miR-15 and miR-16 Are Direct Transcriptional Targets of E2F1 that Limit E2F-Induced Proliferation by Targeting Cyclin E
Matan Ofir, Dalia Hacohen, and Doron Ginsberg

Abstract
microRNAs (miR) are small noncoding RNA molecules that have recently emerged as critical regulators of gene expression and are often deregulated in cancer. In particular, miRs encoded by the miR-15a, miR-16-1 cluster seem to act as tumor suppressors. Here, we evidence that the miR-15a, miR-16-1 cluster and related miR-15b, miR-16-2 cluster comprise miRs regulated by E2F1, a pivotal transcription factor that can induce both proliferation and cell death. E2F1 is a critical downstream target of the tumor suppressor retinoblastoma (RB). The RB pathway is often inactivated in human tumors resulting in deregulated E2F activity. We show that expression levels of the 4 mature miRs, miR-15a, miR-16-1 and miR-15b, miR-16-2, as well as their precursor pri-miRNAs, are elevated upon activation of ectopic E2F1. Moreover, activation of endogenous E2Fs upregulates expression of these miRs and endogenous E2F1 binds their respective promoters. Importantly, we corroborate that miR-15a/b inhibits expression of cyclin E, the latter a key direct transcriptional target of E2F pivotal for the G1/S transition, raising the possibility that E2F1, miR-15, and cyclin E constitute a feed-forward loop that modulates E2F activity and cell-cycle progression. In support of this, ectopic expression of miR-15 inhibits the G1/S transition, and, conversely, inhibition of miR-15 expression enhances E2F1-induced upregulation of cyclin E1 levels. Furthermore, inhibition of both miR-15 and miR-16 enhances E2F1-induced G1/S transition. In summary, our data identify the miR-15 and miR-16 families as novel transcriptional targets of E2F, which, in turn, modulates E2F activity.

Introduction
E2Fs are transcription factors best known for their involvement in the timely regulation of gene expression required for cell-cycle progression (1). The product of the retinoblastoma (RB) tumor suppressor gene, pRB, exerts growth suppression mainly via inhibitory interactions with E2Fs (2). Sequential phosphorylations of RB by the CDK (cyclin-dependent kinase) complexes cyclin D–CDK4/6 and cyclin E–CDK2 lead to release of E2F from the inhibitory grip of RB and activation of E2F-responsive genes that promote cell-cycle progression; one of the pivotal E2F targets being cyclin E itself (3, 4). The critical role of the RB/E2F pathway in normal cellular proliferation is highlighted by the common incidence in human tumors of pathway mutations that result in deregulated E2F activity (5).

In addition to being fundamental regulators of proliferation, E2Fs modulate diverse cellular functions such as DNA repair, differentiation, and development (6). At least 1 member of the E2F family, namely, E2F1, can also mediate apoptosis (1) and autophagy (7–9). Although the effect of E2F1 on transcriptional regulation of protein-encoding genes has been studied extensively, the role of E2F1 as a modulator of noncoding RNA expression, in general, and microRNAs (miRNA), in particular, is less well studied.

The human genome contains several hundred miRNAs. These are noncoding RNAs, typically 20 to 24 nucleotides in length, that are believed to regulate the expression of about 30% of human genes by either inhibiting mRNA translation or inducing its degradation (10, 11). miRNAs are created from primary transcripts, termed pri-miRNAs, via endonuclease cleavage processing. The synthesis of pri-miRNAs closely resembles that of miRNAs, with many miR-encoding regions embedded in regular protein-coding genes. Some miRNAs are clustered and transcribed as polycistrons that contain several mature miRNAs (10). miRNAs influence a variety of key biological processes, including development, differentiation, apoptosis, survival, senescence, metabolism, and signal transduction (12–15). Furthermore, aberrant expression of distinct miRNAs is
often detected in human malignancies and many recent studies indicate that miRNAs can function as tumor suppressors or oncogenes (16). For example, the miRNAs encoded by the miR-15a, miR-16-1 cluster seem to act as tumor suppressors, as deletion or downregulation of these miRNAs is associated with chronic lymphocytic lymphoma (CLL), pituitary adenomas, and prostate carcinoma (17).

