding by the DBD of FOXM1c.

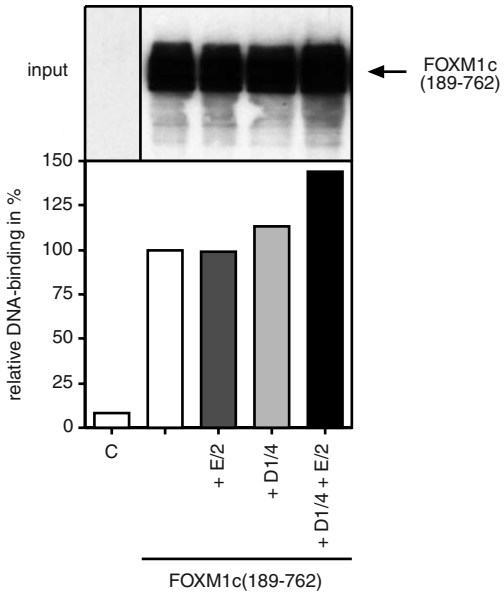

Fig. 4. Neither cyclin E/Cdk2 nor cyclin D1/Cdk4 nor both together increase the DNA-binding by the DBD. (Lower panel) Solid phase DNA-binding assay (SODA): immunoprecipitations were performed with the antibody α-FOXM1c(C-20) and whole cell lysates of COS7 cells transiently transfected with pFOXM1c(189–762) alone or together with expression plasmids for the indicated cyclins and Cdks or as control (C) only with the empty vector. Then the beads were incubated with labelled oligonucleotide hfh-11. After washing the beads the amount of bound hfh-11 was measured in a counter. The amount of hfh-11 bound to FOXM1c(189–762) in the absence of exogenous cyclins or Cdks was set as 100%. In this absence of exogenous cyclins or Cdks about 12-fold more hfh-11 bound to FOXM1c(189–762) than to the control (C) beads. (Upper panel) As input control the FOXM1c(189–762) expression in the COS7 cell lysates used was analyzed in a Western blot with α-FOXM1c(C-20) as primary antibody.

In summary, cyclin E/Cdk2 does not directly target the TAD or the DBD of FOXM1c, but its activating effect strictly depends on the presence of either the NRD-N or the central domain. Cyclin E/Cdk2 reduces the repression of the TAD by these two IDs and thereby increases indirectly the transactivation by the TAD. However, it does not directly increase the transactivation by the TAD. The DNA-binding by the DBD is not at all affected by cyclin E/Cdk2, neither directly nor indirectly.

The activation of FOXM1c by cyclin E/Cdk2 (Figs. 1–4 and Supplementary Fig. 1) showed the same specificity, characteristics, and mechanism as its very strong activation by cyclin D1/Cdk4 [31]. Accordingly, the same was found for their combined action (Fig. 4 and Supplementary Fig. 2).

Cyclin A/Cdk2 and cyclin A/Cdk1 activate FOXM1c via the central domain, but do not directly increase the transactivation by the TAD or the DNA-binding by the DBD

It was reported that transcriptional activity of the splice variant FoxM1B requires the phosphorylation of one threonine residue by cyclin B/Cdk1 to allow recruitment of the coactivator p300/CBP [26]. Activation by p300/CBP or cyclin B/Cdk1 could not be demonstrated for FOXM1c (data not shown) possibly influenced by the use of a different FOXM1 splice variant and a different cell type. Nevertheless, it was analyzed whether besides cyclin D1/Cdk4 and cyclin E/Cdk2 also the two other cyclin/Cdk complexes, which control the cell cycle, activate FOXM1c. Indeed, cyclin A/Cdk2 and cyclin A/Cdk1 activated FOXM1c proteins as well as Gal-FOXM1c fusion proteins demonstrating that they increase the transactivation potential of FOXM1c (Fig. 5 and Supplementary Fig. 3). The detailed characterization of their activating effects by use of a variety of deletion mutants of FOXM1c and Gal-FOXM1c (Fig. 5D) revealed that the activation of FOXM1c by them did not differ in specificity, characteristics or mechanism from its activation by cyclin E/Cdk2 and cyclin D1/Cdk4. Thus the activating effects of cyclin A/Cdk2 and

