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Cyclin E/Cdk2, P/CAF, and E1A regulate the transactivation of the *c-myc* promoter by FOXM1

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Abstract

FOXM1c transactivates the *c-myc* promoter by binding directly to its TATA-boxes. The present study demonstrates that the transactivation of the *c-myc* promoter by FOXM1c is enhanced by the key proliferation signal cyclin E/Cdk2, but repressed by P/CAF and the adenoviral oncoprotein E1A. Furthermore, FOXM1c interacts with the coactivator and histone acetyltransferase P/CAF. This study shows that, on the *c-myc*-P1 TATA-box, FOXM1c does not function simply as normal transcription factor just binding to an unusual site. Moreover, the inhibitory N-terminus of FOXM1c does not inhibit its transrepression domain or its EDA. Others reported that a cyclin/Cdk-binding LXL-motif of the splice variant FoxM1b is required for its interaction with Cdk2, Cdk1, and p27, its phosphorylation by Cdk1 and its activation by Cdc25B. In contrast, we now demonstrate that this LXL-motif is not required for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk and cyclin A/Cdk2 or for the repression of FOXM1c by p27.

Keywords: Human FOXM1; Human *c-myc*; Cyclin D/Cdk4; Cyclin E/Cdk2; Cyclin A/Cdk2; P/CAF; Adenovirus E1A; TAF1; HPV16 E7; RB; p27

FOXM1 (Forkhead box M1), a typical proliferation-associated transcription factor, stimulates proliferation by promoting both G1/S- and G2/M-transition and is implicated in tumorigenesis [1–4].

The human splice variant FOXM1c can transactivate by two different mechanisms depending on the FOXM1c-binding site [5], to which its forkhead/winged helix domain-type DBD (DNA-binding domain) binds [6,7]. On conventional FOXM1c-binding sites (consensus sequence 5′-A-C/T-AAA-C/T-AA-3′), it functions as conventional transcription factor [7–9]. On the P1 (TATAATGC) and P2 (TATAAAAG) TATA-boxes of the human *c-myc* P1 and P2 promoters FOXM1c transactivates by a new mechanism [6,10]. It binds with its DBD directly to these TATA-boxes and with its central domain directly to TBP, TFIIB, and TFIIAα/β as well as possibly indirectly to TFIIAγ and TAF1, i.e. to components of the basal transcription complex [6]. For both transactivation mechanisms the strong acidic C-terminal TAD (transactivation domain) of FOXM1c is required and is completely inhibited by the N-terminal NRD (negative-regulatory domain), which directly binds to the TAD [6,7]. Consequently, N-terminal deletion mutants are often used to examine FOXM1c. The central domain of FOXM1c distinguishes these two mechanisms because it fulfills two opposite functions, which are determined by the FOXM1c-binding site [5]. On the *c-myc* P1 and P2 TATA-boxes, the central domain is essentially required for transactivation of the *c-myc* P1 and P2 promoters by FOXM1c and therefore it was named EDA (essential domain for activation) [5,6]. On conventional FOXM1c-binding sites, the central domain of FOXM1c functions as ID (inhibitory domain) [5], namely as both RB (retinoblastoma protein)-independent TRD (transrepression domain) [7] and RB-recruiting NRD-C [8]. The tumor suppressor RB represses the TAD of FOXM1c indirectly because RB binds directly to the central domain of FOXM1c, but not to its TAD [8]. This switch of the central domain of FOXM1c from the essential EDA on the *c-myc* P1 and P2 TATA-boxes to an ID on conventional FOXM1c-binding

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sites may be mediated by an allosteric control of the conformation of this domain by the FOXM1c-binding site and may involve the direct interaction of the central domain with itself [5,6]. The function of FOXM1c as conventional transcription factor is mimicked by Gal-FOXM1c fusion proteins because its central domain functions as ID in both cases, namely as RB-independent TRD [7] and RB-recruiting NRD-C [8]. Since these Gal-FOXM1c fusion proteins bind with their heterologous GAL4-DBD to GAL4-binding sites of a reporter construct independently of the forkhead/winged helix-type DBD of FOXM1c the *c-myc* P1 and P2 TATA-boxes are required to trigger the conversion of the central domain of FOXM1c into the EDA. Also other promoters with a *c-myc* P2-type TATA-box TATAAAAG are transactivated by FOXM1c, namely those of *c-fos*, *hsp70*, and *histone H2B/a* [6]. However, promoters with other TATA-boxes were not FOXM1c-responsive, e.g. those of *c-jun*, *p21* (*waf1/cip1*), *neutrophil elastase* and the minimal TK (*thymidine kinase*) promoter of HSV (*herpes simplex virus*) [6].

The new transactivation mechanism via TATA-boxes is unprecedented and FOXM1c cannot be classified as general transcription factor, TAF (TBP-associated factor), mediator, general positive co-factor, co-activator, or component of the basal transcription machinery. Therefore, the present study analyzes whether, on the *c-myc*-P1 TATA-box, FOXM1c functions simply as normal transcription factor just binding to an unusual site. Moreover, this study answers the important question whether the N-terminal NRD of FOXM1c inhibits also its central EDA and its central TRD in addition to its C-terminal TAD.

The splice variants FoxM1b and FOXM1c are distinguished only by the absence or presence, respectively, of an exon in their DBD [1,2]. Since this exon affects neither the DNA-binding specificity nor the ability to transactivate FoxM1b and FOXM1c are functionally similar [7,11].

FoxM1b is activated by cyclin B/Cdk (cyclin-dependent kinase) 1 [4,12]. One single leucine within a cyclin/Cdk-binding LXL-motif, which is similar to the known cyclin-binding Cy (RXL) motif [12,13], is required for the interaction of FoxM1b with Cdk1, Cdk2, and p27, for the phosphorylation of FoxM1b by Cdk1, for transcriptional activity of FoxM1b and for the activation of FoxM1b by Cdc (cell division cycle) 25B [3,4,12]. This LXL-motif is identical in FOXM1c.