Recently, a number of E2F-regulated miRNAs have been identified, including the miR-17-92, miR-106a-363, and miR-106b-25 clusters as well as miR-449a and miR-449b (18–24). In line with the complex nature of the RB/E2F pathway, some of these E2F-regulated miRNAs regulate E2Fs and/or other pathway components, thereby inhibiting E2F activity (18–20, 22). Thus, E2F-regulated miRNAs participate in feedback loops that modulate and restrict E2F activity.

Here, we evidence that expression of miR-15a, miR-16-1 and miR-15b, miR-16-2 clusters is regulated by E2F. We show that expression levels of all 4 mature miRNAs, miR-15a, miR-16-1, miR-15b, and miR-16-2, as well as their precursor pri-miRNAs, are elevated upon activation of ectopic E2F1. Moreover, activation of endogenous E2Fs upregulates expression of these miRNAs and endogenous E2F1 binds their promoters. Importantly, we corroborate that miR-15a/b inhibits expression of cyclin E, a bona fide target of E2F, raising the possibility that E2F1, miR-15, and miR-16 participate in feedback loops that modulate and restrict E2F activity.

For miRNA quantification, TaqMan miRNA assays (Applied Biosystems) were used according to the manufacturer’s protocol. Levels were normalized to the U6 control gene. All real-time reverse transcriptase PCR (RT-PCR) reactions were performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems Machine. Results are presented as mean and SD for duplicate runs.

**Western blotting**

Cells were lysed in lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40) in the presence of protease inhibitor and phosphatase inhibitor cocktails I and II (Sigma). Equal amounts of protein, as determined by the Bradford assay, were resolved by electrophoresis in an SDS 12.5% polyacrylamide gel and then transferred onto an Immobilon-P (Millipore) polyvinylidene difluoride membrane. The membrane was incubated overnight with 1 of the following primary antibodies: anti-cyclin E (sc-247; Santa Cruz Biotechnology), anti-p53 (sc-126; Santa Cruz Biotechnology), and anti-actin (sc-1616r; Santa Cruz Biotechnology). Binding of the primary antibody was detected using an enhanced chemiluminescence kit (ECL Amersham).

**Plasmids**

The plasmids pBabe-neo-HA-ER-E2F1, pBABE-puro-16E7, pBABE-puro-E7-dl21-35, and pRETROSUPER-shp53 have been described previously (25).

**Transfection/infection procedures**

To generate retroviruses, cells (2 × 10^6) of the packaging cell line 293T were cotransfected with ecotropic packaging plasmid pSV-EMLV (10 μg), which provides packaging helper function, and the relevant cDNA or short hairpin RNA (shRNA) expression plasmid (10 μg) by using the calcium phosphate method in the presence of chloroquin (Sigma). After 8 hours, the transfection medium was replaced with fresh Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Subsequently, cell supernatants containing retroviruses were collected.

When performing infection, cells were incubated for 5 hours at 37°C in 4.5 mL of retroviral supernatant supplemented with polybrene (8 μg/mL; Sigma H9268). Then, 5.5 mL of medium was added, and after a further 24 hours, the medium was replaced with fresh medium containing puromycin (2 μg/mL; Sigma P7130).

**Materials and Methods**

**Cell culture**

U2OS osteosarcoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. H1299 lung adenocarcinoma cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum. Early passage WI38 human embryonic lung fibroblasts were grown in minimal essential medium supplemented with 15% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and nonessential amino acids. Cells were maintained at 37°C in a humidified atmosphere containing 8% CO2. To induce activation of ER-E2F1, cells were treated with 300 nmol/L 4-hydroxytamoxifen (OHT) for the times indicated.

**Quantitative PCR**

Total RNA was extracted from the cells by the Tri Reagent method. Real-time quantitative PCR (qPCR) was done with Absolute Blue SYBER Green Rox Mix (Thermo) and the following primer pairs:
miRIDIAN miRNA mimics and hairpin antagomiRs were obtained from Dharmacon. For their transfection, Dharmafect transfection reagent (Dharmacon) was employed according to the manufacturer’s instructions. Experiments were carried out 48 hours after transfection with miRNA mimic or hairpin antagomiRs.

