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Regulation of the transcription factor FOXM1c by Cyclin E/CDK2

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Abstract The FOXM1 forkhead proteins, originally identified as M-phase phosphoproteins, are proliferation-associated transcriptional regulators involved in cell cycle progression, genetic stability and tumorigenesis. Here we demonstrate that Cyclin-dependent kinases regulate the transcriptional activity of FOXM1c. This is independent of an N-terminal negative regulatory domain and of the forkhead DNA binding domain. Instead we mapped the responsive sites in the transactivation domain. A combination of three phosphorylation sites mediates the Cyclin E and Cyclin A/CDK2 effects. Our findings provide evidence for a novel Cyclin E/CDK2 substrate that functions in cell cycle control.

Keywords: Transcription; Cell cycle; CDK; FOXM1; p27; Cyclin A

1. Introduction

The forkhead box (FOX) protein family of transcriptional regulators are involved in controlling many distinct aspects of cell behavior [1,2]. FOXM1, originally identified as MPP2, Trident, HFH-11, and WIN [3–7], is expressed in proliferating but not resting or differentiated cells [8]. FOXM1 promotes cell cycle progression by affecting both the G1 to S-phase and the G2 to M-phase transition. In addition FOXM1 is an important regulator of chromosomal stability. These effects are mediated by FOXM1-dependent regulation of target genes that include several *cyclin* genes and the *CENP-F* gene [9–12]. In addition to the *cyclin* genes that are activated, the expression of the CDK inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ are downregulated, together providing an explanation for the ability of FOXM1 to stimulate cell cycle progression. Furthermore forced FOXM1 expression in the liver stimulates hepatocyte proliferation in response to partial hepatectomy while lack of FOXM1 results in a delay of liver regeneration [6,13,14]. The important role of FOXM1 for hepatocyte proliferation is further documented by the finding that this factor is necessary for the development of hepatocellular carcinoma [15]. Recently over-expression of FOXM1 has been observed in human mammary carcinoma and in breast cancer cell lines [10]. While these studies define FOXM1 as a proliferation-associated factor, its down-regulation or knock-out results not simply in inhibition of proliferation but in chromosomal instability [10,12,16]. Together these studies define FOXM1 as an important regulator of cell proliferation and genetic stability.

The progression of cells from the G1 into the S-phase of the cell cycle is controlled by D-type Cyclins associated with CDK4 and CDK6 and by Cyclin E with CDK2 and CDK1 [17,18]. Pocket proteins are the main substrates of Cyclin D/CDK complexes. Indeed these kinases are not required for S-phase entry in retinoblastoma tumor suppressor protein (Rb)-negative cells. In contrast, Cyclin E complexes are essential for S-phase progression also in Rb-negative cells [19]. This indicates that Cyclin E/CDK2 targets additional critical substrates other than Rb. These include p27, Nucleophosmin, Cdc6, NPAT, CP110 and hHR6A that mediate at least in part the effects of Cyclin E/CDK2 on centrosome duplication, initiation of replication and S-phase specific gene transcription [20–25]. The inappropriate activation of these CDKs, including increased expression of Cyclin E, is associated with tumor formation [26,27]. Additionally, the Cyclin E/CDK2 kinase is activated in response to several oncoproteins, including c-Myc and the adenoviral E1A protein, further supporting a role of this kinase in tumorigenesis [28,29]. During S- and G2-phase Cyclin A/CDK2 complexes, in part overlapping with Cyclin E/CDK2, are important to regulate further progression in the cell cycle and many of the Cyclin E/CDK2 substrates are also phosphorylated by Cyclin A/CDK2.