In clear contrast, we have demonstrated that cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2 can activate FOXM1c without phosphorylating it because they release FOXM1c from its repression by RB through phosphorylation of RB, but not FOXM1c [8,9]. To resolve this discrepancy we tested the importance of this LXL-motif for FOXM1c with a new series of FOXM1c deletion mutants lacking this LXL-motif in the present study.

Finally, this study demonstrates for the first time that cyclin E/Cdk2 enhances whereas P/CAF (p300/CBP-associated factor) and E1A repress the transactivation of the *c-myc* promoter by FOXM1c and that P/CAF interacts with FOXM1c.

Materials and methods

Plasmids and antibodies. pFOXM1c(1–232), pGal(GAL4-DBD)-FOXM1c, pGal-FOXM1c(359–762), pGal-FOXM1c(721–762), pGST (glutathione-*S*-transferase)-FOXM1c(1–477) [7], pGal-FOXM1c(359–621; 671–762), pGal-FOXM1c(588–621; 671–762), pGal-FOXM1c(588–621; 671–696) [5], pmyc(−262/−92)luc, pmyc(−224/−92)luc, pmyc(−224/−136)luc, pmyc(−95/+49)luc, pmyc(−262/+49)luc, pmycluc, pmyc(−262/−92)mintkluc, and pmyc(−224/−92)mintkluc [6] were described previously. p(MBS)$_3$-mintk-luc, pXHmintkluc, pP2E7, pFOXM1c [14],and pFOXM1c(189–762) [15] were described before. The cloning of new plasmids is described in the Supplementary Material.

pGal0 (C. Dang), pCMVβ-p300 (R. Eckner, D.M. Livingston), pRSV-HA-CBP (R.H. Goodman), pGal4mintkluc (R. Janknecht), pCMV-HDAC1-myc, pP2E7ΔRB (B. Lüscher), pCX-FLAG-P/CAF (Y. Nakatani), pCMV-E1A-12S (J.R. Nevins), pCMVHAXhTAFII250 (R. Tjian), and the expression plasmids for cyclin D1, cyclin E, cyclin A, Cdk2, Cdk4, dn (dominant-negative) Cdk2, dn Cdk4 (R. Bernards), p27 (R. Bernards, J. Koh), and RB (R. Müller) were generous gifts. pGEX-3X was from Pharmacia.

α-FOXM1c(C-20) (sc-502) was purchased from Santa Cruz. α-Flag(M2) (F3165) was purchased from Sigma–Aldrich. The monoclonal rat antibody α-FOXM1c(1B1) was described before [7].

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 [8].

GST-fusion proteins, GST-pull-down assays, and Western blotting. Preparation of GST-fusion proteins, GST-pull-down assays and Western blotting were done as described previously [8].

Results

FOXM1c bound to the c-myc-P1 TATA-box cannot transactivate a heterologous downstream core promoter

By a new mechanism FOXM1c transactivates the *c-myc*-P1 promoter (pmyc(−224/−92)luc; Fig. 1A) directly via its TATA-box [6]. Normal transcription factors can act via their binding sites positioned 5′ of a core promoter independently of the orientation of these sites. Therefore, to examine whether FOXM1c functions as normal transcription factor at the *c-myc*-P1 TATA-box, the *c-myc*-P1 promoter was inserted 5′ of the non-FOXM1c-responsive minimal *TK* promoter of HSV (pXHmintkluc; Fig. 1A), but in the inverted orientation (pmyc(−92/−224)mintkluc; Fig. 1B), so that transcription of the *TK*-controlled coding sequence of luciferase would only occur if FOXM1c would activate the minimal *TK* promoter. FOXM1c failed to activate pmyc(−92/−224)mintkluc (Fig. 1A) and other similarly inverted reporter constructs (data not shown) demonstrating that FOXM1c, bound to the *c-myc*-P1 TATA-box, cannot transactivate a heterologous downstream core promoter as normal transcription factor. This result is surprising because FOXM1c transactivated the minimal *TK* promoter up to 568-fold from GAL4-binding sites (pGal4mintkluc) and up to 43-fold from conventional FOXM1c-binding sites (p(MBS)$_3$-mintk-luc) (Supplementary Fig. 1; [7]) excluding a general incompatibility of FOXM1c with this core promoter. Additionally, a

