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The HMGA1 Protoncogene Frequently Deregulated in Cancer Is a Transcriptional Target of E2F1

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Reactivation of the HMGA1 protoncogene is very frequent in human cancer, but still very little is known on the molecular mechanisms leading to this event. Prompted by the finding of putative E2F binding sites in the human HMGA1 promoter and by the frequent deregulation of the RB/E2F1 pathway in human carcinogenesis, we investigated whether E2F1 might contribute to the regulation of HMGA1 gene expression. Here we report that E2F1 induces HMGA1 by interacting with a 193 bp region of the HMGA1 promoter containing an E2F binding site surrounded by three putative Sp1 binding sites. Both gain and loss of function experiments indicate that Sp1 functionally interacts with E2F1 to promote HMGA1 expression. However, while Sp1 constitutively binds HMGA1 promoter, it is the balance between different E2F family members that tunes the levels of HMGA1 expression between quiescence and proliferation. Finally, we found increased HMGA1 expression in pituitary and thyroid tumors developed in $Rb^{+/-}$ mice, supporting the hypothesis that E2F1 is a novel important regulator of HMGA1 expression and that deregulation of the RB/E2F1 path might significantly contribute to HMGA1 deregulation in cancer.

Key words: HMGA1 promoter; carcinogenesis; Sp1; transcription

INTRODUCTION

Together with HMGA2, the three splicing variants of the High Mobility Group A1 gene (HMGA1a, HMGA1b, and HMGA1c) belong to a family of small nonhistone proteins binding AT-rich DNA sequences with relatively low affinity, by means of so-called ''AT-hooks'' [1]. Although HMGA proteins have no transcription-activating domain, their ability to interact with multiple partners allows them to coordinate the assembly and disassembly of high order multiprotein complexes known as enhanceosomes and to regulate the transcription of a large array of genes [2–4].

HMGA genes are strongly and widely expressed during development, but they are very little or not expressed in most adult differentiated tissues. This initially suggested their implication in governing proliferation and differentiation during development. Indeed HMGA1 and HMGA2 mouse models highlight their role in adipogenesis, spermatogenesis, cardiac development, lymphoematopoietic differentiation, and diabetes [5–12].

Importantly, HMGA proteins are also involved in carcinogenesis, as shown in vitro and in animal models. Their expression was initially discovered in He-La and in virally transformed rat thyroid (FRTL5) cells [1314]. Their overexpression transformed Rat1a fibroblasts and lymphoid cells [15]. Furthermore the injection of breast epithelial cells conditionally expressing HMGA1 in nude mice led to both primary and metastatic tumors by promoting epithelial–mesenchymal transition [16]. Conversely, impairment of HMGA1 activity by either antisense or dominant negative constructs inhibits several features of the transformed phenotype [1617]. Consistent with the in vitro data, HMGA2 overexpression induces benign mesenchymal tumors, pituitary tumorigenesis, and lymphomas in transgenic animals expressing the wild type or the truncated active form [18–20]. Development of lymphoid malignancies and pituitary adenomas were typically reported in the HMGA1 transgenic mouse models [2122]. In vitro and in vivo studies demonstrated that HMGA proteins contribute to carcinogenesis at different levels, by transcriptionally regulating the expression

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Abbreviations: siRNA, strand interfering RNA; ChIP, chromatin immunoprecipitation.

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of genes involved in inflammation, epithelial–mesenchymal transition, extracellular matrix degradation, angiogenesis, and DNA repair (reviewed in [12324]). Importantly they may also interfere with important oncosuppressive pathways such as those orchestrated by p53 and pRB [1925–28].

The prompt for all the studies on the role of HMGA proteins in transformation largely came from the well-known deregulation of their expression in human cancer. Chromosomal translocation involving HMGA2 locus frequently occur in benign tumors of mesenchymal and epithelial origin and often lead to overexpression of chimeric or truncated proteins that loose the acidic C-terminal tail, but maintain the three AT-hooks DNA binding domains. In contrast, HMGA1 gene structure is usually spared from rearrangements (reviewed in [24]), and its very frequent overexpression in human cancer is more typically associated with reactivation of transcription. Indeed, wild type HMGA1 expression characterizes malignant lesions of most epithelial neoplasia (including thyroid, colon, pancreas, breast, cervix, and prostate) while being absent in the corresponding normal epithelium/mucosa and even in hyperplastic lesions [29–36]. Furthermore, its expression is more frequently associated to advanced, metastatic stages, and to poorer prognosis. Even in tumors of the nervous system, HMGA1 expression is deregulated and associated with more aggressive/advanced stages and/or treatment failure [37–41].

