Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 352 (2007) 61–68
BBRC
www.elsevier.com/locate/ybbrc

FOXM1c and Sp1 transactivate the P1 and P2 promoters of human *c-myc* synergistically

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

[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*

Received 25 October 2006
Available online 3 November 2006

Abstract

We have previously shown that FOXM1c transactivates the *c-myc* P1 and P2 promoters via their TATA-boxes by a new transactivation mechanism, namely by directly binding to the P1 and P2 TATA-boxes and to TBP, TFIIA, and TFIIB. We now confirm this surprising mechanism by demonstrating that FOXM1c transactivates the human *c-myc* P1 and P2 promoters synergistically with Sp1, a transcription factor known to bind and transactivate these two promoters. This synergism requires the P1 or P2 TATA-boxes as well as the respective Sp1-binding sites. Moreover FOXM1c binds directly to Sp1. Cooperative DNA binding, if it should occur, is not sufficient for synergism of Sp1 and FOXM1c at P1, but their contacts to multiple components of the basal transcription complex (TFIID, TFIIA, TFIIB) seem to be essential. However, FOXM1c does not synergize with Sp1 if it transactivates via its conventional binding site.

Keywords: FOXM1; Sp1; *c-myc*; Synergism; Cooperativity

c-Myc, a key factor in cell growth control, drives proliferation, inhibits differentiation, and can induce apoptosis [1]. It affects also cell growth, energy metabolism, cell adhesion, angiogenesis, immortality, metastasis, and genomic instability. Deregulated *c-myc* expression possesses a high transformation potential. Consequently, the *c-myc* promoter is tightly regulated by many transcription factors, signalling pathways, oncogenes, and tumor suppressors [2].

We have shown that FOXM1c transactivates the human *c-myc* P1 and P2 promoters via their TATA-boxes by a new transactivation mechanism [3]. Thereby its forkhead domain directly binds to the P1 and P2 TATA-boxes and its central domain directly binds to TATA-binding protein (TBP), transcription factor IIA (TFIIA), and TFIIB. We postulate that each promoter with a *c-myc*-P2 TATA-box TATAAAAG is a FOXM1c target gene. This was evidenced for the human *c-fos*, *hsp70*, and *histone H2B/a* promoters [3]. Additionally, FOXM1c can also function as conventional transcription factor [4–6]. These two different transactivation mechanisms of FOXM1c are clearly distinguished by its central domain, which is essentially required for the transactivation via TATA-boxes [3] but acts as inhibitory domain if FOXM1c transactivates via conventional FOXM1c-binding sites [4–6]. FOXM1 stimulates proliferation by promoting entry into S- and M-phase, regulates genes that control G1/S- and G2/M-transition, is regulated by proliferation and antiproliferation signals, and appears to be implicated in tumorigenesis [3,4,6,7].

Sp1 also transactivates the human *c-myc* P1 and P2 promoters [8,9]. The human and murine *c-myc* promoters possess five Sp1-binding sites: CT-element, distal and −44 upstream of P1, ME1a2 and CT-I_2 (ME1a1) upstream of P2 [8–11]. −44 is sufficient for strong transactivation of P1 by Sp1 [8]. CT-I_2 is essential for transactivation of P2 by Sp1 [9].

Sp1 possesses two strong transactivation domains (TADs), which both directly bind to TBP and TBP-associ-

* Corresponding author. Fax: +49 511 883536.
E-mail address: iwiwiwi@web.de (I. Wierstra).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.151

ated factor (TAF) 4 [12,13]. These domains A and B are required for transactivation via one Sp1-binding site and for synergistic transactivation via two or more Sp1-binding sites [14–16]. Domain D, which lacks any own transactivation potential, is only required for the latter. Domain C has a very low transactivation potential, but is never required. Sp1 binds also directly to TAF7 [17]. The ubiquitous Sp1, which is regulated by phosphorylation, glycosylation, and acetylation, is targeted by key regulators of proliferation (E2F-1, c-Myc, p53, TGF-β/Smads) to regulate genes with central functions in proliferation (*p21*, *p15*, *dhfr*, *tk*) and tumorigenesis (*tert*) [18].

Transcription factors can synergize in gene activation in two ways [19]: (1) They can cooperatively bind to their binding sites. (2) They can synergistically recruit subcomplexes of the basal transcription complex by simultaneously contacting multiple of its components.

The new transactivation mechanism, by which FOXM1c transactivates the *c-myc* P1 and P2 promoters via their TATA-boxes [3], is provocative. To verify its biological importance, we show in the present study that FOXM1c transactivates both P1 and P2 synergistically with Sp1, a factor known to bind and transactivate these two promoters. Furthermore, FOXM1c and Sp1 interact directly.

Materials and methods

Plasmids and antibodies. pFOXM1c(189–263; 297–762), pFOXM1c(189–348; 573–762)NLS, pFOXM1c(189–425; 568–762), pGal-FOXM1c(195–762), pBS-FOXM1c(189–762), pGST-FOXM1c(195–596), pGST-FOXM1c(1–477), pGST-FOXM1c(1–379) [5], pGST-FOXM1c(359–565), pmyc(−224/−136)luc, pmyc(−95/+49)luc, pTATA-P1-luc, pTATA-P2-luc, p(−44)mintkluc [3], and expression plasmids for FOXM1c, GST-FOXM1c(233–334) [20], FOXM1c(189–762), FOXM1c(189–587; 744–762), and FOXM1c(1–347; 574–762) [21] were described previously. pCIneo-HA-Sp1, pCIneo-HA-Sp1(1–611), pCIneo-HA-Sp1(612–778), and the expression plasmid for GST-Sp1 were gifts of H. Rotheneder and C. Seiser. pCIneo is from Promega, pGEX-3X from Pharmacia. Cloning of plasmids is described in the Supplementary Material.

α-FOXM1c(C-20) (sc-502) was purchased from Santa Cruz Biotech, α-HA(3F10) from Boehringer. α-GST is a monoclonal rat antibody. α-Sp1 was a gift of G. Suske.

Cell culture, transient transfections, luciferase reporter gene assays, and whole cell lysates. Cell culture, transient transfections, luciferase reporter gene assays, and whole cell lysates were performed as described before [4].