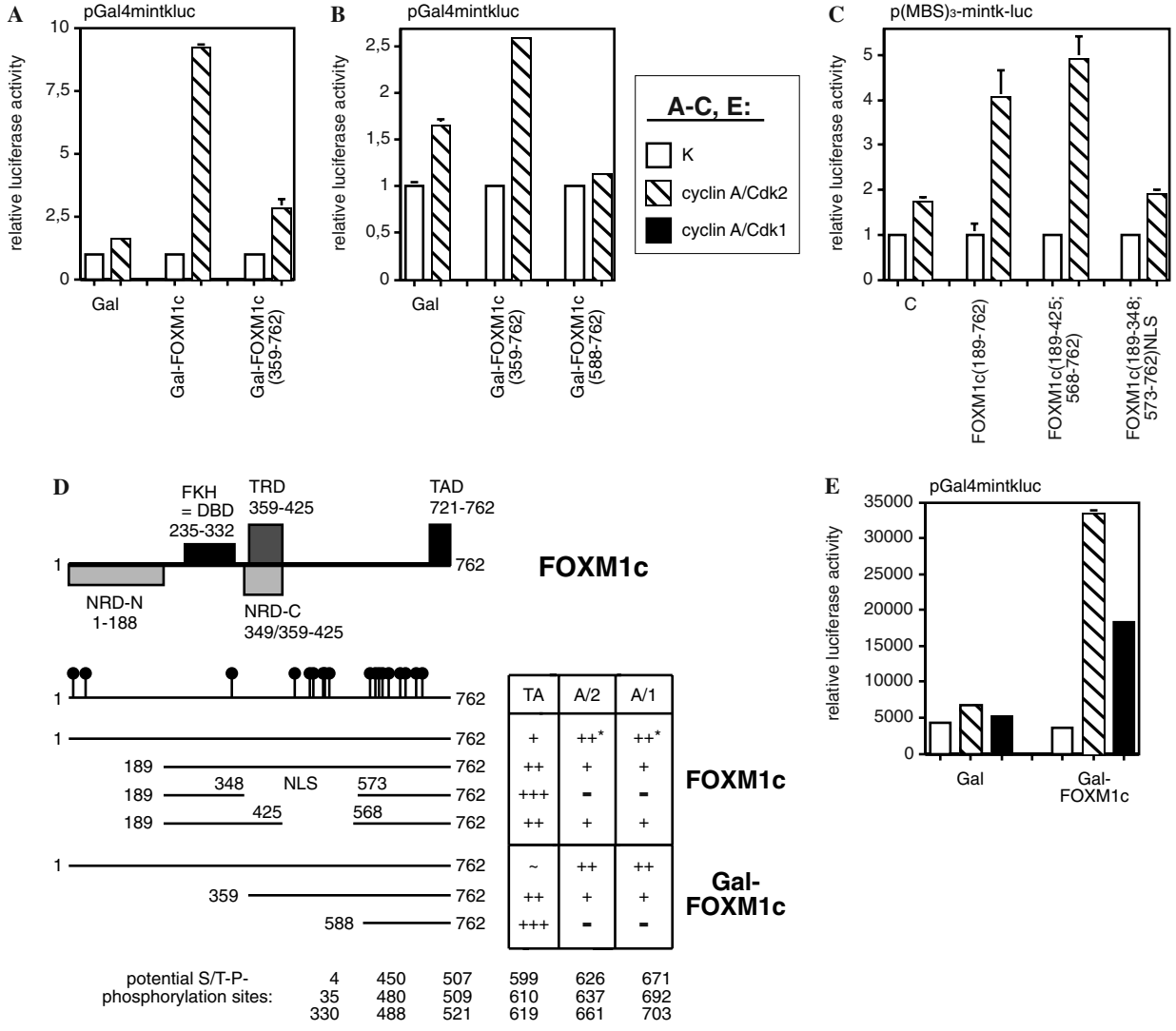

Fig. 5. Cyclin A/Cdk2 activates FOXM1c via the central domain, but does not directly increase the transactivation by the TAD or the DNA-binding by the DBD. (A, B, and E) RK-13 cells were transiently transfected with pGal4mintkluc and expression plasmids for Gal or Gal-FOXM1c fusion proteins. Either expression plasmids for cyclin A and the indicated Cdks or as control (K) the empty vector were cotransfected. In (A,B) the relative luciferase activity of pGal4mintkluc in the control (K) was set as 1. (C) RK-13 cells were transiently transfected with $p(MBS)_3$-mintk-luc and expression plasmids for FOXM1c proteins or as control (C) the empty vector. Either expression plasmids for cyclin A and Cdk2 or as control (K) the empty vector were cotransfected. The relative luciferase activity of $p(MBS)_3$-mintk-luc in the control (K) was set as 1. (D) Map of functional domains and potential cyclin/Cdk phosphorylation sites S/T-P (needle heads) of the FOXM1c proteins and Gal-FOXM1c fusion proteins. TA (=transactivation): it is shown whether these FOXM1c proteins transactivate very strongly (=+++), strongly (=++), weakly (=+) or not (=∼). A/2 and A/1: it is indicated whether cyclin A/Cdk2 (=A/2) and cyclin A/Cdk1 (=A/1; see Supplementary Fig. 3) activate these FOXM1c proteins strongly (=++), medium (=+) or not (=−). *Data not shown.