Such an ubiquitous HMGA1 deregulation in human cancer suggests that its promoter might be very responsive to most common transformation pathways. Although this hypothesis has been raised long ago [42], surprisingly few articles have addressed the molecular mechanisms governing HMGA1 transcriptional regulation. We and others have studied human HMGA1 gene promoter in silico, and found putative binding sites for transcription factors involved in proliferation and transformation [43]. Among them only AP1, Sp1, c-MYC, and MYCN have been experimentally shown to bind HMGA1 promoter and to regulate its gene expression, thus providing a molecular explanation to the high HMGA1 levels found in KRAS transformed cells, Burkitt lymphoma and MYCN amplified neuroblastomas [15424445]. However it is clear that these data cannot account for all cases of HMGA1 deregulation. In example, we observed that high HMGA1 expression can also occur in human neuroblastomas with no MYCN amplification and/or overexpression [45].

Here we have addressed whether the E2F1 oncogene, whose activity is frequently deregulated in carcinogenesis, might control HMGA1 expression. Indeed, we report that E2F1 binds to HMGA1 promoter and cooperates with Sp1 to regulate its expression.

MATERIALS AND METHODS

Cell Cultures

HEK293 and T98G cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 U/ml of penicillin G sodium, and 100 μg/ml streptomycin sulfate in a humidified atmosphere containing 5% CO_2 at 37°C.

DNA Constructs, Transfection and Luciferase Assay

The HMGA1 promoter/luciferase reporter constructs and their 5′ and 3′ progressive deletions generated using the Erase-a-Base System (Promega Corporation, Madison, WI) were previously described [45]. The P1p73-luc reporter was previously described [46]. The CMV-Sp1 vector expressing the human Sp1 protein was from Add-Gene (Cambridge) while PCDNA-E2F1 vector expressing the human E2F1 protein was a kind gift of Dr. A. Ianari. HEK293 and T98G cells (1×10^6 cells plated in dish 60 mm dishes) were transfected with the different HMGA1 firefly luciferase reporters (2.5 μg/dish) along with the indicated amount of E2F1 and Sp1 expression vectors and the pRL-TK vector expressing the *Renilla* luciferase (0.125 μg/dish) for normalization of the transfection efficiency (Promega Corporation) by the calcium phosphate method (HEK293) or by Lipofectamine Plus reagent (Promega Corporation for T98G). Forty-eight hours after transfection, cells were lysed and luciferase activity was determined using a TD-20/20 automatic dual injector luminometer (Turner Designs, Sunnyvale, CA) and the Dual-Luciferase Reporter Assay System (Promega Corporation).

Double strand interfering RNA (siRNA) targeting human Sp1 and a control nonspecific siRNA (Sigma Life Science, St. Louis, MO) were transfected by means of the DharmaFECT 2 Transfection Reagent (Dharmacon Res., Inc., Lafayatte, CO). Two days after siRNA administration, cells were detached and split in replicate wells. Twenty-four hours later they were eventually transfected with luciferase reporter constructs as above.

Growth arrested or proliferating asynchronous T98G cells were infected with a recombinant adenovirus expressing human E2F1 (a kind gift of Dr. Marco Crescenzi) or with an empty control (approximately 100 pfu/cell) 24 h after plating. Subsequently cells were harvested and used for RNA and/or protein extraction.

RNA Preparation and Real-Time Quantitative PCR Analysis

Total RNA from human cell lines was extracted using TRIzol reagent (Invitrogen, San Diego, CA). For Q-PCR analysis 1 μg of total RNA was transcribed using the GeneAmp Gold RNA PCR Reagent Kit pAW109 (Applied Biosystems, Warrington, UK) according to the manufacturer. $Rb^{+/-}$ mRNAs were

kindly provided by Dr. T. Parisi. Gene expression analysis was carried out by Q-PCR employing commercially available TaqMan® Assay Reagents for GAPDH and an home made assay for HMGA1 previously described [45]. HMGA1 levels were normalized on GAPDH values.

Protein Extraction and Western Blot

For protein extraction, cell pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, and Bromo-Phenol Blue) sonicated at maximum power for 20 min and centrifuged at 13 000 rpm for 30 min, and the supernatant was then collected. Protein extracts (20 μg) were separated on 8% SDS–PAGE gel, blotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and probed with rabbit anti-E2F1, rabbit anti-Sp1, and rabbit anti-CREB polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by enhanced chemoluminescence (Pierce Chemical Co., Rockford, IL).