GST fusion proteins, in vitro transcription/translation, GST pull downs, co-immunoprecipitations, and Western blotting. Preparation of GST fusion proteins, *in vitro* transcription/translation, GST pull downs, co-immunoprecipitations, and Western blotting were done as described previously [4].

Electrophoretic mobility shift assays (EMSAs). EMSAs are described in the Supplementary Material.

Results

FOXM1c and Sp1 transactivate the c-myc P1 and P2 promoters synergistically

FOXM1c transactivates the minimal human *c-myc* P1 and P2 promoters, including only TATA-box and transcription start, via their TATA-boxes by directly binding to these TATA-boxes as well as to TBP, TFIIA, and TFIIB [3]. To confirm this new surprising transactivation mechanism we tested whether FOXM1c synergizes in transactivation of the *c-myc* P1 and P2 promoters with Sp1 (Fig. 1), which is known to bind and transactivate both promoters [8–11].

pmyc(−224/−136)luc, i.e. the P1 promoter, possesses the Sp1-binding site −44 [8–10] (Supplementary Fig. 3A). pmyc(−95/+49)luc, i.e. the P2 promoter, possesses the Sp1-binding site CT-I_2 [9–11] and the potential Sp1-binding site −66, a typical GC-box (GGCGGG). Thus Sp1 could bind to CT-I_2 or/and −66. pTATA-P1-luc, i.e. the minimal *c-myc*-P1 promoter, lacking any Sp1-binding site (Supplementary Fig. 3A) was used as negative control. Accordingly, HA-Sp1 transactivated (−224/−136)luc and pmyc(−95/+49)luc (in correlation with previous studies [8,9]), but not pTATA-P1-luc (Fig. 1). The transactivation by HA-Sp1 was rather low because of the high amounts of endogenous Sp1 in RK-13 cells. All three reporter constructs were strongly transactivated by FOXM1c(189–762) (Fig. 1). This deletion mutant was used because the N-terminal negative-regulatory domain (NRD; amino acid (aa) 1–188) of FOXM1c inhibits the transactivation via the *c-myc* P1 and P2 TATA-boxes [3].

Synergism of transcriptional activators occurs if two activators give transcriptional levels that are greater than the sum of the transcriptional levels from the individual activators, which would indicate their functional independence [19]. FOXM1c(189–762) and HA-Sp1 together transactivated pmyc(−224/−136)luc and pmyc(−95/+49)luc considerably greater than the sum of the transactivations by each factor alone (Fig. 1A and C). Consequently, both pmyc(−224/−136)luc and pmyc(−95/+49)luc were synergistically transactivated by FOXM1c(189–762) and HA-Sp1. In contrast, the transactivation of pTATA-P1-luc by these two transcription factors together was approximately equal to the sum of their individual (non-)transactivations indicating their functional independence (Fig. 1B).

Thus in fact, FOXM1c(189–762) and HA-Sp1 transactivated the human *c-myc* P1 and P2 promoters synergistically (Fig. 1A and C). This finding strongly verifies the biological importance of the new transactivation mechanism of FOXM1c via the *c-myc* P1 and P2 TATA-boxes [3].

Herschlag and Johnson [19] defined three different energetic classes of synergism: (1) Synergism with independent energetic effects occurs if two activators give transcriptional levels that are equal to the product of the transcriptional levels from the individual activators, i.e. if transcription is multiplicative. (2) Positive cooperativity occurs if two activators give transcriptional levels that are greater than the product of the transcriptional levels from the individual activators, i.e. if transcription is greater than multiplicative. (3) Negative cooperativity occurs if transcription is smaller than multiplicative but greater than additive. According to this definition of Herschlag and Johnson [19] the synergism of FOXM1c(189–762) and HA-Sp1 in transactivation of