FOXM1 was originally identified as a protein that reacts with the monoclonal antibody MPM-2 that recognizes phospho-epitopes in mitotic cells [3]. This suggested that the phosphorylation of FOXM1 is cell cycle and/or signal regulated. Indeed recent evidence suggests phosphorylation-dependent regulation of FOXM1 [30,31]. We identified FOXM1c in a yeast two-hybrid screen as an interaction partner for the human papilloma virus 16 E7 protein and cloned one of the several splice variants referred to as FOXM1c [4]. E7 induces S-phase in part by binding to the Rb protein, thereby blocking interaction with E2F transcription factors. However additional activities of E7 are required to promote S-phase entry [32]. These findings, together with the fact that FOXM1 contains several potential CDK phosphorylation sites, led us to hypothesize that Cyclin E/CDK2 might regulate FOXM1c. Indeed we observed that the transcriptional activity of FOXM1c is stimulated in response to Cyclin E/CDK2. This was dependent on the C-terminal transactivation domain. We identified a combination of three phosphorylation sites that are phosphorylated by Cyclin E/CDK2 and mediate the transcriptional response.

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2. Materials and methods

2.1. Cell culture, plasmids and transient transfection assays

Cultivation and transfection of NIH3T3, RK-13, HEK293, HeLa and COS7 cells has been described previously [4]. For serum stimulation experiments, transiently transfected NIH3T3 cells were starved in 0.1% fetal calf serum (FCS) for 24 h prior to stimulation with 10% FCS. S-phase progression was monitored by incubating the cells in 100 μM bromodeoxyuridine (BrdU) for 1 h and staining with an anti-BrdU antibody coupled to fluoresceine (Boehringer, Mannheim). Roscovitine (25 μM) was added to the transfected cells 6 h prior to harvesting. The expression plasmid for FOXM1c and Gal4-FOXM1c and the reporter plasmids $(MBS)_3$-mintk-luc and $(Gal4)_4$-mintk-luc have been described previously [4]. FOXM1c mutants were cloned using standard techniques. Point mutations were generated using Quick-change (Stratagene). Plasmids encoding Cyclin E, Cyclin A, CDK2, CDK2dn, $p27^{KIP1}$, and $p21^{WAF1}$ were kindly provided by R. Bernards. FOXM1c and mutants were detected using SC-502 (Santa Cruz) that recognizes the C-terminus of the protein. For Western blots cells were lysed in F-buffer (10 mM Tris–HCl, pH 7.05, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 μM $ZnCl_2$, 100 μM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 5 U/ml α2-macroglobulin, 2.5 U/ml pepstatin A, 2.5 U/ml leupeptin, 0.15 mM benzamidin) as described before [33].

2.2. Preparation of active cyclin E/CDK2 complexes, kinase assays and metabolic labeling

Expression and purification of human cyclin E/CDK2 complexes from insect cells infected with recombinant baculoviruses were carried out as described [34]. For kinase assays FOXM1c and mutants were expressed in COS7 cells, immunoprecipitated and incubated with Cyclin E/CDK2 in kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.01% Tween-20, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM orthovanadate, 0.01 % BSA) containing 25 μM ATP (specific activity 2 mCi/μmol) for 30 min. For in vivo labeling, COS7 cells were transiently transfected with plasmids encoding FOXM1c or FOXM1c (T599A/T610A/S637A) together with CDK2 and Cyclin E. Two days post transfection, cells were metabolically labeled in phosphate-free DMEM supplemented with 10% dialyzed FCS, 20 mM sodium bicarbonate, 18 mM HEPES, pH 7.5, and 2 mCi ^{32}P-orthophosphate for 2 h and lysed in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 7 μg/ml aprotinin and 20 mM β-glycerophosphate).

2.3. GST pull-down assays and phosphatase treatment

The FOXM1c proteins were immunoprecipitated and analyzed by SDS–PAGE. GST pull-down assays were performed as described previously [4]. For phosphatase treatment FOXM1c was immunoprecipitated using SC-502 from F-buffer lysates of HeLa cells. The immunoprecipitates were washed three times in F-buffer, once in PPase buffer (0.5 M Tris, pH 8.5, 1 mM EDTA) and then incubated in 20 μl PPase buffer with 1U calf intestinal phosphatase. The proteins were analyzed by SDS–gel electrophoresis and Western blotting.