Chromatin Immunoprecipitation (ChIP) Assay

Protein complexes were crosslinked to DNA in living nuclei by adding formaldehyde (Merck, Darmstadt, Germany) directly to tissue culture medium to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine to a final concentration of 0.125 M. Crosslinked cells were scraped, washed with phosphate-buffered saline. Cells were pelleted by centrifugation and lysed by incubation in nuclear lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris-chloride [pH 8.1], 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin per ml, and 100 ng of aprotinin per ml).

The resulting chromatin solution was sonicated (Bioruptor sonicator, Diagenode, Liege, Belgium) to generate 500–1500 bp DNA fragments. After microcentrifugation, the supernatant was precleared with blocked protein G plus (Pierce Chemical Co., Rockford, IL), diluted 1:10 with dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-chloride [pH 8.1], 167 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin per ml, and 100 ng of aprotinin per ml), and divided into aliquots. Five micrograms of antibody was added to each aliquot of chromatin and incubated on a rotating platform for 12–16 h at 4°C. Antibodies used were directed against E2F-1, E2F-4, and the Sp1 were purchased from Santa Cruz Biotechnology, Inc.

Antibody–protein–DNA complexes were isolated by immunoprecipitation with blocked protein G plus. Following extensive washing, bound DNA fragments were eluted and analyzed by Real Time PCR using primers specific for the HMGA1 promoter. Primer sequences related to the experiment shown in Figure 5D are: A1-for 5′-TCTGAGCGCCTCTGCTCTC-3′ and A1-rev 5′-CGCCGCTGGTAGCAAATG-3′.

RESULTS

A Matinspector scan revealed the presence of several putative E2F binding sites scattered on the TATA-less promoter of the human HMGA1 gene [43]. Therefore we set out to study whether E2F1 could stimulate HMGA1 transcription. To this end we used a set of fragments of the human HMGA1 promoter we had previously described [45] encompassing the region between nucleotide −2381 and nucleotide +1214 (Figure 1A,C). This region contains the first three transcription start sites (TS) and a few cis-acting elements known to be involved in basal or induced HMGA1 transcriptional regulation, including AP-1, c-MYC, MYCN, and Sp1 binding sites (Figure 1A,C) [15424445]. In a luciferase assay, E2F1 clearly promoted HMGA1 activation, being the shorter HMGA1-5#1 construct more efficiently activated than the longer HMGA1-5′LE-box construct (Figure 1A,B), suggesting that a putative E2F binding site previously identified in this region did not significantly contribute to HMGA1 promoter regulation [43]. Further analysis of a panel of 5′- and 3′-end deletion mutants of the HMGA1-5#1 construct indicated that the first 478 bp of this region (including TS1 and the previously described Sp1A and Sp1B binding sites [44] were not relevant for E2F1 responsiveness (Figure 1C), while deletion of 453 and 479 bp from the 3′-end of the construct progressively increased E2F1 responsiveness up to 12 times over the control (Figure 1C), suggesting the presence of potentially inhibitory cis-acting elements in this region. Interestingly, loss of additional 193 bp from the 3′-end (construct 3′LΔ672) strongly reduced responsiveness to E2F1, indicating that an E2F1 response region maps between nucleotide 542 and 735 of the human HMGA1 promoter. Indeed, the 3′LΔ479 construct was strongly activated even when low amounts of E2F1 DNA were transfected (Figure 1D). A closer look into this region revealed the presence of a putative E2F binding site (gcggCGCGagccggc), flanked by three putative Sp1 binding sites (Figure 2A). Mutagenesis of the conserved CGCG core motif of this putative E2F binding site to CATG within the 3′LΔ479 construct (3′LΔ479MUT) led to a consistent drop in luciferase activity at low amounts of E2F1 DNA, while being ineffective at higher E2F1 DNA concentrations (Figures 2B,C and S1). These results indicate that an E2F1 direct interaction with its putative binding site within the 3′LΔ479 construct is probably required for HMGA1 promoter activation at low E2F1 concentration; in addition, at higher concentrations, E2F1 might contribute to HMGA1 promoter activation either through a low affinity binding to additional site/s eventually present between nucleotide