cyclin A/Cdk1 strictly depend on the presence of aa 349/359–425 of FOXM1c demonstrating that they do not directly increase the transactivation by the TAD or the DNA-binding by the DBD, but that they reduce the repression of the TAD by the central domain thereby indirectly increasing the transactivation by the TAD (Fig. 5B–D and Supplementary Fig. 3B and C). The significant transactivation of the otherwise inactive Gal-FOXM1c in the presence of exogenous cyclin A/Cdk2 or cyclin A/Cdk1 (Fig. 5E) showed that they also reduce the inhibition of the TAD by the NRD-N thereby again indirectly increasing the transactivation by the TAD. Accordingly, cyclin A/Cdk2 (Fig. 5A) and cyclin A/Cdk1 (Supplementary Fig. 3A) activated Gal-FOXM1c considerably more than its N-terminal deletion mutant with only the central ID suggesting that they exert two additive activating effects on FOXM1c. However, they do not directly target the TAD or the DBD of FOXM1c.

Finally, since all four cyclin/Cdk complexes that activate FOXM1c increase its transactivation potential the magnitude of their activating effects on the inactive Gal-FOXM1c and its strongly transactivating N-terminal deletion mutant (Fig. 3A) was analyzed (Supplementary Fig. 3D). This comparison showed that FOXM1c is by far predominantly activated by cyclin D1/Cdk4 whereas the activating effects of cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1 are all considerably smaller, even if among each other in the same range. Thereby the possibility remains that cyclin E/Cdk2 may enhance further the already very strong activation of FOXM1c by cyclin D1/Cdk4.

FOXM1c is repressed by GSK-3α

FOXM1 stimulates proliferation by promoting G1/S- as well as G2/M-transition [14–18,20–25]. In addition, it is assumed to be implicated in tumorigenesis [21–23,25,27,29]. Transcription factors with similar functions, e.g., E2F-1/2/3, c-Myc, AP-1, NF-κB, and STAT3, are known to be subject to complex regulation by a variety of kinases and signal transduction pathways. Therefore we tested whether FOXM1c is affected by glycogen syn-