**Fluorescence-activated cell-sorting analysis**
Cells were trypsinized and then fixed by incubating in 70% ethanol at 4°C overnight. After fixation, cells were centrifuged for 4 minutes at 1,500 rpm before being incubated for 30 minutes at 4°C in 1 mL of PBS. Then, the cells were centrifuged again and resuspended in PBS containing 5 mg/mL propidium iodide (PI) and 50 μg/mL RNase A. After incubation for 20 minutes at room temperature, fluorescence intensity was analyzed by a Beckton Dickinson flow cytometer.

**Chromatin immunoprecipitation**
DNA–protein complexes were immunoprecipitated from U2OS cells by using the ChIP (chromatin immunoprecipitation) assay kit (Upstate Biotechnology) according to the manufacturer’s protocol with the following polyclonal antibodies: anti-E2F1 (sc-193; Santa Cruz), anti-E2F4 (sc-866), and anti-HA (sc-805; Santa Cruz); the latter served as a control for nonspecific DNA binding. The precipitated DNA was subjected to PCR analysis by using specific primers corresponding to the Dleu2 promoter, the SMC4 (structural maintenance of chromosomes 4) promoter, and primers that served as a negative control for E2F1 binding (β-tubulin):

- **DLEU2:**
  5’GGTTATCCTCTCTCCGCTAGATTGA and 5’CATGCCGAAAATGTCG GGA
  SMC4: 5’GCA-GAGGGACAATAAGATGG and 5’ CGCTCTCAGCCGTTCTCGβ-
  Tubulin: 5’GGAAGCTGATGG and 5’ CAGCTCTCAGCCGTTCTCG

**Results**
To identify miRNAs regulated by E2F, we took advantage of the fact that many miRNAs are located within genes, and, in such cases, expression of the resident miRNA(s) and host gene is often coregulated. With this in mind, we screened the miRbase database for miRNAs located within E2F-regulated genes. This screen picked up 2 miRNA clusters, namely, the miR-106b-25 cluster, which resides in intron 13 of the minichromosome maintenance complex component 7 gene (Mcm7), already reported to be E2F regulated (20), and the miR-15b, miR-16-2 cluster, which resides in intron 5 of the SMC4 gene. The latter encodes for a protein that functions mainly in M phase and is essential for mitosis-specific chromatin condensation (26) and has been suggested to be an E2F-regulated gene, as its mRNA levels are elevated in RB-deficient cells (27) and its promoter binds E2F1 and E2F4 (28). Of note, the miR-15b, miR-16-2 cluster is homologous to the miR-15a, 16-1 cluster, which is located in the Dleu2 locus and considered to be a tumor suppressor (29) as its downregulation is associated with a number of human tumors, including CLL, pituitary adenomas, and prostate carcinoma (17).

Given the high-throughput data concerning E2F regulation of SMC4 and the tumor suppressor activity attributed to the Dleu2-encoded miRs, we focused our attention on the miR-15 and miR-16 families. We took advantage of a conditionally active E2F1 (ER-E2F1), which is activated by the addition of OHT (30), to validate that SMC4 expression is E2F-regulated and to examine whether Dleu2 expression is influenced similarly by E2F. Indeed, activation of this conditional E2F1 in both U2OS human osteosarcoma cells and H1299 human lung carcinoma cells was associated with significantly increased SMC4 and Dleu2 RNA levels (Fig. 1A and B). Next, we examined directly whether E2F1 activation affects expression levels of Fig. 1. Activation of ectopic E2F1 upregulates expression of the miR-15a, miR-16-1 and miR-15b, miR-16-2 clusters. H1299 cells (A) and U2OS cells (B) expressing ER-E2F1 were incubated with OHT (300 nmol/L) for 18 hours (+) or left untreated (−). Then, RNA was extracted, and Dleu2 and SMC4 RNA levels were determined by real-time RT-PCR and normalized to actin or HPRT levels. C: H1299 cells expressing ER-E2F1 were incubated with OHT (300 nmol/L) for 18 hours (+) or left untreated (−). Then, RNA was extracted and mature miR-15a, miR-15b and miR-16 expression levels determined by real-time RT-PCR and normalized to RNU6B levels.
mature miRs-15 and miRs-16. As predicted from their location, the expression levels of mature miR-15a, miR-15b and miR-16 were induced significantly upon activation of conditional E2F1 in H1299 cells (Fig. 1C).