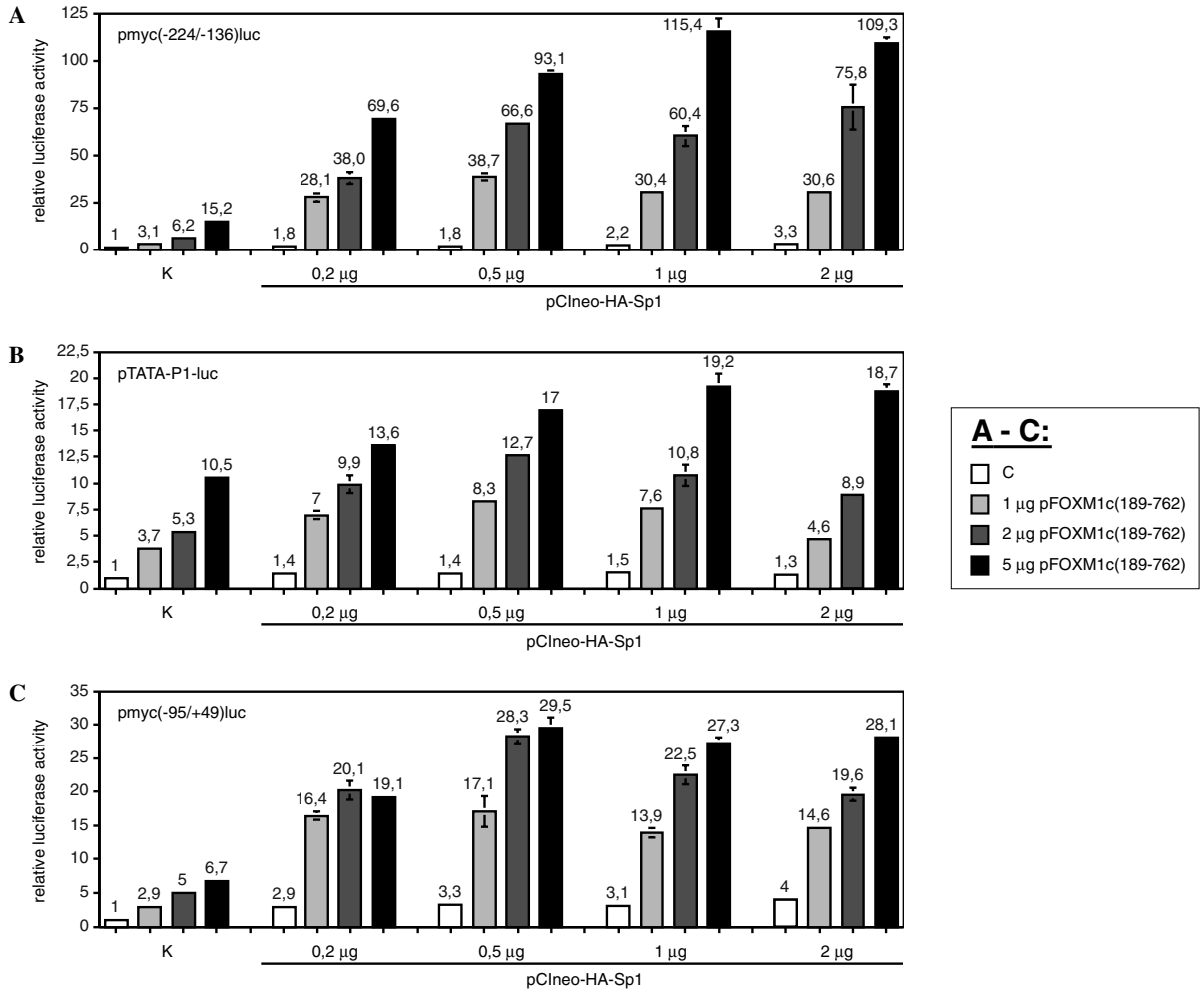

Fig. 1. Synergistic transactivation of the *c-myc* P1 and P2 promoters by FOXM1c and Sp1. RK-13 cells were transiently transfected with reporter constructs, pFOXM1c(189–762) and pCIneo-HA-Sp1, as indicated. The relative luciferase activity of each reporter construct in the combination of control (C) with control (K), i.e. in the absence of both pFOXM1c(189–762) and pCIneo-HA-Sp1, was set as 1. An overview of the proximal human *c-myc* promoter and these reporter constructs is shown in Supplementary Fig. 3A.

the *c-myc* P1 and P2 promoters belongs to two different energetic classes: positive cooperativity at P1 versus synergism with independent energetics effects at P2 (Supplementary Fig. 1). This finding that the transactivation by FOXM1c(189–762) and HA-Sp1 together was even considerably greater than (P1) or approximately equal to (P2) the product of the individual transactivations by each factor alone demonstrates that their synergism in transactivation of the *c-myc* P1 and P2 promoters is very strong.

Binding of Sp1 to the c-myc-P1 promoter

The Sp1-binding site −44 of the *c-myc*-P1 promoter (−44) was bound by HA-Sp1, HA-Sp1(612–778) and endogenous Sp1 (Fig. 2A). Specific DNA binding was demonstrated by supershifts with specific (α-Sp1, α-HA) but not control (α-GST) antibodies (Fig. 2A) and by competitions with unlabeled specific (−44) but not control (CMD) oligonucleotides (Fig. 2B). HA-Sp1 bound also to the Sp1-binding site GC-box III of the simian virus 40 (SV40) *early* promoter (SV40), but neither to a point mutated version of −44 (−44mut) nor to the *c-myc*-P1 (P1) or *c-myc*-P2 (P2) TATA-boxes (Fig. 2B; Supplementary Fig. 2).

In summary, in the *c-myc*-P1 promoter Sp1 bound to −44 [8–10] but not to the P1 TATA-box (Fig. 2) while vice versa FOXM1c bound to the P1 TATA-box [3] but not to −44 (data not shown). In the *c-myc*-P2 promoter Sp1 bound to CT-I_2 [9–11] or/and −66 but not to the P2 TATA-box (Fig. 2B) while FOXM1c bound to the P2 TATA-box, but not to −66 [3].

The Sp1-binding site −44 and the minimal P1 promoter are required for synergism of FOXM1c and Sp1 in transactivation of P1

To study the importance of −44 and the minimal P1 promoter for synergism of FOXM1c(189–762) and HA-Sp1 at P1 these two elements were varied (Fig. 3A–D; Supplementary Fig. 3B and C). HA-Sp1 bound to the Sp1-binding site GC-box III (Fig. 2B), which functions in oppo-