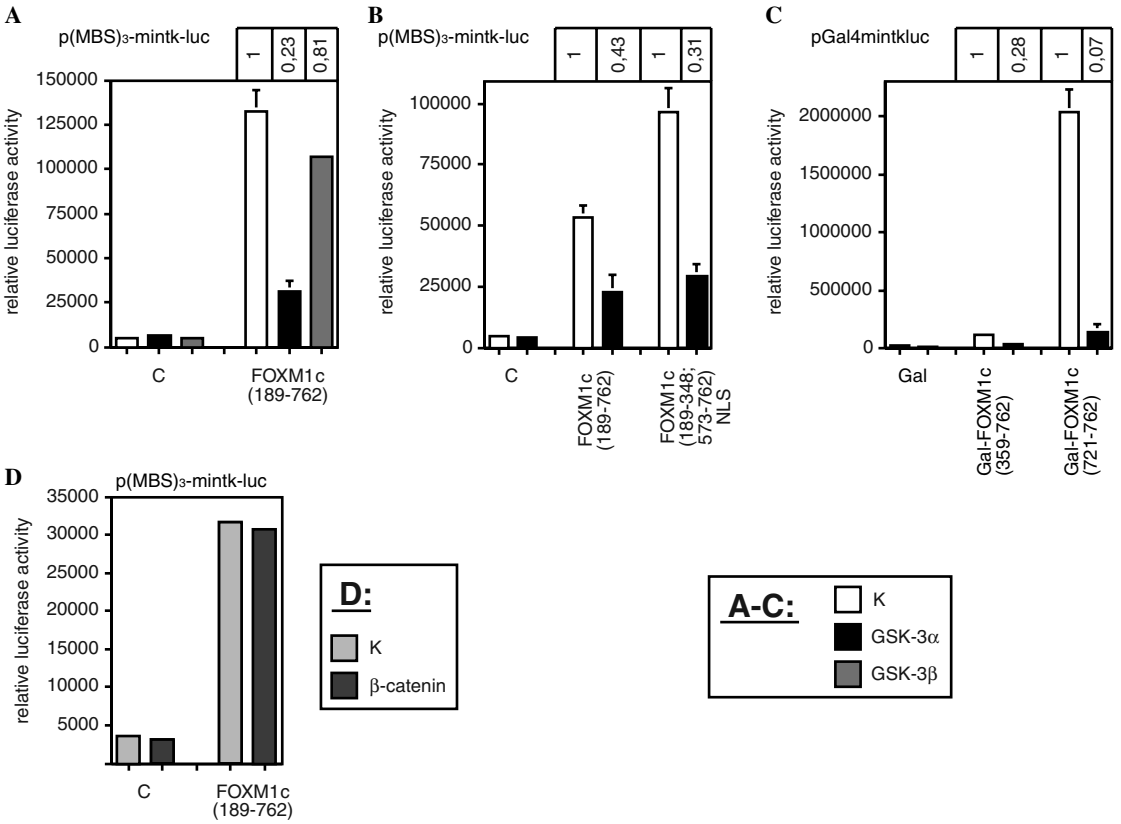

Fig. 6. GSK-3α represses FOXM1c. (A, B) RK-13 cells were transiently transfected with p(MBS)$_3$-mintk-luc and expression plasmids for FOXM1c proteins or as control (C) the empty vector. Either expression plasmids for GSK-3α or GSK-3β or as control (K) the empty vector were cotransfected. For each FOXM1c protein the relative repression by GSK-3α or GSK-3β is indicated by numbers above the columns. For this the relative luciferase activity of p(MBS)$_3$-mintk-luc in the control (K), i.e., in the absence of exogenous GSK-3α and GSK-3β, was set as 1. (C) RK-13 cells were transiently transfected with pGal4mintkluc and expression plasmids for Gal or Gal-FOXM1c fusion proteins. Either the expression plasmids for GSK-3α or as control (K) the empty vector were cotransfected. For each Gal-FOXM1c fusion protein the relative repression by GSK-3α is indicated by numbers above the columns. For this the relative luciferase activity of pGal4mintkluc in the control (K), i.e., in the absence of exogenous GSK-3α, was set as 1. (D) RK-13 cells were transiently transfected with p(MBS)$_3$-mintk-luc and pFOXM1c(189–762) or as control (C) the empty vector. Either the expression plasmids for β-catenin or as control (K) the empty vector were cotransfected.

thase kinase-3 (GSK-3), a proliferation-associated kinase whose inhibition in response to Wnt [37] or phosphatidylinositol 3-kinase (PI3K) [38] signalling is known to mediate proliferation signals [39–41].

GSK-3α, but not GSK-3β, repressed FOXM1c and FOXM1c(189–762) (Fig. 6A; data not shown). GSK-3α repressed also the maximal transactivator FOXM1c(189–348; 573–762)NLS and the isolated TAD (aa 721–762) of FOXM1c suggesting that it targets the TAD (Fig. 6B and C). A consensus recognition motif for GSK-3α (**S/T**-X-X-X-S/T(P)) might be the perfectly conserved sequence GLVLD**T**$_{725}$MNDSLSKIL. Phosphorylation by GSK-3 requires priming of its substrates through prior phosphorylation by another kinase at position N_{+4} [38,39]. Yet, it is unknown whether this S_{+4} is phosphorylated in FOXM1c so that also other possibilities have to be considered. Alternatively, GSK-3α could phosphorylate another protein (e.g., component of basal transcription complex or coactivator), with which the TAD has to interact in order to transactivate, and thereby GSK-3α could either interrupt this interaction or inhibit the function of this other protein. GSK-3 phosphorylates β-catenin, a coactivator of TCF/LEF transcription factors, thereby targeting it for ubiquitination and proteasomal degradation [37,39,41]. However, co-expression of β-catenin did not affect FOXM1c or FOXM1c(189–762) (Fig. 6D; data not shown). Thus, the mechanism for repression of FOXM1c by GSK-3α remains to be elucidated. The inhibition of FOXM1c by GSK-3α correlates well not only with its function in stimulation of S- and M-phase entry but also with its role in tumorigenesis because both the Wnt and the PI3K pathways are often deregulated in cancer [37–39,42].