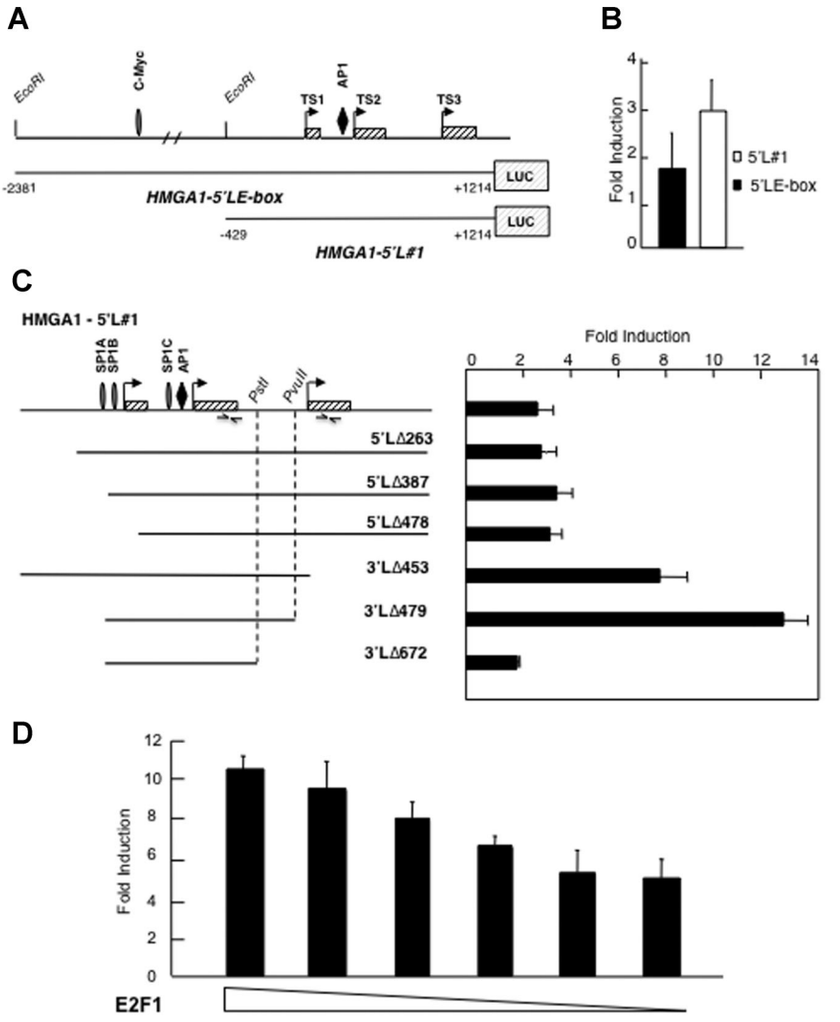

Figure 1. E2F1 stimulates HMGA1 promoter activity. (A) Schematic representation of the HMGA1 promoter and luciferase reporter constructs. (B) Luciferase reporter assay in HEK293 cells showing HMGA1 promoter response to E2F1 (2.5 μg/dish). (C) Detailed characterization of the luciferase reporter activity of a series of deletion mutants of the HMGA1-5′L#1 reporter construct; the position of the three TSs, of the Sp1 and AP1 binding sites, and of the CHIP amplicons (arrows) are given; dashed lines indicate the E2F1 response region whose sequence is given in Figure 2A. (D) Analysis of the dose-response (from 2.5 to 0.25 μg/dish) activity of E2F1 on the 3′LΔ479 reporter construct. For all luciferase reporter assays, data are reported as averages (±SD) of the fold induction (E2F1-containing versus empty control vector) of triplicate samples.

542 and 735 and/or via a functional interaction with additional transcription factor/s. Indeed, E2F1 may directly bind to GC-rich sequences present in the majority of human promoters [47] and also in this region of the HMGA1 promoter. Furthermore the TATA-less configuration with close E2F1 and Sp1 biding sites of this specific region of the HMGA1 promoter is highly reminiscent of cell cycle genes transcriptionally regulated by an E2F1–Sp1 interaction [48–52]. Therefore we checked whether Sp1 overexpression played any role in the regulation of HMGA1, either alone or in cooperation with E2F1. Sp1 cotransfection with the 3′LΔ479 construct did not affect luciferase activity and failed to show any cooperative activity with E2F1 on the E2F1 responsive construct p73LUC. In contrast, it potently cooperated with low amounts of E2F1 DNA in inducing luciferase activity from both 3′LΔ479 and 3′LΔ479MUT constructs (Figures 3A and S2A), while only modestly affecting the activity of E2F1 when used at higher concentration (Figure S3C). These results suggest that E2F1 might stimulate HMGA1 promoter also via its functional interaction with Sp1. Indeed, Sp1 RNAi reduced by 30% the promoter activation due to high amounts of E2F1 DNA on the 3′LΔ479, but did not impair the E2F1 induced transcription of the p73LUC (Figures 3B and S2B). Importantly, under the same conditions, Sp1 RNAi caused a significantly stronger decrease in luciferase activity (about 60%) from the 3′LΔ479MUT construct