3. Results

3.1. FOXM1c is activated by Cyclin E/CDK2

To address whether Cyclin E/CDK2 could affect FOXM1c transcriptional activity, we performed reporter gene assays. We observed that Cyclin E/CDK2 strongly enhanced FOXM1c-dependent transcription of the $(MBS)_3$-mintk-luc reporter gene that contains three binding sites for FOXM1c while Cyclin E or CDK2 alone had little effect (Fig. 1A). A reporter gene without binding sites was not activated. The coexpression of a kinase dead mutant of CDK2 (CDK2dn) did not activate but rather repressed FOXM1c transactivation indicating that CDK2 kinase activity is required for basal as well as stimulated FOXM1c activity (Fig. 1A). In addition the Cyclin E/CDK2 inhibitors $p21^{WAF1}$ and $p27^{KIP2}$ abolished and roscovitine, a CDK2 inhibitor, suppressed the Cyclin E/CDK2 effect on FOXM1c-dependent transactivation (Fig. 1B and C). Furthermore a Gal4-FOXM1c fusion protein was also stimulated by Cyclin E/CDK2 and repressed by $p27^{KIP2}$ suggesting that the kinase is not targeting the DNA binding domain (Fig. 1D). Together these findings define Cyclin E/CDK2 as an efficient activator of the transcriptional activity of FOXM1c.

3.2. Cell cycle regulation of FOXM1c(190–763)

As demonstrated above the transcriptional activity of FOXM1c is strongly stimulated by Cyclin E/CDK2, which is activated in the late G1-phase. Therefore we addressed whether FOXM1c might be activated at the G1 to S-phase border. NIH3T3 cells were transiently transfected with an expression plasmid for FOXM1c(190–763) and the $(MBS)_3$-mintk-luc reporter gene and serum starved for 24 h prior to stimulation with 10% FCS. An induction of FOXM1c(190–763)-dependent transactivation was observed when cells entered S-phase as determined by BrdU incorporation (Fig. 2A and B). Similar experiments were performed with FOXM1c. However due to the low activity of the full length protein only small activations were measured after serum stimulation (data not shown). These observations are consistent with an activation of FOXM1c by endogenous Cyclin E/CDK2.

3.3. The C-terminal transactivation domain is important to mediate the Cyclin E/CDK2 effect

FOXM1c possesses 18 Ser/Thr-Pro motifs that represent potential Cyclin E/CDK2 modification sequences. Particularly obvious are two clusters of potential sites in the C-terminal half of the protein (Fig. 4A). In order to identify Cyclin E/CDK2 responsive regions in FOXM1c deletion mutants were generated. Truncation of the N-terminal 189 amino acids resulted in a protein, FOXM1c(190–763), that showed significantly enhanced transcriptional activity (Fig. 3A). Since this protein was expressed equally to the wild-type FOXM1c (Fig. 3B), the N-terminal domain represents a negative regulatory domain (NRD, Fig. 3). Although two potential phosphorylation sites are deleted in FOXM1c(190–763), this protein was still efficiently activated by Cyclin E/CDK2 (Fig. 3A). Both the basal and the Cyclin E/CDK2-stimulated activity of FOXM1c(190–763) was efficiently repressed by $p27^{KIP2}$ suggesting that endogenous CDK2 activity is required for transactivation by FOXM1c(190–763) (Fig. 3C). In contrast deletion of the acidic transactivation domain (TAD) near the C-terminus in FOXM1c(1–588/745–763) completely abolished both transactivating activity and Cyclin E/CDK2 responsiveness, indicating that the TAD is important for the Cyclin E/CDK2 effect (Fig. 3A). Deletion of the N-terminal domain in addition to the TAD in FOXM1c(190–588/745–763) was not sufficient to activate the transcription factor, despite efficient expression of this mutant, and this mutant remained insensitive to Cyclin E/CDK2 (Fig. 3B and data not shown). FOXM1c(1–348/575-763) with a deletion of a central portion was poorly expressed (Fig. 3B). Nevertheless this protein was activated by Cyclin E/CDK2 with a similar potency as FOXM1c and FOXM1c (190–763) (Fig. 3A). Thus neither the N-terminal NRD nor the central region of FOXM1c is important to mediate the Cyclin E/CDK2 effect. Instead the C-terminal quarter of the protein that includes the TAD appears to be the target of this kinase.