Discussion

In this study, the activation of FOXM1c by cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1 as well as by cyclin D1/Cdk4 + cyclin E/Cdk2 (Supplementary Fig. 2) was analyzed: Cyclin E/Cdk2 reduces the repression of the TAD by the central domain and the NRD-N, and thereby increases indirectly the transactivation by the TAD (Figs. 1 and 2A, C, and D and 3A and C). However, it does not directly increase the transactivation by the TAD (Figs. 2C and 3C). The DNA-binding by the DBD was not affected by cyclin E/Cdk2, neither directly nor indirectly (Fig. 4). Since cyclin E/Cdk2 does not directly target the TAD or the DBD, its activating effect on FOXM1c strictly depends on the presence of either the central domain or the NRD-N and thus was completely lost if these two IDs for the TAD were deleted (Figs. 2B and 3B). The two activating effects of cyclin E/Cdk2 on FOXM1c via the central domain and via the NRD-N are additive (Figs. 2A and D, and 3A). Cyclin A/Cdk2 and cyclin A/Cdk1 showed similar characteristics (Fig. 5 and Supplementary Fig. 3).

How can cyclin E/Cdk2 activate FOXM1c via the NRD-N and via the central domain? (Since the activating effects of cyclin A/Cdk2 and cyclin A/Cdk1 on FOXM1c did not differ in specificity, characteristics or mechanism from its activation by cyclin E/Cdk2, the considerations for cyclin E/Cdk2 are also valid for them.)

The NRD-N functions as ID for the TAD, which inhibits the TAD completely by directly binding to it [30]. Thus cyclin E/Cdk2 could reduce this interaction. Thereby it could theoretically phosphorylate the NRD-N, but not the TAD, because the NRD-N (aa 1–188) contains two potential cyclin/Cdk phosphorylation sites S/T-P at the serines 4 and 35 whereas the TAD (aa 721–762) lacks any such site (Fig. 2B). S4 (MKT**S**PRRP) conforms to the consensus motif for cyclin E-, A-, and B-dependent kinases **S/T**-P-X-K/R [43,44] while S35 (PKR**S**PAQQ) does not so that only S4 should represent a good target site for cyclin E/Cdk2, cyclin A/Cdk2 and cyclin A/Cdk1.

The central domain functions as ID for the TAD by two different mechanisms, namely as strong RB-independent TRD [30] and as RB-recruiting NRD-C [31]. The transrepression by the TRD was not affected by cyclin E/Cdk2, cyclin A/Cdk2 or cyclin A/Cdk1 (data not shown). The activation of N-terminal deletion mutants of FOXM1c by cyclin E/Cdk2 depends specifically on aa 349–425 or aa 359–425, respectively, which do not contain any potential cyclin/Cdk phosphorylation site (Figs. 2B and C, and 3B and C). Consequently, cyclin E/Cdk2 cannot activate them by phosphorylation of aa 349–425 directly. Alternatively, aa 349/359–425 could bind to another domain of FOXM1c and cyclin E/Cdk2 could interrupt their interaction by phosphorylation of this other domain. However, in pull-down assays we could not detect any such interaction of aa 348–573 with other parts of FOXM1c (data not shown). Consequently, the activating effect of cyclin E/Cdk2 on FOXM1c, which depends on aa 349/359–425, is no direct effect on FOXM1c. This conclusion was also supported by other FOXM1c and Gal-FOXM1c deletion mutants (data not shown). Therefore it has to be caused by the phosphorylation of a second protein that binds to aa 359–425 of FOXM1c. In fact, RB binds directly to aa 359–425 of FOXM1c and thereby represses indirectly the transactivation by the TAD [31]. Cyclin D1/Cdk4 strongly activates FOXM1c dependent on aa 349/359–425 by interrupting this interaction and thus releasing the TAD from its repression by RB [31]. Thereby cyclin D1/Cdk4 phosphorylates RB, but not FOXM1c [31]. Since RB is also phosphorylated by cyclin E/Cdk2 during G1-phase [1–5,7–12] it could, like cyclin D1/Cdk4, activate FOXM1c dependent on aa 349/359–425 by phosphorylation of RB. Similarly, phosphorylation of RB by cyclin A/Cdk2 and cyclin A/Cdk1 could explain how they activate FOXM1c dependent on the central domain. Phosphorylation of RB by them in later cell cycle phases is thought to keep RB inactive after its initial inactivation by cyclin D1/Cdk4 and cyclin E/Cdk2 during G1-phase [1,7,10–12].