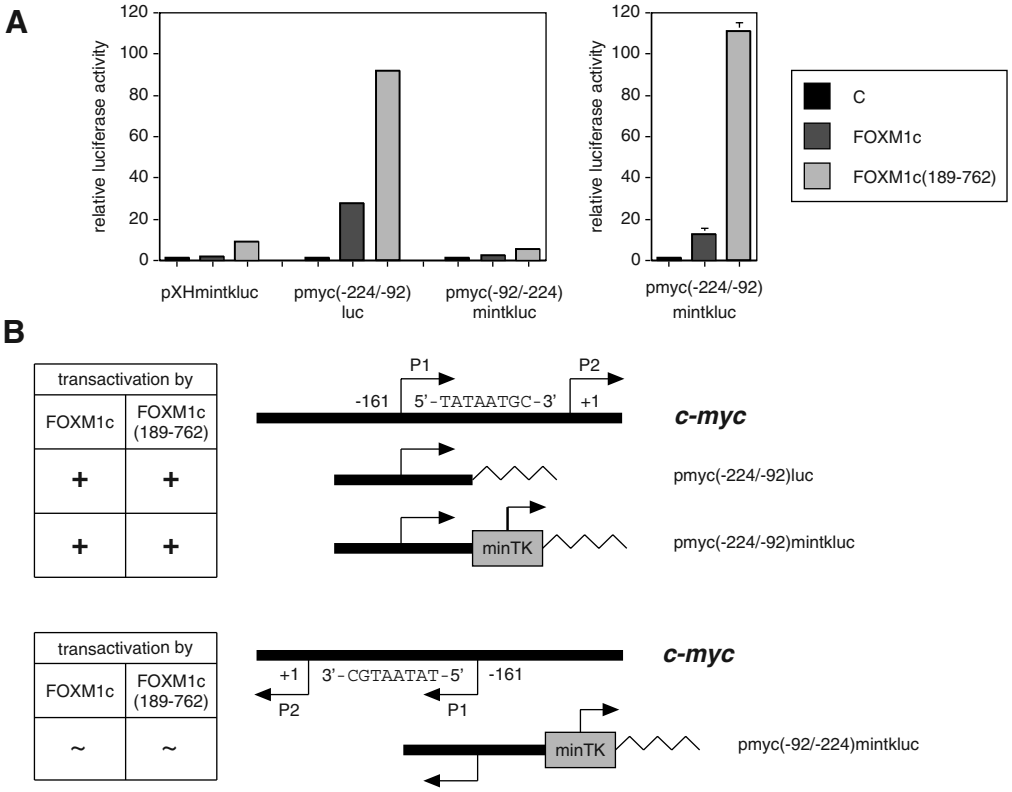

Fig. 1. FOXM1c bound to the *c-myc*-P1 TATA-box cannot transactivate a heterologous downstream core promoter. (A) RK-13 cells were transiently transfected with the indicated reporter constructs and pFOXM1c, pFOXM1c(189–762) or the empty vector (control C). The relative luciferase activity of each reporter construct in control C was set to 1. Each panel shows a different experiment. (B) Map of reporter constructs. *c-myc* promoter sequences are depicted as thick black lines, the minimal (min) *TK* promoter (−32 to +51) of HSV as light gray box, transcription start sites as arrows and the coding sequence of luciferase as zigzag line. The sequence of the *c-myc*-P1 TATA-box is indicated in letters. Numbers refer to the transcription start (+1) of the *c-myc*-P2 promoter. It is indicated whether the reporter constructs are transactivated (= +) or not (= ∼).

downstream minimal *TK* promoter (pmyc(−224/−92) mintkluc; Fig. 1B) did not interfere with the FOXM1c-mediated transactivation of the *c-myc*-P1 promoter (Fig. 1A). Thus, FOXM1c fulfills a special function in transactivation of the *c-myc*-P1 promoter via its TATA-box, but it does not function simply as normal transcription factor just binding to an unusual site.

The N-terminus of FOXM1c does not inhibit the EDA or the TRD

Next, it was examined whether the N-terminus FOXM1c inhibits also its central TRD (Fig. 2A and B) and its central EDA (Fig. 2C and D) in addition to its C-terminal TAD [6,7].

First, we analyzed whether full-length FOXM1c, in which the C-terminal TAD is completely inhibited by the N-terminus, can still transrepress through its central TRD (Fig. 2B). To distinguish active transrepression from passive competition with endogenous FOXM1 Gal-FOXM1c fusion proteins and a reporter construct with GAL4-binding sites (pGal4mintkluc) were used. To exclude a participation of RB the experiments were performed in RB-negative SAOS-2 cells. Full-length Gal-FOXM1c transrepressed efficiently in these cells (Fig. 2A) demonstrating that the N-terminus does not inhibit the RB-independent TRD of FOXM1c.

Second, to analyze whether the N-terminus inhibits the EDA we determined the transactivation by FOXM1c relative to that by FOXM1c(189–762) for all performed experiments (Fig. 2C; Supplementary Fig. 2). On each reporter construct, FOXM1c(189–762) transactivated strongly because it lacked the inhibitory N-terminus whereas the N-terminus inhibited the C-terminal TAD completely in FOXM1c [5–7]. Consequently, if the EDA is not inhibited by the N-terminus, full-length FOXM1c should retain the active central EDA on the *c-myc* P1 and P2 TATA-boxes (Fig. 2D) whereas it retained the active central TRD if it functioned as conventional transcription factor (Fig. 2A and B). Therefore, the residual transactivation by FOXM1c compared to that by FOXM1c(189–762) should be higher on the *c-myc* P1 and P2 promoters than on the conventional reporter construct p$(MBS)_3$-mintk-luc. Moreover, Sp1, which directly binds to the EDA of FOXM1c (Fig. 2D), synergizes with FOXM1c in transactivation of the *c-myc* P1 and P2 promoters, but not on conventional FOXM1c-binding sites [10]. Fig. 2C shows that the transactivation by FOXM1c was in average only 12% of the