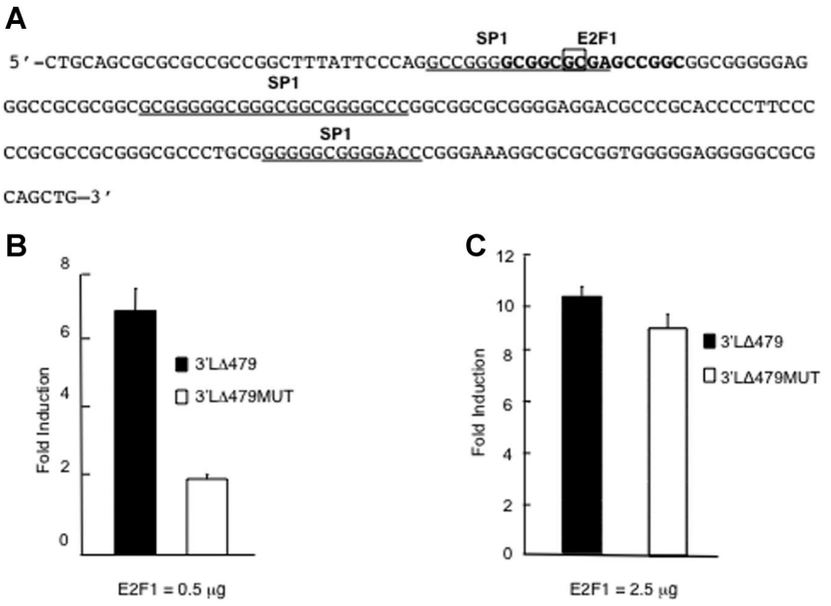

Figure 2. Effects of the mutagenesis of the putative E2F binding site. (A) Sequence of the E2F1 response region of the HMGA1 promoter containing the putative E2F (bold) and Sp1 (underlined) binding sites. The box indicates the two bases mutated in the 3′LΔ479 MUT construct. (B,C) Luciferase reporter assay in HEK293 cells showing the responses of the WT (3′LΔ479) or mutant (3′LΔ479 MUT) HMGA1 reporters to different amounts of exogenous E2F1.

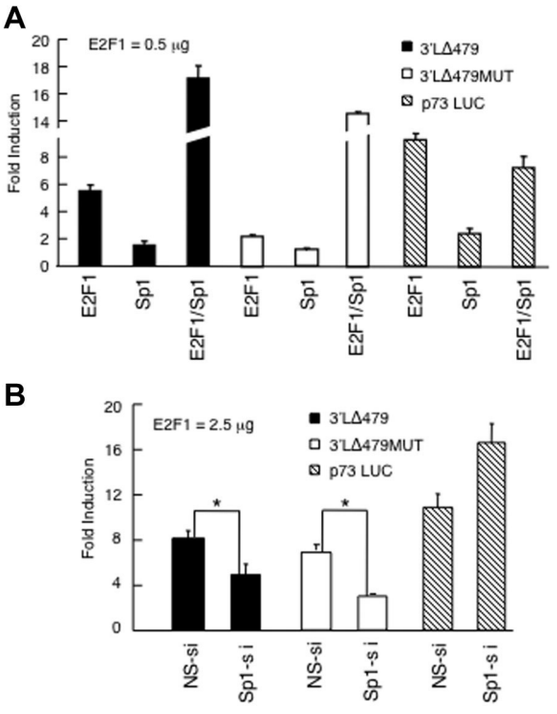

Figure 3. Sp1 cooperates with E2F1 in stimulating HMGA1 promoter. (A) Luciferase reporter assay in HEK293 cells showing the responses of the indicated reporters to E2F1 (0.5 μg) and/or Sp1 (2.5 μg). (B) Luciferase reporter assay in HEK293 cells showing the responses of the indicated reporters to E2F1 (2.5 μg) in the presence of control or Sp1 specific siRNA (NS-si and Sp1-si, respectively). *Statistically significant difference, $P = 0.0048$.

(Figures 3B and S2B). Overall these data provide support to the hypothesis that E2F1 exerts both a direct and an Sp1-mediated activity on HMGA1 promoter.