Recently, Bernhard Lüscher and co-workers published a paper [28] on the activation of FOXM1c by cyclin E/Cdk2 and cyclin A/Cdk2, which comes to different conclusions. One major point is their statement that neither the N-ter-

minal NRD nor the central region of FOXM1c would be important for the cyclin E/Cdk2 effect [28]. Instead they map three cyclin E/Cdk2 phosphorylation sites (T600, T611, and S638), whose phosphorylation should directly influence the TAD [28]. Here we show that cyclin E/Cdk2 does not directly target the TAD or the DBD but that its activating effect on FOXM1c strictly depends on the presence of either the central domain or the NRD-N so that it is completely lost if both IDs are deleted although the TAD is still present (see FOXM1c(189–348; 573–762)NLS in Figs. 2B and C and Gal-FOXM1c(588–762) in Fig. 3B and C). Cyclin E/Cdk2 reduces the repression of the TAD by these two IDs but it does not directly increase the transactivation by the TAD (Figs. 2 and 3). FOXM1c(189–348; 573–762)NLS and Gal-FOXM1c(588–762) each possess the three phosphorylation sites T600, T611 and S638 (Figs. 2B and 3B). If these three sites mediated the cyclin E/Cdk2 and cyclin A/Cdk2 effects these cyclin/Cdk complexes would activate both FOXM1c(189–348; 573–762)NLS and Gal-FOXM1c(588–762). However, these two deletion mutants are activated neither by cyclin E/Cdk2 (Figs. 2C and 3C) nor by cyclin A/Cdk2 (Fig. 5B and C). Thus this decisive test of the hypothesis that the three sites T600, T611, and S638 mediate the cyclin E/Cdk2 and cyclin A/Cdk2 effects revealed that they do not. An additional discrepancy is the size of the TAD, which Lüscher and co-workers enlarge down to aa 589 [28]. We have shown recently [30] that a C-terminal deletion starting from aa 697 abolishes any transactivation potential of FOXM1c. The highly conserved fragment aa 721–762 alone is sufficient to work as TAD as well as to be bound and inhibited by the NRD-N of FOXM1c [30]. Therefore the three cyclin E/Cdk2 phosphorylation sites T600, T611, and S638 are far outside the TAD (aa 721–762) of FOXM1c (Fig. 2B). Instead they are positioned in the fragment aa 588–696, which lacks any transactivation potential [30]. Why do Lüscher and co-workers then find that the activation by cyclin E/Cdk2 and cyclin A/Cdk2 is lower for the triple alanine mutant T600A/T611A/S638A than for wildtype FOXM1c [28]? Major et al. [26] have already shown for the splice variant FoxM1B that one of these sites, namely T596 (corresponds to T611 of FOXM1c), has to be phosphorylated by cyclin B/Cdk1 to allow recruitment of the co-activator p300/CBP. Thus it may well be that the lower transactivation activity of the triple alanine mutant in the presence as well as in the absence of exogenous cyclin E/Cdk2 or cyclin A/Cdk2 [28] results from the failure of this mutant to recruit p300/CBP in response to cyclin B/Cdk1 and not from less activation by the two former Cdks.

In summary, we have shown that the proliferation-specific transcription factor FOXM1c is activated by several Cdks, especially cyclin D1/Cdk4 and cyclin E/Cdk2 as well as cyclin A/Cdk2 and cyclin A/Cdk1. These Cdks counteract the intrinsic inhibition of the TAD of FOXM1c by its central domain and its NRD-N but do not directly influence DNA-binding or transactivation theirselves. Our previous results on the mechanism of the activating effect of cyclin D1/Cdk4 on FOXM1c [31] let us conclude that the three other Cdks discussed here in a similar manner abolish the recruitment of RB to the central domain and the direct interaction of the NRD-N with the TAD, which results in the uncovering of the high transactivation potential of FOXM1c. This suggests that after the initial activation of FOXM1c by cyclin D1/Cdk4 in early G1-phase its subsequent activation by cyclin E/Cdk2, cyclin A/Cdk2, and cyclin A/Cdk1 may serve to keep FOXM1c active during later stages of the cell cycle until its final activation by cyclin B/Cdk1 at the end of G2-phase. The repression of FOXM1c by GSK-3α provides an additional possibility for its regulation in addition to its activation by several Cdks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.07.008.

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