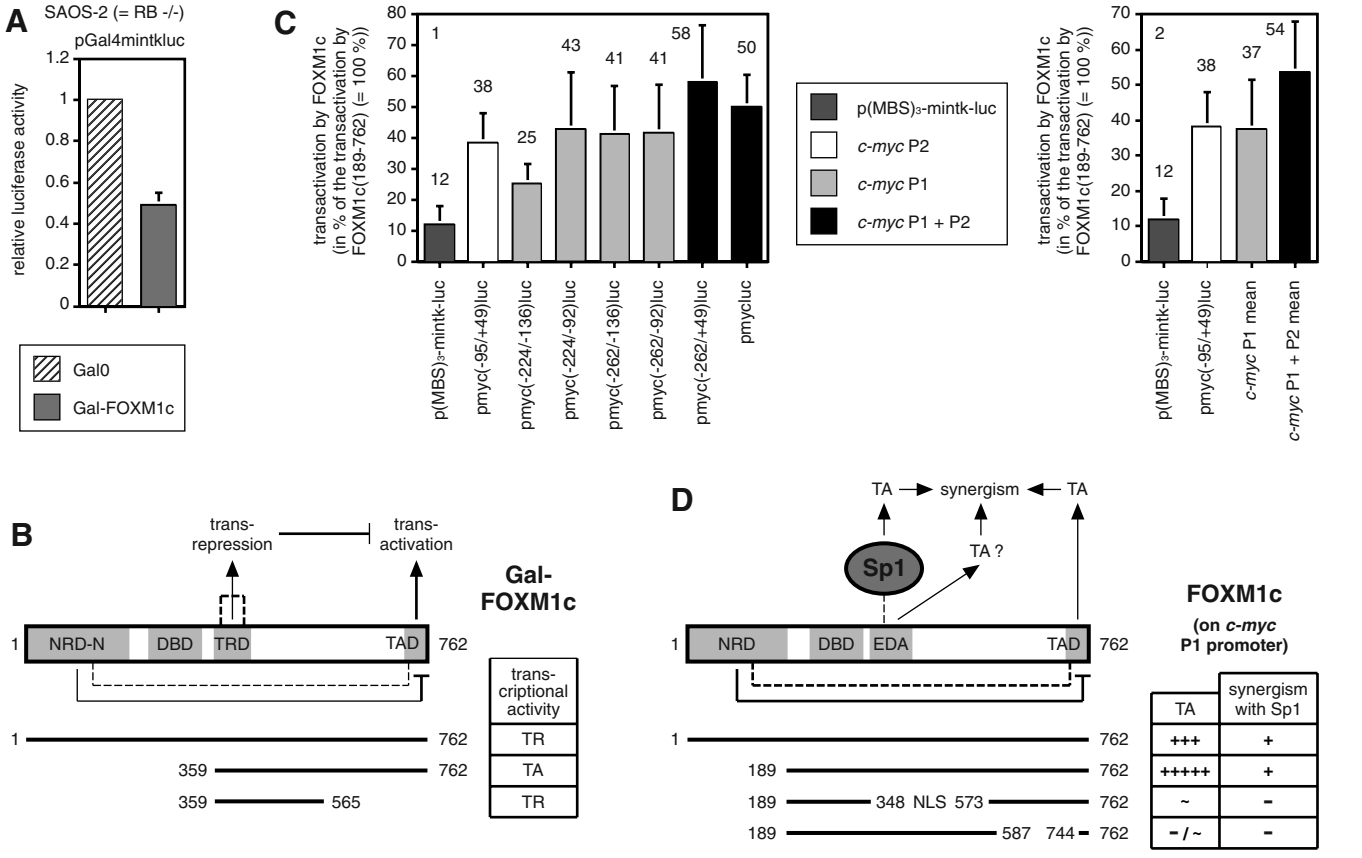

Fig. 2. The N-terminus of FOXM1c does not inhibit the EDA or the TRD. (A) The N-terminus does not inhibit the RB-independent TRD. RB-negative SAOS-2 cells were transiently transfected with pGal4mintkluc and pGal-FOXM1c or pGal0 (control). The relative luciferase activity of pGal4mintkluc in control Gal0 was set to 1. (B) Functional domains of FOXM1c as conventional transcription factor. The N-terminal GAL4-DBD of the Gal-FOXM1c fusion proteins is not shown. The table (data of panel A and [7,8]) summarizes whether the Gal-FOXM1c fusion proteins transactivated (TA) or transrepressed (TR) in RB-negative SAOS-2 cells. Broken lines indicate direct DNA-independent protein-protein interactions. DBD, DNA-binding domain; TAD, transactivation domain; TRD, transrepression domain; NRD, negative-regulatory domain. (C) The N-terminus does not inhibit the EDA. Shown is the transactivation by FOXM1c, displayed as percentage of the transactivation by FOXM1c(189–762), for the conventional FOXM1c reporter construct p(MBS)$_3$-mintk-luc (dark gray) and different *c-myc* promoter reporter constructs (see Supplementary Fig. 2) comprising only the *c-myc*-P1 promoter (light gray) or only the *c-myc*-P2 promoter (white) or both the *c-myc* P1 and P2 promoters (black). For measurements and calculations see legend to Supplementary Fig. 2. (D) Functional domains of FOXM1c on the *c-myc*-P1 promoter. In the left column (TA) (data of Fig. 1A and [6]), it is indicated whether the FOXM1c proteins transactivated the *c-myc*-P1 promoter very strongly (+++++) or strongly (+++) or repressed it (−) or neither transactivated nor repressed it (∼). In the right column (synergism with Sp1) (data of [10]), it is indicated whether the FOXM1c proteins transactivated the *c-myc*-P1 promoter synergistically with HA-Sp1 (+) or not (−). Broken lines indicate direct DNA-independent protein-protein interactions. TA, transactivation; EDA, essential domain for activation; NLS, heterologous nuclear localization signal of SV40 large T antigen.

transactivation by FOXM1c(189–762) for p(MBS)$_3$-mintk-luc. In contrast, the FOXM1c-mediated transactivation reached in average 37%, 38%, and 54% of the FOXM1c(189–762)-mediated transactivation for the *c-myc*-P1 or *c-myc*-P2 or *c-myc*-P1 + *c-myc*-P2 promoters, respectively (Fig. 2C; Supplementary Fig. 2). This significantly higher residual transactivation by full-length FOXM1c on the *c-myc* P1 and P2 TATA-boxes indicates that the EDA is not inhibited by the N-terminus of FOXM1c. In accordance, full-length FOXM1c transactivated pmyc(−224/−92)luc up to 30-fold (Fig. 1A), but p(MBS)$_3$-mintk-luc only maximally 3.3-fold (data not shown).

In summary, the N-terminus of FOXM1c inhibits its C-terminal TAD [5–7] but neither its central EDA (Fig. 2C) nor its central TRD (Fig. 2A).