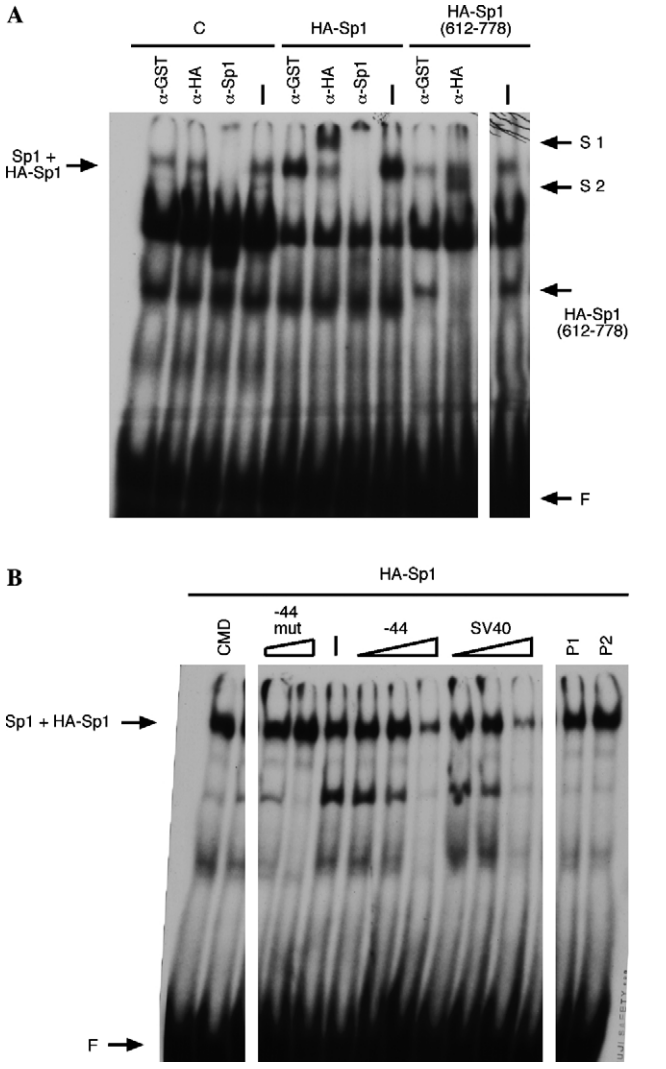

Fig. 2. Binding of Sp1 to the *c-myc*-P1 promoter. EMSAs were performed with radioactively labeled oligonucleotide −44 and with total cell lysates of COS-7-cells transiently transfected with expression plasmids for HA-Sp1 or HA-Sp1(612–778) or as control (C) with the empty vector. (A) For supershifts the antibodies α-GST, α-HA, and α-Sp1 were used. The protein–DNA-complexes of HA-Sp1, HA-Sp1(612–778) and endogenous Sp1 with −44 were supershifted by the specific antibodies α-Sp1 (recognizing HA-Sp1 and endogenous Sp1) and α-HA (recognizing the two exogenous HA-tagged Sp1 proteins), but not by the control antibodies α-GST (not recognizing all three Sp1 proteins) or α-HA (not recognizing endogenous Sp1). HA-Sp1(612–778) possesses the complete DNA-binding domain (DBD) of Sp1, i.e. the three zinc fingers (Supplementary Fig. 5A). (B) For competitions unlabeled oligonucleotides were used in excess: −44 and SV40: 5-, 20-, 100-fold; −44mut: 20-, 100-fold; CMD, P1 and P2: 100-fold. S, supershift; F, free probe. A summary of this DNA binding by HA-Sp1 is shown in Supplementary Fig. 2.

site orientations in both the SV40 *early* (Supplementary Fig. 2) and *late* (Supplementary Fig. 3C) promoters. Fig. 3A and B shows that FOXM1c(189–762) and HA-Sp1 together transactivated pmyc(−224/−136)luc and pGCIII-P1-luc synergistically. In contrast, they functioned independent in transactivation of p(−44)mintkluc (Fig. 3C), which has a non-FOXM1c-responsive minimal promoter [3], as well as of pSp1mut-P1-luc (Fig. 3D) and pTATA-P1-luc (Fig. 1B), which have no Sp1-binding site (Supplementary Fig. 3B and C). Consequently, synergism at P1 requires both −44 and the FOXM1c-responsive minimal *c-myc*-P1 promoter. pGCIII-P1-luc demonstrated that −44 is exchangable against another Sp1-binding site without loss of synergism at P1 (Fig. 3B).

The Sp1-binding sites CT-I_2 or/and −66 and the minimal P2 promoter are required for synergism of FOXM1c and Sp1 in transactivation of P2

Similarly, also the importance of CT-I_2 or/and −66 and the minimal P2 promoter for synergism of FOXM1c(189–762) and HA-Sp1 at P2 was examined (Fig. 3E; Supplementary Fig. 3B and C). In the hybrid promoter p(−44)-P2-luc the P2 region with CT-I_2 and −66 was replaced by the P1 region with −44 (Supplementary Fig. 3B and C). pTATA-P2-luc, i.e. the minimal *c-myc*-P2 promoter, lacking any Sp1-binding site (Supplementary Fig. 3) was used as negative control. Both p(−44)-P2-luc and pTATA-P2-luc were strongly transactivated by FOXM1c(189–762) whereas HA-Sp1 transactivated only the former one (Fig. 3E).

Like pmyc(−224/−136)luc (Fig. 3A) and pmyc(−95/+49)luc, p(−44)-P2-luc was synergistically transactivated by FOXM1c(189–762) and HA-Sp1 demonstrating that their synergism at P2 is not lost if CT-I_2 or/and −66 are exchanged against another Sp1-binding site (Fig. 3E). In contrast, FOXM1c(189–762) and HA-Sp1 functioned independent in transactivation of pTATA-P2-luc (Fig. 3E), which has no Sp1-binding site, and of p(−44)mintkluc (Fig. 3C), which has a non-FOXM1c-responsive minimal promoter [3], but the same Sp1-binding site as p(−44)-P2-luc (Supplementary Fig. 3B and C). Thus, synergism at P2 requires both CT-I_2 or/and −66 and the FOXM1c-responsive minimal *c-myc*-P2 promoter.

Since each promoter with a *c-myc*-P2 TATA-box TATAAAAG is postulated to be transactivated by FOXM1c [3] it can be postulated that each promoter with both a Sp1-binding site and a *c-myc*-P2 TATA-box is synergistically transactivated by FOXM1c and Sp1. A database search revealed more than 100 such target genes (Supplementary Fig. 7).

FOXM1c as a conventional transcription factor does not transactivate synergistically with Sp1

Next, we tested whether FOXM1c transactivates also synergistically with Sp1 if it functions as a conventional transcription factor [4–6] (Fig. 4). FOXM1c(189–762) transactivated via its conventional binding site HFH-11 (Fig. 4A; Supplementary Fig. 3D) as a conventional transcription factor [5]. The transactivation by Gal-FOXM1c(195–762) via GAL4-binding sites (Fig. 4B; Supplementary Fig. 3D) mimicked this mechanism [5]. The FOXM1c proteins did not transactivate pSp1-(HFH-11)-mintkluc (Fig. 4A) or p(−44)-Gal-mintkluc (Fig. 4B) syn-