HMGA1 is a delayed early growth regulated gene and its expression is differentially regulated between quiescence and proliferation [53]. Since E2F1 is deeply involved in cell cycle dependent transcription, our data raise the possibility that E2F1 might contribute to HMGA1 growth-regulated transcription, via the cis-acting elements located between nucleotides 542 and 735 of its promoter region, possibly in concert with Sp1. A cell model where both the RB-E2F1 pathway and HMGA1 can be easily modulated in concert with the quiescent or proliferative state obtained by serum deprivation or re-stimulation, is represented by T98G cells [28]. Indeed, in asynchronous T98G cells, E2F1 induced a small, but reproducible, increase in the endogenous HMGA1 and Sp1 further potentiated this effect (Figure 4A). Conversely, Sp1 RNAi efficiently impaired E2F1-induced HMGA1 increase (Figure 4B). Similar data have been obtained also in Hek293 cells (Figure S3B).

Upon serum deprivation, T98G cells efficiently reach a pRB-E2F1 dependent quiescent state, associated with reduced HMGA1 expression [28]. By adding serum, they re-enter cell cycle and upregulate HMGA1 expression [28] (see also Figure 5A). Interestingly, serum deprivation of adenovirally transduced (Ad-E2F1) cells failed to repress HMGA1 expression and Ad-E2F1 infection of quiescent cells was sufficient to induce HMGA1 upregulation

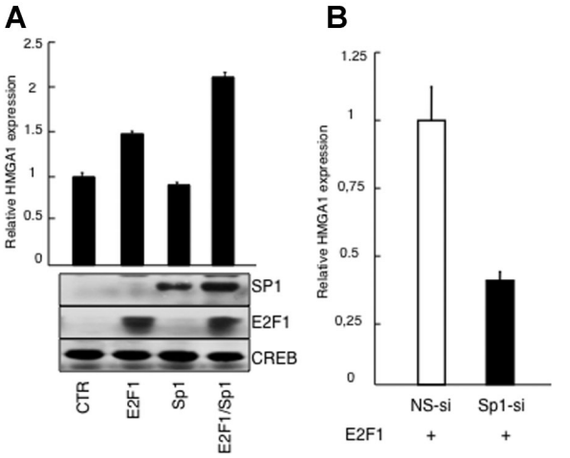

Figure 4. Sp1 cooperates with E2F1 to stimulate endogenous HMGA1 transcript expression. (A) Q-RT-PCR analysis of endogenous HMGA1 expression (upper panel) and Western blot analysis of the indicated proteins (lower panel), in T98G cells transfected with E2F1 and/or Sp1. For Q-RT-PCR, data were normalized on GAPDH expression and reported as fold induction with respect to the empty vector transfected sample. (B) Q-RT-PCR analysis of the endogenous HMGA1 expression in E2F1 transfected T98G cells in the presence of control or Sp1 specific siRNA.

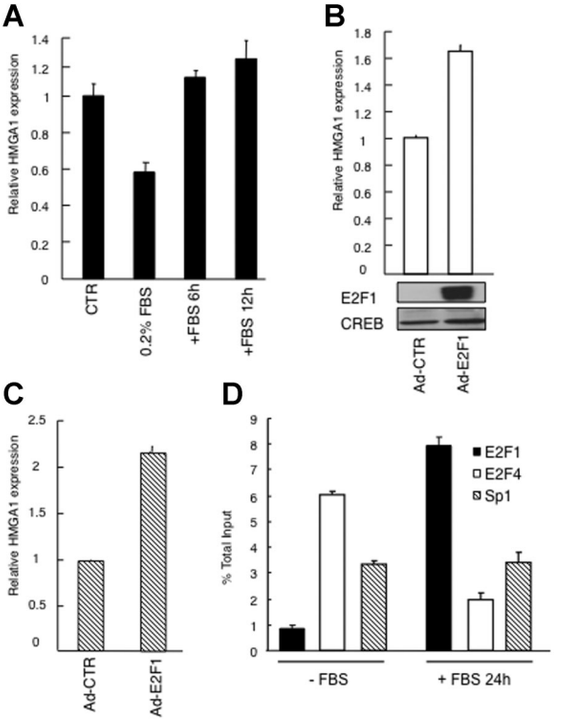

Figure 5. E2F1 promotes HMGA1 expression through a direct interaction with HMGA1 promoter. (A) Q-RT-PCR analysis of endogenous HMGA1 expression in asynchronously cycling (CTR) quiescent (0.2% FBS) and FBS re-stimulated (+FBS 6h, +FBS12h) T98G cells. (B) Quiescent T98G were infected with control (Ad-CTR) or E2F1 (Ad-E2F1) adenoviruses and endogenous HMGA1 expression was analyzed by Q-RT-PCR. (C) T98G infected with control (Ad-CTR) or E2F1 (Ad-E2F1) adenoviruses were subjected to serum deprivation and endogenous HMGA1 expression was analyzed by Q-RT-PCR. (D) HMGA1 promoter chromatin immunoprecipitation by the antibodies directed against E2F1, E2F4, and Sp1 in quiescent or serum stimulated T98G cells (ChIP amplicons are given in Figure 1C).