P/CAF and E1A repress the transactivation of the human c-myc promoter by FOXM1c and P/CAF interacts with FOXM1c

The oncoprotein E7 of HPV (human papillomavirus) 16 binds directly to FOXM1c [14] and enhances the transactivation of the *c-myc* promoter by FOXM1c (Fig. 3A) [6]. HPV16 E7 disrupts E2F-pocket protein complexes and targets pocket proteins (RB, p107, and p130) for proteolytic degradation [16,17]. Therefore we asked whether binding of HPV16 E7 to RB is required for its positive effect on FOXM1c. Indeed, the mutant E7ΔRB, which does not bind RB, had no effect on the FOXM1c-mediated transactivation of the *c-myc* promoter (Fig. 3A) although it retained the FOXM1c-binding domain. Thus, binding of

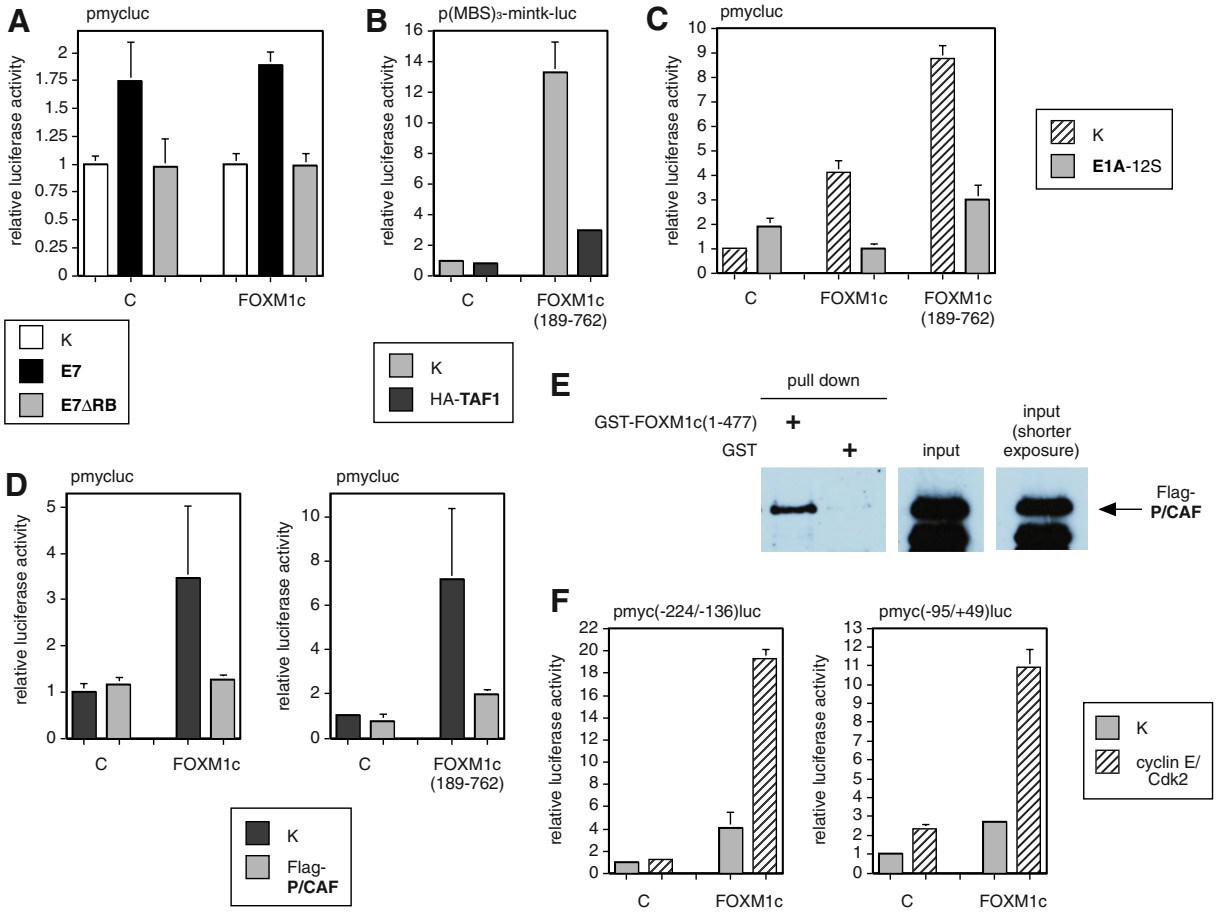

Fig. 3. Cyclin E/Cdk2, P/CAF and E1A regulate the transactivation of the *c-myc* promoter by FOXM1c. (A–D, F) RK-13 cells were transiently transfected with the indicated reporter constructs and pFOXM1c, pFOXM1c(189–762) or the empty vector (control C). Either expression plasmids for HPV16 E7, HPV16 E7ΔRB, HA-TAF1, E1A-12S, Flag-P/CAF or cyclin E/Cdk2 or the empty vectors (control K) were cotransfected. (A) The relative luciferase activity of pmycluc in control K was set to 1. (B–D, F) The relative luciferase activity of each reporter construct in the combination of control C with control K was set to 1. (F) The two panels show part of the same experiment. (E) P/CAF interacts with FOXM1c. GST-pull-down assays were performed with purified GST or GST-FOXM1c(1–477) and total cell lysates of COS-7 cells transiently transfected with the expression plasmid for Flag-P/CAF. Bound P/CAF was detected in a Western blot using α-Flag(M2) as primary antibody. The input control represents 1/40 of the volume used in the GST-pull-down assays.

HPV16 E7 to RB seems to be required for its stimulating effect on FOXM1c.

Like HPV16 E7, the related oncoprotein E1A of human adenovirus 5 also disrupts E2F-pocket protein complexes [16,17]. Therefore, we tested whether E1A enhances the FOXM1c-mediated transactivation of the *c-myc* promoter, too. Surprisingly, E1A-12S (Fig. 3C) and the larger splice variant E1A-13S (data not shown) considerably repressed the transactivation of the *c-myc* promoter by FOXM1c. In contrast to HPV16 E7 [14], an interaction between E1A and FOXM1c was not observed (data not shown), which may explain these opposite effects of the related viral oncoproteins E1A and E7 on FOXM1c.