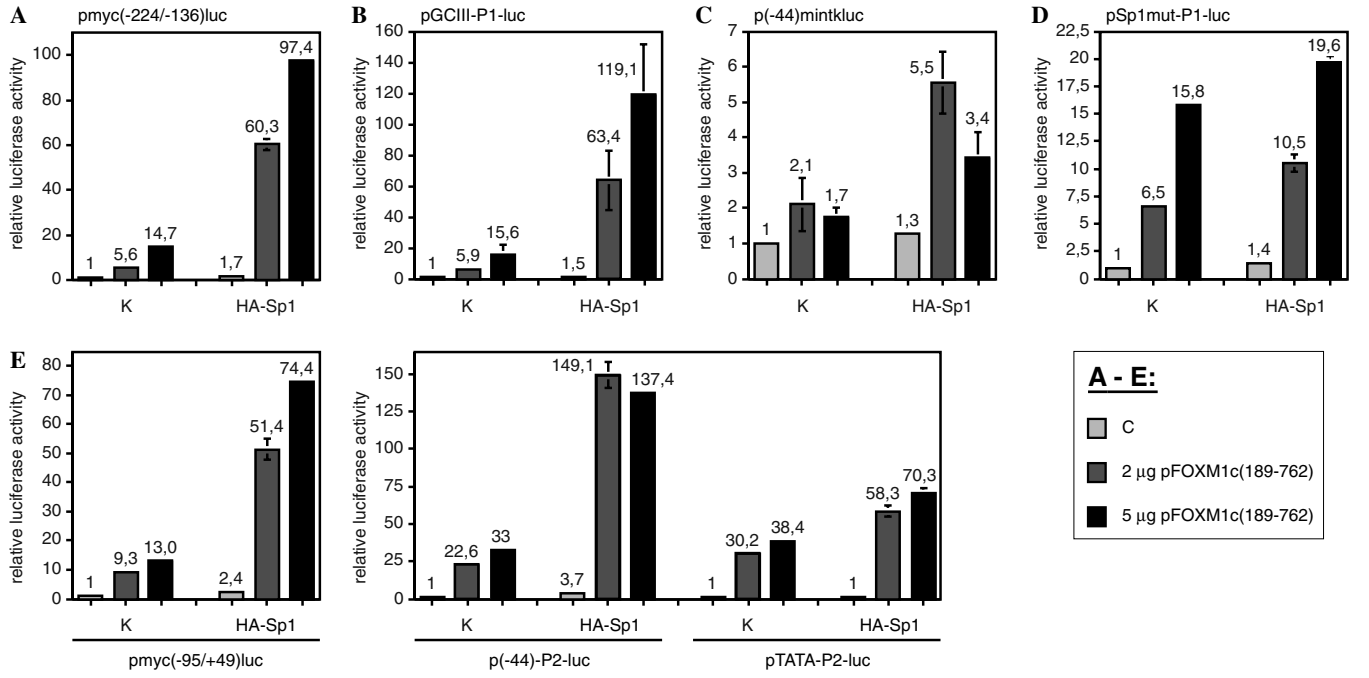

Fig. 3. *c-myc* promoter sequences required for synergism of FOXM1c and Sp1 in P1 and P2 transactivation. RK-13 cells were transiently transfected with reporter constructs, pFOXM1c(189–762) and the expression plasmid for HA-Sp1, as indicated. The relative luciferase activity of each reporter construct in the combination of control (C) with control (K), i.e. in the absence of both pFOXM1c(189–762) and HA-Sp1, was set as 1. Sp1-binding sites, minimal promoters, and nucleotide sequences of these reporter constructs are shown in Supplementary Fig. 3B and C.

ergistically with HA-Sp1, but were functionally independent. Consequently, FOXM1c does not synergize with Sp1 if it functions as a conventional transcription factor, i.e. if it transactivates via conventional FOXM1c-binding sites.

FOXM1c binds directly to Sp1

Synergism of two transcriptional activators can be caused by their direct interaction [19]. Indeed, FOXM1c bound directly to Sp1 (Fig. 5A). Their interaction was also demonstrated *in vivo* (Supplementary Fig. 4A). Sp1 bound to aa 380–425 of FOXM1c, but neither to aa 1–379 nor to aa 574–762 (Fig. 5B, C, E; Supplementary Fig. 4B). Thus the Sp1 interaction domain of FOXM1c (aa 380–425) is positioned in the central domain, namely in the essential domain for activation (EDA) (Supplementary Fig. 4B). FOXM1c bound to aa 612–778 of Sp1, but not to aa 1–611 (Fig. 5D). Thus the FOXM1c interaction domain of Sp1 (aa 612–778) is positioned in the C-terminus, i.e. in the DNA-binding domain (DBD) or/and domain D (Supplementary Fig. 5A).

Sp1 domains required for synergism with FOXM1c at P1

Two transcriptional activators, which interact directly, can synergize through cooperative DNA binding. But even if they do not interact directly, they can transactivate synergistically by simultaneously binding to multiple components of a single basal transcription complex [19]. Therefore we tested whether cooperative DNA binding by FOXM1c and Sp1 is sufficient for their synergism on pmyc(−224/−136)luc or whether the two TADs A and B of Sp1 and thus its direct interaction with TAF4 and TBP [12–16] is required (Fig. 6A; Supplementary Fig. 5A). HA-Sp1(612–778) should be able to bind DNA cooperatively with FOXM1c because it bound to both FOXM1c (Fig. 5D and E) and −44 (Fig. 2A). However, because of deletion of the two TADs A and B and of domain C it has no transactivation potential (Supplementary Fig. 5A), which was demonstrated with the respective GAL4-DBD fusion protein (data not shown) and Sp1(611–778) [14,15]. In contrast to HA-Sp1, HA-Sp1(612–778) did not synergize with FOXM1c(189–762) on pmyc(−224/−136)luc (Fig. 6A; Supplementary Fig. 5A). This could not be explained by slightly different expression levels (Supplementary Fig. 5B). Consequently, cooperative DNA binding of HA-Sp1 and FOXM1c(189–762), suggested by their direct interaction (Fig. 5A), is not sufficient for their synergism at P1, but direct binding of the Sp1-TADs A and B to TAF4 and TBP is required. Nevertheless, cooperative DNA binding of Sp1 and FOXM1c may occur and contribute to their synergism.

FOXM1c domains required for synergism with Sp1 at P1

Several FOXM1c proteins, whose expression levels were compared previously [5], were tested for their ability to