(Figure 5B,C). These data therefore support the hypothesis that E2F1 is an important regulator of HMGA1 expression under growth factor stimulation.

At quiescence, E2F inhibitory species (i.e., E2F4) recruit inhibitory pocket proteins of the RB family onto the promoters of cell cycle regulated E2F-responsive genes in order to repress their transcription. At induction of cell cycle entry, E2F active species (i.e., E2F1) typically bind the specific promoter elements, thus leading to transcriptional activation [54]. To test whether E2F1 binds HMGA1 promoter and whether this may eventually occur with the described prototypic switch between E2F1 and E2F4, we performed ChIP assays in quiescent and serum stimulated T98G cells. Interestingly, we detected constitutive Sp1 binding in both conditions (Figure 5D). In contrast, we revealed high E2F4 and low E2F1 binding at quiescence and low E2F4 and high E2F1 binding in cycling cells (Figure 5D), consistent with a typical cell cycle and E2F-dependent regulation of the HMGA1 gene.

Increased HMGA1 expression and deregulation of the RB-E2F1 pathway are common findings in human carcinogenesis. Therefore our data raise the hypothesis that, in tumors with deregulated RB-E2F1 pathway, the increased E2F1 activity may be responsible for sustained HMGA1 expression. To start addressing this hypothesis we investigated on HMGA1 expression in pituitary and thyroid c-cell tumors arisen in the $Rb^{+/-}$ mouse models [55]. Importantly, we detected higher levels of HMGA1 expression in pituitary and thyroid tumors developed in the $Rb^{+/-}$ mice compared to thyroid and pituitary tissue from wild type animals (Figure 6), which suggests that an activated RB/E2F1 pathway contributes to HMGA1 deregulation during carcinogenesis.

DISCUSSION

HMGA1 deregulation is a common event in carcinogenesis, but so far relatively few oncogenic pathways were shown to contribute to this phenomenon. Activation of the WNT pathway seems to sustain the elevated expression of HMGA1 in gastric cancer, largely but not exclusively through c-MYC induction [56]. c-MYC and MYCN were also shown to support high HMGA1 expression in Burkitt lymphoma and neuroblastoma, respectively [1545]. EGF and the constitutively active mutant KRAS are also known inducers of HMGA1 expression [5357] and thus deregulation of the EGFR pathway might further account for HMGA1 deregulation, in cancer of epithelial origin. Nevertheless, the widespread occurrence of HMGA1 overexpression in human

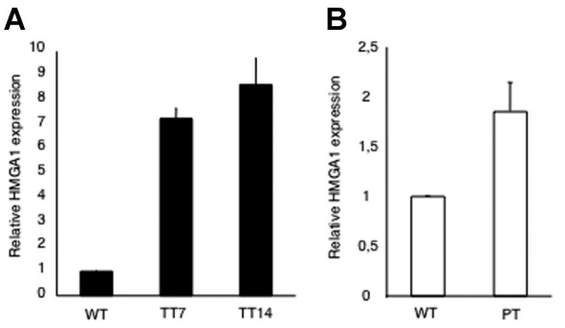

Figure 6. HMGA1 expression is deregulated in thyroid and pituitary tumors arising in $Rb^{+/-}$ mice. Q-RT-PCR analysis of endogenous HMGA1 expression in (A) thyroid (TT7 and TT14) and (B) pituitary gland (PT) tumors compared to the corresponding normal tissue from WT animals (WT).

cancer cannot be entirely explained by this knowledge. By combining luciferase reporter assays, ChIP experiments and analysis of the HMGA1 transcript expression, here we have shown that E2F1 binds to a regulatory sequence on the HMGA1 gene and sustains its expression at transition between quiescence and proliferation.