Consequently, we asked how E1A could indirectly exert its negative effect on FOXM1c. E1A blocks the function of the coactivator and HAT (histone acetyltransferase) CBP (CREB (cAMP response element-binding protein)-binding protein) [18] that interacts with the splice variant FoxM1b [12]. However, CBP did not affect the FOXM1c-mediated transactivation of the *c-myc* promoter (Supplementary Fig. 3A). CBP recruits the HAT P/CAF, which binds to the same region of CBP as E1A, so that E1A displaces P/CAF from CBP [18,19]. If E1A represses FOXM1c through this displacement of P/CAF from CBP P/CAF should stimulate FOXM1c. In contrast, P/CAF significantly repressed the transactivation of the *c-myc* promoter by FOXM1c (Fig. 3D). In addition, P/CAF interacted with FOXM1c (Fig. 3E) suggesting that P/CAF might inactivate FOXM1c by acetylating it. Conversely, removal of this acetylation by a HDAC (histone deacetylase) would be expected to activate FOXM1c. The effect of HDAC1 on FOXM1c was tested with $p(MBS)_3$-mintk-luc because *c-myc* is one of the HDAC inhibitor-susceptible genes and because P/CAF and CBP had similar effects on the FOXM1c-mediated transactivation of both the *c-myc* promoter and $p(MBS)_3$-mintk-luc (Fig. 3D; Supplementary Fig. 3A and B). Surprisingly, HDAC1 reduced the transactivation of $p(MBS)_3$-mintk-luc by FOXM1c (Supplementary

Fig. 3B) suggesting that P/CAF may not acetylate FOXM1c to suppress its transcriptional activity, but may act through its interaction with FOXM1c.

Nevertheless, the negative effect of HDAC1 on FOXM1c could point to an activation of FOXM1c by another HAT. One obvious candidate is TAF1, which interacts with FOXM1c [6] and possesses HAT activity [20]. However, TAF1 significantly repressed the transactivation of p(MBS)$_3$-mintk-luc by FOXM1c (Fig. 3B).

Cyclin E/Cdk2 enhances the transactivation of the human c-myc P1 and P2 promoters by FOXM1c

Cyclin E/Cdk2 increased the FOXM1c-mediated transactivation of the conventional reporter construct p(MBS)$_3$-mintk-luc [8,9]. Therefore, we tested whether cyclin E/Cdk2 stimulates also the FOXM1c-mediated transactivation of the *c-myc* promoter. In fact, the key proliferation signal cyclin E/Cdk2 significantly enhanced the transactivation of the *c-myc* P1 and P2 promoters by FOXM1c (Fig. 3F).

The cyclin/Cdk-binding LXL-motif of FOXM1 is not required for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2

To test the importance of the cyclin/Cdk-binding LXL-motif of FOXM1 [12] for FOXM1c a new series of FOXM1c deletion mutants (Δ622–670) lacking this LXL-motif (aa (amino acid) 639–641 of FoxM1b, aa 653–655 of FOXM1c) and thus its essential second leucine (L-641 of FoxM1b, L-655 of FOXM1c) was analyzed (Supplementary Fig. 4D). Cyclin D1/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2 activate FOXM1c by releasing its TAD from repression by both its own N-terminus and RB so that they indirectly increase the transactivation potential