It has been reported that miR-15 and miR-16 are regulated by p53 (21, 31, 32). Since E2F1 activates p53 via numerous pathways (33), it is possible that E2F1 regulation of miR-15 and miR-16 expression is mediated by p53. However, since H1299 cells are p53 deficient (34), induction of miR-15 and miR-16 levels upon E2F1 activation in this cell line is p53 independent. In line with this, we found that activation of conditional E2F1 induced miR-15 expression levels in the p53-proficient U2OS cell line even in the presence of shRNA directed against p53 (Supplementary Fig. S1). Therefore, at least in some settings, E2F1 upregulates expression of miR-15 and miR-16 in a p53-independent manner.

Having established that activation of ectopic E2F1 induces expression of miRs-15 and miR-16, we checked whether endogenous E2Fs are capable of influencing their expression. To this end, we took advantage of the human papilloma virus oncoprotein E7, which disrupts RB/E2F complexes resulting in the activation of endogenous E2Fs. In line with our ectopic E2F2 data, expression of E7 in WI38 human fibroblasts elevated the levels of mature miR-15a, miR-15b and miR-16 whereas expression of a mutant E7, which does not disrupt RB/E2F complexes, had no effect (Fig. 2A). To discover whether endogenous E2Fs directly regulate miRs-15 and miRs-16, we investigated whether E2Fs bind the human promoters of the Dleu2 pri-miR and the SMC4 gene that contain miR-15a, miR-16-1 and miR-15b, miR-16-2, respectively. In silico analysis revealed that the human Dleu2 promoter contains an evolutionary conserved E2F binding site at position −4 to +4 and the human SMC4 promoter contains 2 evolutionary conserved E2F binding site at positions −1 to +7 and −41 to −33 (Fig. 2B). Importantly, ChIP using chromatin from proliferating U2OS cells and antibodies directed against E2F1 and E2F4 showed that endogenous E2F1 is associated detectably with both promoters in vivo (Fig. 2C). In addition, binding of endogenous E2F4 to the SMC4 promoter was also detected (Fig. 2C; refs. 28, 35). Taken together, these data support that expression of the miR-15 and miR-16 families is regulated by endogenous E2F. To test whether the expression of miRs-15 and miRs-16 is cell-cycle regulated, we examined their RNA levels in WI38 cells that were growth arrested at G1 by serum starvation and induced to reenter the cell cycle by addition of 15% FBS. Under these conditions, there were no significant changes in the levels of miR-15 and miR-16 whereas levels of another E2F-responsive gene, cd25A, were increased (Supplementary Fig. S2). Thus, our data suggest that endogenous deregulated E2F elevates miR-15 and miR-16 expression, yet these miRs are not regulated by E2F during normal cell-cycle progression. A similar pattern of expression was reported for other E2F-regulated genes such as ARF.

Expression of miR-15 and miR-16 has been reported to inhibit cell proliferation and induce apoptosis (36, 37). This ability to inhibit proliferation is attributed to their targeting multiple cell-cycle genes, including cyclin D1, cyclin D3, cyclin E1, and Cdk6 (37–41), while the effect on apoptosis is through targeting antiapoptotic genes such as Bcl-2 and