Deletion of a short (193 bp) genomic region mapping between nucleotides 542 and 735 of the human HMGA1 promoter abolished HMGA1 induction by E2F1 in luciferase assays, indicating that an E2F response element maps within this region. Although we failed to find a typical TTTSSCGC E2F binding motif in this area, a Matinspector scan indicated that a putative binding site might be represented by the gcggCGCGagccggc motif. Mutagenesis of its CGCG core sequence impaired transactivation at low E2F1 concentrations. Furthermore, we confirmed E2F1 direct binding at this region, by ChIP assay. In sharp contrast, higher E2F1 concentration, such as those employed for the analysis of the deletion mutants, efficiently induced luciferase activity from the mutant construct, thus suggesting that, at higher concentration, E2F1 might also activate transcription by binding at a lower affinity site/s or by forcing its functional interaction with additional transcription factors. A genome-wide ChIP-seq approach revealed a very broad binding of E2F1 at GC-rich sequences (consensus CGCGC) present in the majority of human promoters in vivo [47]. Interestingly, the 193 bp E2F1 responsive region also comprises three GC-rich putative Sp1 binding sites, the first of which largely overlaps the E2F binding site. This finding, together with the TATA-less configuration of the HMGA1 promoter, makes it very similar to a number of cell cycle regulated genes, where E2F1 and Sp1 cooperate for the positive regulation of gene expression [48–52]. Indeed we could show that Sp1 functionally interacts with E2F1 to promote HMGA1 expression, by both luciferase reporter assay and direct examination of the endogenous HMGA1 transcript. However, while Sp1 constitutively binds HMGA1 promoter, it is the balance between different E2F family members (i.e., E2F1 vs. E2F4) that tunes the levels of HMGA1 expression between quiescence and proliferation, similar to other cell cycle regulated genes. Whether this cooperation also involves E2F1 binding to additional GC-rich sites in the HMGA1 promoter needs to be further addressed in future work. Cao et al. [47] revealed E2F1 binding at a TTTCCCGC site located 776 bp downstream of the main element identified in this work, in MCF7 cells. In our ChIP assay we did not find E2F1 binding at this site, in T98G cells. The reason for this apparent discrepancy is not known at the present time, but it might be related to the different nature of the precipitated target (endogenous E2F1 in our assay versus HA-tagged overexpressed E2F1 in Cao's article) or to the different cell system. At this site, however, we revealed E2F4 binding selectively under quiescent cell conditions (Figure S4), suggesting it may possibly mediate a repressive function. Unfortunately, this element maps out of the HMGA1-5#1 luciferase reporter construct and its role in the regulation of HMGA1 expression by different E2Fs could not be investigated further.

The pRB/E2F1 pathway is very frequently deregulated in cancer by either pRB inactivation (due to mutations, interaction with viral oncoproteins or cyclin dependent kinase hyperactivation) or by E2F1 overexpression [58]. Supporting the idea that this pathway might contribute to HMGA1 deregulation during carcinogenesis, we found that pituitary and thyroid tumors developed in an $Rb^{+/-}$ background display increased HMGA1 expression. Therefore, the identification of E2F1 as a transcriptional regulator of HMGA1 significantly broadens our understanding of the mechanisms leading to HMGA1 deregulation in cancer and leads to novel hypothesis that need to be tested in future work. In example, the high expression of HMGA1 commonly detected in cervical cancer appears to be supported by HPV E6/E7 oncoproteins through yet undescribed molecular mechanisms [345859]. The data presented here open the possibility that the activation of E2F1 consequent to E7 binding to pRB might be responsible for HMGA1 deregulated expression.

Fedele and coworkers [19] showed that HMGA2 interacts with pRB, displacing HDAC1 from the pRB/E2F1 complex and leading to E2F1 acetylation and activation. Subsequently, also HMGA1 was shown to interact with pRB and to inhibit its function in cell cycle arrest and differentiation due to E2F1 activation [2728]. Therefore, not only primary activation of the pRB/E2F pathway may lead to HMGA1 overexpression, but it appears that HMGA1 and the pRB/E2F1 pathway are involved in an autoregulatory loop, whereby overexpression of HMGA1 (such as that induced by c-MYC, MYCN, or mutant

KRAS) may enhance E2F1 activity due to its ability to displace HDAC1 from the pRB/E2F1 complexes, further contributing to carcinogenesis.

Importantly HMGA1 seems to impact on transformation at multiple levels. Indeed, by regulating the expression of specific target genes, it enhances inflammation, extracellular matrix degradation, angiogenesis, and impairs DNA repair [24]. Very importantly, HMGA1 also limits apoptosis by inhibiting the induction of p53 target genes and by inducing mislocalization of the p53 proapoptotic activator HIPK2 [252660]. Since E2F1 might also promote apoptosis through p53 dependent and independent mechanisms, its ability to induce HMGA1 might contribute to constrain the p53-dependent E2F1 intrinsic apoptotic potential, while potentiating cell proliferation. At the same time, E2F1-induced HMGA1 expression might inhibit HIPK2 proapoptotic potential recruited by replication stress-inducing oncogenes, such as MYCN [61].

In conclusion, we have reported here that E2F1 is a novel important regulator of HMGA1 expression, thus significantly expanding our understanding of HMGA1 deregulation in cancer.

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