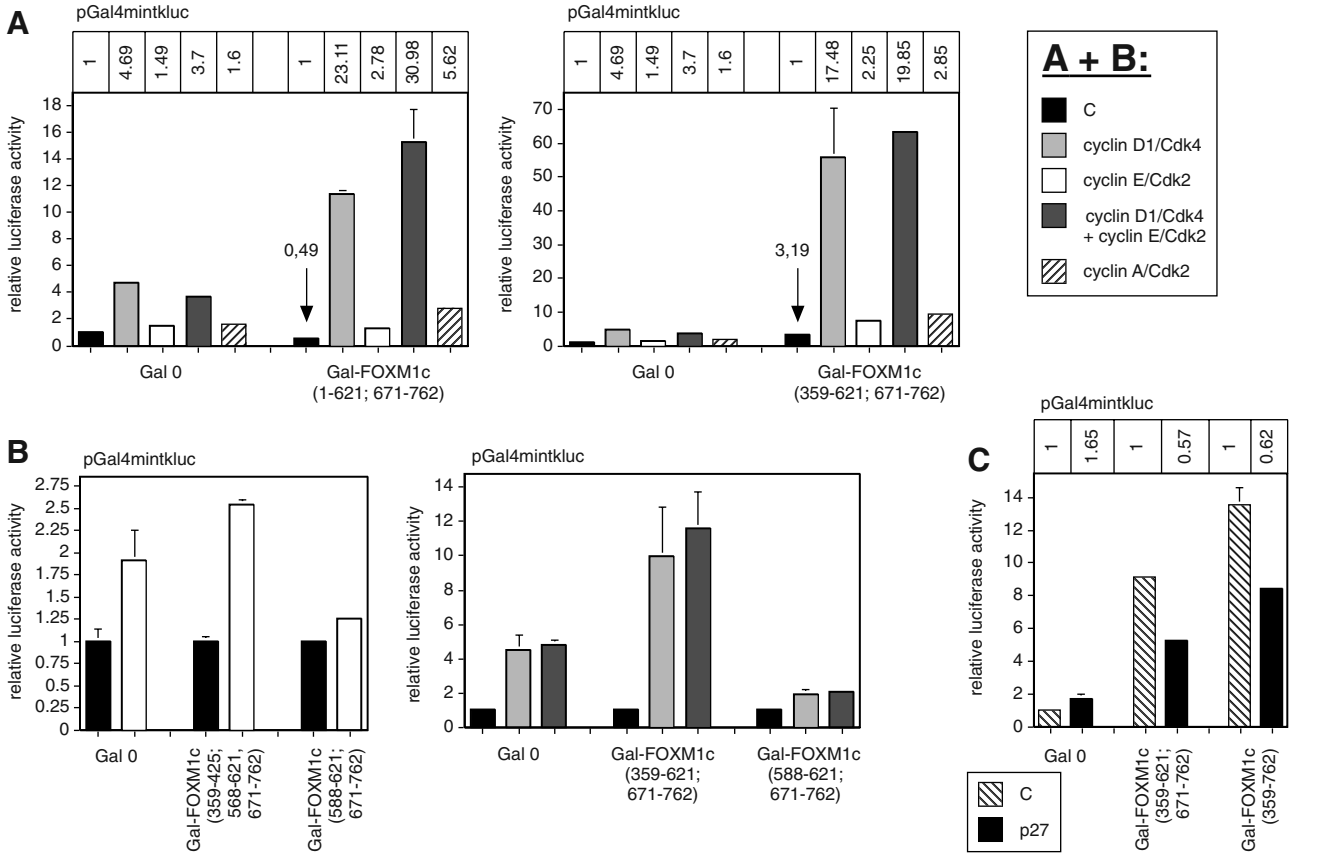

Fig. 4. (A and B) The cyclin/Cdk-binding LXL-motif of FOXM1 is not required for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2. RK-13 cells were transiently transfected with pGal4mintkluc and the indicated Gal-FOXM1c fusion proteins or pGal0 (control). Either expression plasmids for the indicated cyclins and Cdks or the empty vector (control C) were cotransfected. (A) The relative luciferase activity of pGal4mintkluc in the combination of control Gal0 with control C was set to 1. The numbers above the columns indicate the "fold activation by cyclin/Cdk complexes", i.e. the resulting values if the relative luciferase activity of pGal4mintkluc in control C is set to 1. The two panels show part of the same experiment. (B) The relative luciferase activity of pGal4mintkluc in control C was set to 1. The two panels show part of the same experiment. (C) The cyclin/Cdk-binding LXL-motif of FOXM1 is not required for the repression of FOXM1c by p27. RK-13 cells were transiently transfected with pGal4mintkluc and the indicated Gal-FOXM1c fusion proteins or pGal0 (control). Either the expression plasmid for p27 or the empty vector (control C) were cotransfected. The relative luciferase activity of pGal4mintkluc in the combination of control Gal0 with control C was set to 1. The numbers above the columns indicate the repression of these Gal-FOXM1c fusion proteins by p27, i.e. the resulting values if the relative luciferase activity of pGal4mintkluc in control C is set to 1.

of the TAD of FOXM1c [8,9]. Accordingly, neither cyclin D1/Cdk4 nor cyclin E/Cdk2 nor both together increased the DNA-binding by the DBD of FOXM1c [9]. Therefore, Gal-FOXM1c fusion proteins were used.

In all respects tested this new series of deletion mutants (Fig. 4; Supplementary Figs. 4 and 5) exhibited the same characteristics as their "wild-type" counterparts before [7–9]:

- The N-terminus and the central domain of FOXM1c repressed the TAD and thus functioned as IDs (Supplementary Fig. 4A). The N-terminus, which binds directly to the TAD [7], inhibited the TAD also in *trans* (Supplementary Fig. 4B). The TAD of FOXM1c was repressed by the tumor suppressor RB (Supplementary Fig. 4C), which binds directly to the central domain of FOXM1c [8].
- LXL-motif deletion mutants with (Gal-FOXM1c(1–621; 671–762)) and without (Gal-FOXM1c(359–621; 671–762)) the N-terminus were activated strongly by cyclin D1/Cdk4 as well as moderately by cyclin E/Cdk2 and cyclin A/Cdk2 (Fig. 4A) demonstrating that the cyclin/Cdk-binding LXL-motif of FOXM1 [12] is not required for the activation of FOXM1c by these three cyclin/Cdk complexes.
- Cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2 released the TAD of FOXM1c from its inhibition by the N-terminus of FOXM1c (Gal-FOXM1c(1–621; 671–762) in Fig. 4A) indicating that the cyclin/Cdk-binding LXL-motif of FOXM1 [12] is not required for this release.
- If the N-terminus was deleted (Gal-FOXM1(359–621; 671–762), Gal-FOXM1c(359–425; 568–621; 671–762)) the activation of FOXM1c by cyclin D1/Cdk4 and cyclin E/Cdk2 depended specifically on the presence of the central domain (aa 359–425) of FOXM1c (Fig. 4B; Supplementary Fig. 5A and B), which lacks any potential cyclin/Cdk phosphorylation site S/T-P (Supplementary Fig. 5D), and was lost in RB-negative SAOS-2 cells (Supplementary Fig. 5C). These findings demonstrate that cyclin D1/Cdk4 and cyclin E/Cdk2 activate FOXM1c, dependently on its central domain, by releasing the TAD of FOXM1c from its repression by RB through phosphorylation of RB, but that they do not directly activate FOXM1c or phosphorylate it. Thus, cyclin D1/Cdk4 and cyclin E/Cdk2 do not require the cyclin/Cdk-binding LXL-motif of FOXM1 [12] to release FOXM1c from its repression by RB.
- The stimulating effect of each cyclin/Cdk complex on Gal-FOXMc(1–621; 671–762) was higher than that on the N-terminally truncated Gal-FOXM1c(359–621; 671–762) (Fig. 4A) because the two stimulating effects of them, which depend on the N-terminus and the central domain of FOXM1c, are additive.

The cyclin/Cdk-binding LXL-motif of FOXM1 is not required for the repression of FOXM1c by p27

Finally, p27 (Fig. 4C), p21 and p16 (data not shown) repressed Gal-FOXM1c(359–621; 671–762) as efficiently as its "wild-type" counterpart Gal-FOXM1c(359–762) indicating that the cyclin/Cdk-binding LXL-motif of FOXM1 [12] is not required for the inhibition of FOXM1c by these three CKIs (cyclin-dependent kinase inhibitors).