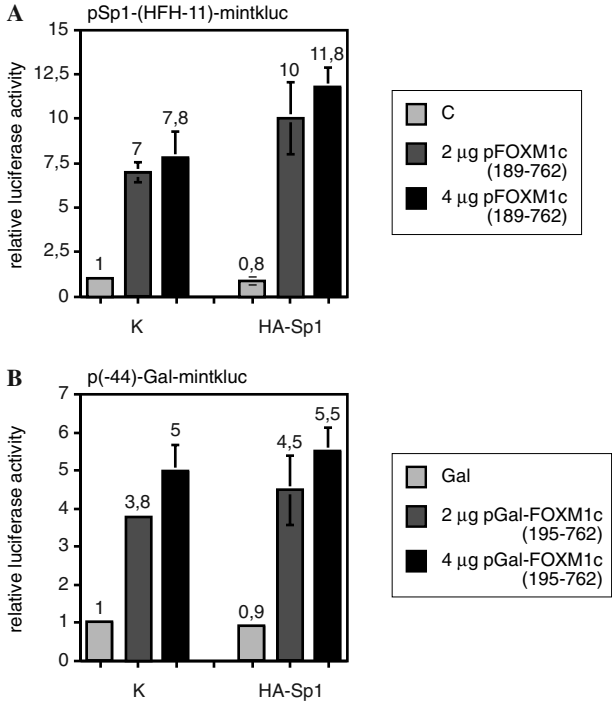

Fig. 4. FOXM1c does not transactivate synergistically with Sp1 if it functions as a conventional transcription factor. RK-13 cells were transiently transfected with reporter constructs, the expression plasmid for HA-Sp1 and (A) pFOXM1c(189–762) or (B) pGal-FOXM1c(195–762) or the expression plasmid for Gal as indicated. (A) The relative luciferase activity of pSp1-(HFH-11)-mintkluc in the combination of control (C) with control (K), i.e. in the absence of both pFOXM1c(189–762) and HA-Sp1, was set as 1. (B) The relative luciferase activity of p(−44)-Gal-mintkluc in the combination of control (Gal) with control (K), i.e. in the absence of both pGal-FOXM1c(195–762) and HA-Sp1, was set as 1. Gal-FOXM1c(195–762) is a fusion protein of the GAL4-DBD and aa 195–762 of FOXM1c. (A,B) A map of these reporter constructs is shown in Supplementary Fig. 3D.

transactivate pmyc(−224/−136)luc synergistically with HA-Sp1 (Fig. 6B and C; Supplementary Fig. 5C). Both transactivation of pmyc(−224/−136)luc by FOXM1c and its synergism with HA-Sp1 on pmyc(−224/−136)luc were abolished by either deletion of part of the DBD or deletion of part of the TAD (Supplementary Fig. 5C; data not shown) or deletion of the EDA (aa 349–425; Fig. 6B; Supplementary Fig. 5C), which is essential for transactivation via the *c-myc* P1 and P2 TATA-boxes [3]. The wildtype FOXM1c transactivated pmyc(−224/−136)luc synergistically with HA-Sp1 (Fig. 6C) demonstrating that the NRD, which inhibits the transactivation via the P1 and P2 TATA-boxes [3] but not the interaction with Sp1 (Fig. 5C and E; Supplementary Fig. 4B), does not affect the synergism with Sp1 at P1 (Supplementary Fig. 5C). Consequently, the same three components of FOXM1c are required for transactivation of the minimal *c-myc*-P1 promoter [3] and for synergism with Sp1 at P1 (Fig. 6B and C; Supplementary Fig. 5C): (a) the intact DBD, (b) the intact TAD, (c) the EDA, which directly binds to Sp1 (Fig. 5; Supplementary Fig. 4B), TBP, TFIIB, and (probably) TFIIA [3].

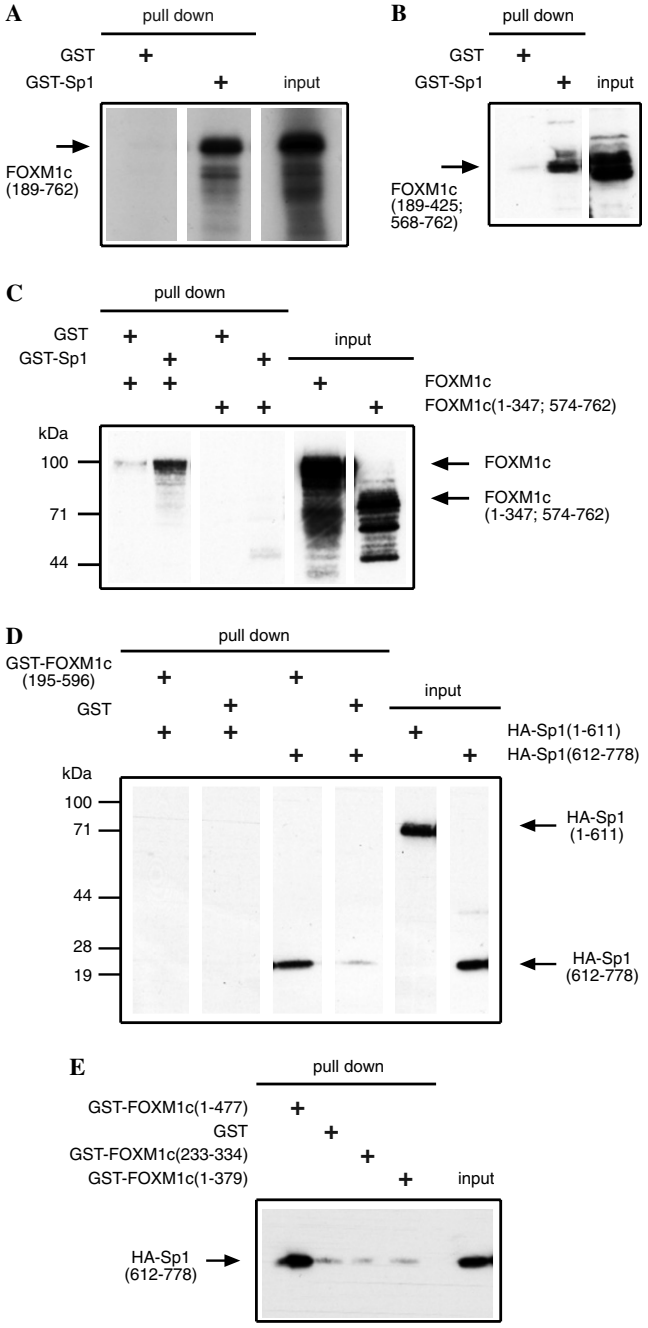

Fig. 5. Direct binding of FOXM1c to Sp1. Pull down-assays were performed with purified GST or the indicated GST-fusion proteins and *in vitro*-translated FOXM1c(189–762) (in the presence of ethidium bromide) (A) or total cell lysates of COS-7-cells transiently transfected with expression plasmids for the indicated proteins (B–E). Bound proteins were detected by autoradiography (A) or in Western blots using α-FOXM1c (B,C) or α-HA (D,E) as primary antibody. The input control represents 1/10 (A), 1/40 (B,C) or 1/50 (D,E) of the volume used in the pull down-assays. A map of FOXM1c and a summary of this mapping of the Sp1-interaction domain of FOXM1c are shown in Supplementary Fig. 4B.