In summary, the cyclin/Cdk-binding LXL-motif of FOXM1 [12] does not play any role for FOXM1c and is dispensable for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2.

Discussion

The potent proto-oncogene c-Myc drives cells through G1-phase and induces S-phase entry [21]. Accordingly, the key G1-phase proliferation signal cyclin E/Cdk2 enhanced the transactivation of the *c-myc* P1 and P2 promoters by FOXM1c (Fig. 3F).

On the *c-myc*-P1 TATA-box, FOXM1c does not function simply as normal transcription factor just binding to an unusual site (Fig. 1). Remarkably, the very strong acidic TAD of FOXM1c [7] was unable to transactivate a heterologous downstream core promoter if FOXM1c bound to the *c-myc*-P1 TATA-box (Fig. 1) whereas it transactivated the same core promoter very strongly if FOXM1c was recruited via conventional FOXM1c or GAL4 binding sites (Supplementary Fig. 1) [7]. We have previously suggested that the EDA may represent a TATA-box-specific proline-rich second TAD of FOXM1c [5] in addition to this acidic TAD. This view is strongly supported by the high residual transactivation of the *c-myc* P1 and P2 promoters by full-length FOXM1c (Fig. 2C), in which the acidic C-terminal TAD is inhibited by the N-terminus, so that only the central EDA is free to transactivate and to synergize with Sp1 (Fig. 2D). Nevertheless, neither the TAD nor the EDA of FOXM1c can separately transactivate the *c-myc* P1 and P2 promoters because FOXM1c mutant proteins with deletions of/in either domain failed to transactivate them (Fig. 2D) [5,6,10]. Since TADs normally represent independent functional domains this interdependence of the central EDA and the C-terminal TAD of FOXM1c confirms that FOXM1c does not function simply as normal transcription factor on the *c-myc* P1 and P2 TATA-boxes.

The N-terminus of FOXM1c, which inhibits its C-terminal TAD completely by binding to it directly [5–7], inhibits neither its central EDA (Fig. 2C) nor its central TRD (Fig. 2A and B). Consistently, the N-terminus did not interact with the central domain of FOXM1c [5,7] and the interaction of the N-terminus with the C-terminal TAD seems not to abolish the binding of the central domain to other proteins and itself because full-length FOXM1c bound efficiently to TBP, TFIIB, TAF1 [6], Sp1 [10], RB [8], and the central domain of FOXM1c [5].

HPV16 E7 enhances the FOXM1c-mediated transactivation of the *c-myc* promoter [6], which seems to require binding of HPV16 E7 to RB (Fig. 3A). In contrast, the related adenoviral oncoprotein E1A [16,17] repressed the transactivation the *c-myc* promoter by FOXM1c

(Fig. 3C). This was not due to inhibition of the coactivators and HATs CBP (Supplementary Fig. 3A) or P/CAF (Fig. 3D), two targets of E1A [18,19]. Instead, P/CAF interacted with FOXM1c (Fig. 3E) and repressed the FOXM1c-mediated transactivation of both the *c-myc* promoter (Fig. 3D) and the conventional reporter construct p(MBS)$_3$-mintk-luc (Supplementary Fig. 3B) suggesting that P/CAF might inactivate FOXM1c by acetylating it. However, three findings argue against acetylation of FOXM1c as cause for its repression by P/CAF. First, HDAC1 reduced the transactivation of p(MBS)$_3$-mintk-luc by FOXM1c (Supplementary Fig. 3B). Second, E1A, which inhibits the HAT-activity of P/CAF [19], diminished the transactivation of the *c-myc* promoter by FOXM1c (Fig. 3C) and the transcriptional activity of the splice variant FoxM1b [12]. Third, HPV16 E7 inhibits the HAT-activity of P/CAF and binds directly to its HAT-domain [22,23], but the mutant E7ΔRB failed to increase the FOXM1c-mediated transactivation of the *c-myc* promoter (Fig. 3A) although it retained both the zinc finger-region and amino acid 2 that are required for binding to and/or inhibition of the HAT P/CAF [22,23]. Also the HAT TAF1 inhibited the transactivation of p(MBS)$_3$-mintk-luc by FOXM1c (Fig. 3B). However, again, the negative effect of HDAC1 on the FOXM1c-mediated transactivation of p(MBS)$_3$-mintk-luc argues against acetylation of FOXM1c as cause for its inhibition by TAF1.

The cyclin/Cdk-binding LXL-motif of FOXM1 [12] is not required for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2 (Fig. 4A and B; Supplementary Fig. 5). This finding verifies our previous result that cyclin D1/Cdk4, cyclin E/Cdk2 and cyclin A/Cdk2 can activate FOXM1c without phosphorylating it because they release FOXM1c from its repression by RB through phosphorylation of RB, but not FOXM1c [8,9]. In addition, this finding rules out any requirement of the LXL-motif of FOXM1c to recruit these three cyclin/Cdk complexes for the phosphorylation of RB bound to the central domain of FOXM1c. Point mutation of leucine 641 of FoxM1b to alanine abolished its interaction with both Cdk2 [12] and p27 [3]. In clear contrast, the corresponding leucine 655 of FOXM1c is not required for its repression by p27 (Fig. 4C) or for its activation by cyclin E/Cdk2 and cyclin A/Cdk2 (Fig. 4A and B; Supplementary Fig. 5A). In summary, in contrast to the reports on FoxM1b [3,4,12], the cyclin/Cdk-binding LXL-motif of FOXM1 and thus its essential second leucine [12] do not play any role for FOXM1c and are completely dispensable for the activation of FOXM1c by cyclin D1/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2.

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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.2008.01.039.

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