Discussion

FOXM1c transactivates the human *c-myc* P1 and P2 promoters via their TATA-boxes by binding to them and to TBP, TFIIA, and TFIIB [3]. To confirm this surprising

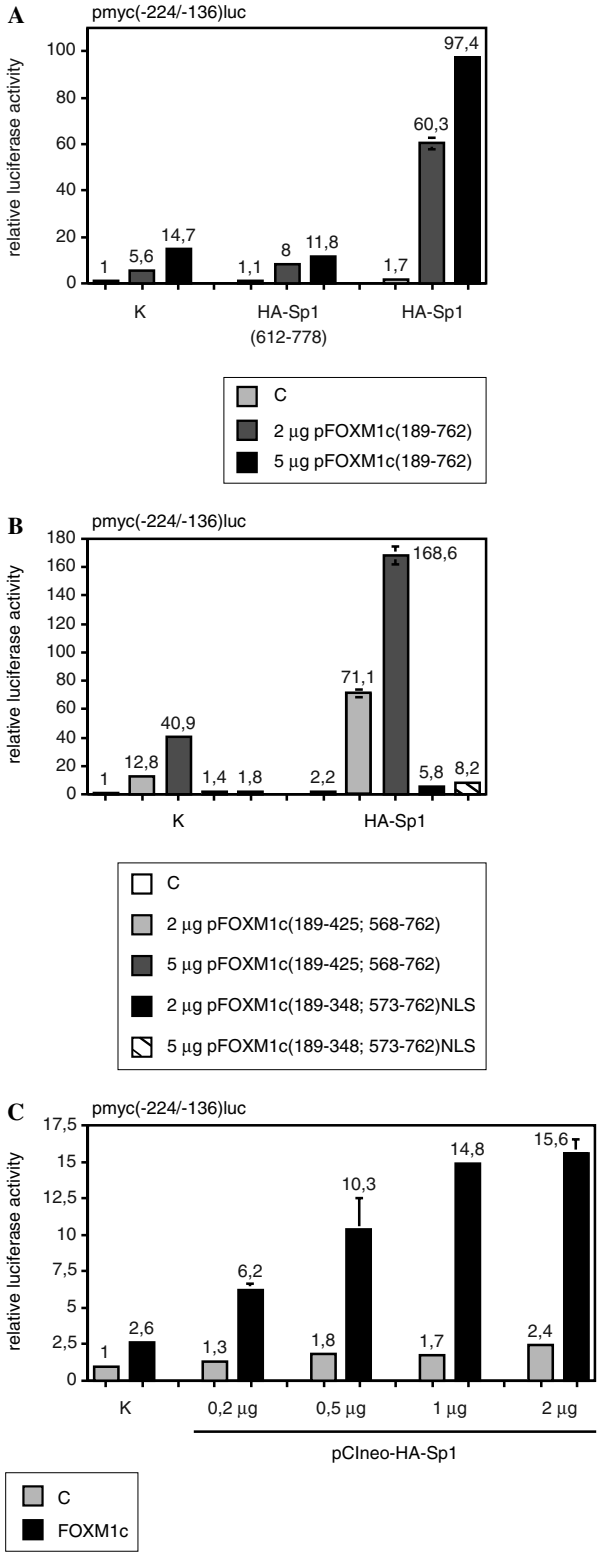

Fig. 6. Domains of Sp1 and FOXM1c required for their synergism at P1. RK-13 cells were transiently transfected with pmyc(−224/−136)luc and expression plasmids for FOXM1c proteins and HA-Sp1 proteins as indicated. The relative luciferase activity of pmyc(−224/−136)luc in the combination of control (C) with control (K), i.e. in the absence of both HA-Sp1 proteins and exogenous FOXM1c proteins, was set as 1. Maps of these HA-Sp1 and FOXM1c proteins and summaries of these experiments with them are shown in Supplementary Fig. 5A and C.

new transactivation mechanism we tested in the present study whether FOXM1c synergizes in transactivation of P1 and P2 with Sp1, which binds and transactivates both promoters [8–11]. Indeed, FOXM1c and Sp1 transactivated the human *c-myc* P1 and P2 promoters synergistically (Fig. 1A and C). This required binding of Sp1 to the Sp1-binding sites −44 (Fig. 2) or CT-I_2 or/and −66, respectively, and binding of FOXM1c to the P1 or P2 TATA-boxes [3], respectively (Fig. 3). In addition, FOXM1c and Sp1 interacted directly (Fig. 5A). These findings together verify the biological importance of the new transactivation mechanism of FOXM1c via the *c-myc* P1 and P2 TATA-boxes and the identification of *c-myc* as a new direct FOXM1c target gene [3].

The Sp1-binding sites in front of the minimal P1 and P2 promoters could be exchanged against other Sp1-binding sites without loss of synergism of FOXM1c and Sp1 at P1 or P2 (Fig. 3A, B, E; Supplementary Fig. 3B and C). Since each promoter with a P2 TATA-box is postulated to be transactivated by FOXM1c [3], it can be postulated that each promoter with a *c-myc*-P2 TATA-box TATAAAAG and a Sp1-binding site is synergistically transactivated by FOXM1c and Sp1. More than 100 target genes with this TATA-box and a potential Sp1-binding site were found in databases (Supplementary Fig. 7). Since transactivation by FOXM1c via the P1 TATA-box required an appropriate sequence context [3] it is uncertain whether other promoters with this TATA-box and a Sp1-binding site are synergistically transactivated by FOXM1c and Sp1.

In contrast, if FOXM1c functions as a conventional transcription factor, i.e. if it transactivates via conventional FOXM1c-binding sites [4–6], it does not synergize with Sp1 (Fig. 4) offering more possibilities of combinatorial gene regulation.

How can FOXM1c and Sp1 synergize in transactivation of the *c-myc* P1 and P2 promoters? FOXM1c directly binds to TBP, TFIIA, and TFIIB [3]. Sp1 directly binds to TBP, TAF4, and TAF7 [12,13,17]. Thus FOXM1c and Sp1 could synergistically recruit these components of the basal transcription complex and stabilize the preinitiation complex by simultaneous contacts to multiple of its components (Supplementary Fig. 6). In addition, the direct interaction of FOXM1c with Sp1 (Fig. 5A) suggests the possibility of cooperative DNA binding. However, if it should occur, it is not sufficient for their synergism at P1, because this requires transactivation of P1 by the two TADs A and B of Sp1 (Fig. 6A; Supplementary Fig. 5A) and thus direct binding of Sp1 to TAF4 and/or TBP. Synergism with Sp1 at P1 (Fig. 6B and C; Supplementary Fig. 5C) requires like transactivation of the minimal *c-myc*-P1 promoter [3] three components of FOXM1c: (1) binding of the forkhead domain to the P1 TATA-box, (2) transactivation by the TAD, (3) direct binding of the EDA to TBP, TFIIB, and (probably) TFIIA. Consequently, for synergism of FOXM1c and Sp1 at P1 their simultaneous binding to TFIID, TFIIA, and/or TFIIB seems to be essential (Supplementary Fig. 6).

The synergism of Sp1 and FOXM1c at P1 may be important for the severe deregulation of *c-myc* transcription in human Burkitt's lymphomas with a chromosome translocation of the *c-myc* gene to the Igκ immunglobulin locus: Both the Sp1-binding site −44 and the P1 TATA-box are essential for activation of P1 and induction of the characteristic promoter shift in transcription initiation from P2 to P1 by the κ intron and 3′ enhancers (κEi plus κE3′) [8].

In summary, we have shown that FOXM1c and Sp1 synergistically transactivate the *c-myc* P1 and P2 promoters. This requires the respective Sp1-binding sites and the P1 and P2 TATA-boxes. In addition, FOXM1c and Sp1 interact directly. These findings together confirm the biological significance of the new transactivation mechanism of FOXM1c via the *c-myc* P1 and P2 TATA-boxes and the identification of *c-myc* as a new direct FOXM1c target gene. For synergism of FOXM1c and Sp1 at P1 their cooperative DNA binding, if it should occur, is not sufficient but their binding to TFIID, TFIIA, and/or TFIIB is required. Thus, they seem to recruit the basal transcription complex synergistically by simultaneous contacts to multiple of its components.

Acknowledgments

We thank H. Rotheneder and C. Seiser for providing plasmids, G. Suske for providing antibody, and H. Burckhardt for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.151.

References

[1] S.K. Oster, C.S.W. Ho, E.L. Soucle, L.Z. Penn, The myc Oncogene: MarvelouslY Complex, Adv. Cancer Res. 84 (2002) 81–154.
[2] J. Liu, D. Levens, Making Myc, Curr. Topics Microbiol. Immunol. 302 (2006) 1–32.
[3] I. Wierstra, J. Alves, FOXM1c transactivates the human c-myc promoter directly via the two TATA-boxes P1 and P2, FEBS J. 273 (2006) 4645–4667.
[4] I. Wierstra, J. Alves, Transcription factor FOXM1c is repressed by RB and activated by cyclin D1/Cdk4, Biol. Chem. 387 (2006) 949–962.
[5] I. Wierstra, J. Alves, Despite its strong transactivation domain transcription factor FOXM1c is kept almost inactive by two different inhibitory domains, Biol. Chem. 387 (2006) 963–976.
[6] I. Wierstra, J. Alves, FOXM1c is activated by cyclin E/Cdk2, cyclin A/Cdk2 and cyclin A/Cdk1, but repressed by GSK-3α, Biochem. Biophys. Res. Commun. 348 (2006) 99–108.
[7] J. Laoukili, M. Stahl, R.H. Medema, FoxM1: at the crossroads of ageing and cancer, Biochim. Biophys. Acta (2006), Epub.
[8] C. Geltinger, K. Hörtnagel, A. Polack, TATA-box and Sp1 sites mediate the activation of c-myc-promoter P1 by immunoglobulin κ enhancers, Gene Expr. 6 (1996) 113–127.
[9] B. Majello, P. De Luca, G. Suske, L. Lania, Differential transcriptional regulation of c-myc-promoter through the same DNA binding sites targeted by Sp1-like proteins, Oncogene 10 (1995) 1841–1848.
[10] C. Asselin, A. Nepveu, K.B. Marcu, Molecular requirements for transcriptional initiation of the murine c-myc gene, Oncogene 4 (1989) 549–558.
[11] E. Des Jardins, N. Hay, Repeated CT elements bound by zinc finger proteins control the absolute and relative activities of the two principal human c-myc promoters, Mol. Cell. Biol. 13 (1993) 5710–5724.
[12] A. Emili, J. Greenblatt, C.J. Ingles, Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA-box-binding protein, Mol. Cell. Biol. 14 (1994) 1582–1593.
[13] T. Hoey, R.O.J. Weinzierl, G. Gill, J.L. Chen, B.D. Dynlacht, R. Tjian, Molecular cloning and functional analysis of *Drosophila* TAFII110 reveal properties expected of coactivators, Cell 72 (1993) 247–260.
[14] A.J. Courey, R. Tjian, Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif, Cell 55 (1988) 887–898.
[15] A.J. Courey, D.A. Holtzman, S.P. Jackson, R. Tjian, Synergistic activation by the glutamine-rich domains of human transcription factor Sp1, Cell 59 (1989) 827–836.
[16] E. Pascal, R. Tjian, Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism, Genes Dev. 5 (1991) 1646–1656.
[17] C.M. Chiang, R.G. Roeder, Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators, Science 267 (1995) 531–536.
[18] S. Safe, M. Abdelrahim, Sp transcription factor family and its role in cancer, Eur. J. Cancer 41 (2005) 2438–2448.
[19] D. Herschlag, F.B. Johnson, Synergism in transcriptional activation: a kinetic view, Genes Dev. 7 (1993) 173–179.
[20] J.M. Lüscher-Firzlaff, J.M. Westendorf, J. Zwicker, H. Burkhardt, M. Henriksson, R. Müller, F. Pirollet, B. Lüscher, Interaction of the fork head domain transcription factor MPP2 with the human papilloma virus 16 E7 protein: enhancement of transformation and transactivation, Oncogene 18 (1999) 5620–5630.
[21] J.M. Lüscher-Firzlaff, R. Lilischkis, B. Lüscher, Regulation of transcription factor FOXM1c by Cyclin E/CDK2, FEBS Lett. 580 (2006) 1716–1722.