Figure 2. Expression of miR-15 and miR-16 is regulated by endogenous E2F. A, WI38 cells were infected with a retrovirus expressing either wild-type E7 (E7) or an RB-binding-deficient mutant of E7 (E7mut). Total RNA was extracted from the cells, and expression levels of the mature miR-15a, miR-15b and miR-16 were determined by RT-PCR and normalized to RNU6B levels. B, sequences of SMC4 and Dleu2 promoter regions surrounding their putative E2F binding sites, which are shown in bold and underlined. The sequences of SMC4 and Dleu2 promoter region of human, rhesus, mouse, and dog were obtained using UCSC genome browser and analyzed for the presence of E2F binding sites using TRANSFAC matrices. C, ChIP analysis was performed using U2OS cells. Cross-linked chromatin was precipitated using antibodies specific to E2F1, E2F4, and HA. Levels of SMC4 and Dleu2 promoter fragments were determined using PCR. Input DNA represents 0.5% of total chromatin.
Mcl-1 (36, 42). Since the subset of genes targeted by a given miR can differ between tissues and cell lines, we investigated the consequences of miR-15 expression in U2OS human osteosarcoma cells. miR-15 was transfected into U2OS cells and Western blot analysis was done to determine protein levels of cyclin E and Bcl-2. In agreement with the presence of 2 evolutionary conserved binding sites for miR-15/16 in the 3' UTR (untranslated region) of cyclin E (40), ectopic miR-15 expression resulted in markedly reduced cyclin E protein levels (Fig. 3A). Levels of Bcl-2 protein were mildly reduced. Notably, this miR-15–dependent decrease in cyclin E levels was associated with a reduction in the percentage of S-phase cells and a concomitant increase in the percentage of G, cells (Fig. 3B), indicating that miR-15 inhibits the G1/S transition. In line with the mild effects of miR-15 expression on Bcl-2 levels, no significant change in apoptosis levels was detected (data not shown).

Cyclin E1 is transcriptionally regulated by E2F (3) and, as shown by us here and previously by others, posttranscriptionally regulated by the miR-15 and miR-16 families (37, 39–41). In addition, as shown here, E2Fs transcriptionally regulate the miR-15 and miR-16 families. We noted that this network of regulation constitutes an incoherent type 1 FFL, whereby a transcription activator activates a gene directly and also activates a repressor of the gene (Fig. 4D; ref. 43). To test whether such an FFL is operating, we examined the effect of reducing miR-15a and miR-15b levels on E2F1-induced cyclin E expression. To this end, miR-15a and miR-15b expression was inhibited in U2OS cells expressing conditionally active E2F1 by introducing antagomiRs directed against either miR-15a or miR-15b. As expected, introduction of a nonspecific antagomiR did not affect either the basal level of miR-15a and miR-15b or their induction by E2F1. In contrast, introducing an antagomiR directed against miR-15a or miR-15b resulted in reduced basal levels of these miRs and inhibited their upregulation by E2F1 (Fig. 4A). We observed that the antagomiR directed against miR-15a also significantly inhibited miR-15b expression and an antagomiR directed against miR-15b also reduced to some extent the expression of miR-15a, most probably due to the high sequence similarity between these 2 related miRs. Having established the activity of the antagomiRs, we examined their effects on E2F regulation of cyclin E1 expression. As previously reported, activation of E2F1 resulted in an increase in cyclin E1 mRNA and protein levels (Fig. 4B and C). Importantly, antagomiR-mediated inhibition of miR-15a and miR-15b expression resulted in significantly enhanced E2F1-induced upregulation of cyclin E1 levels, reflected at both the RNA and protein levels (Fig. 4B and C). Thus, these data support the existence of an incoherent FFL comprising E2F1, miR-15, and cyclin E1.

To test whether miR-15 and miR-16 affect E2F1-induced G1/S transition, we examined the effect of reducing miR-15 and miR-16 levels on E2F1-induced G1 exit of serum-starved cells. To this end, antagomiRs directed against miR-15a and miR-16 were introduced into U2OS cells expressing conditionally active E2F1. Next, cells were serum starved to enrich for cells in G1 and then E2F1 was activated to induce S-phase entry. As previously reported, activation of ectopic E2F1 resulted in G1 exit. Importantly, inhibition of miR-15 and miR-16 expression (Fig. 5B) resulted in significantly enhanced E2F1-induced G1 exit (Fig. 5A). These data indicate that miRs-15 and miRs-16 may attenuate E2F1-mediated cell-cycle progression.

In summary, our data identify the miR-15 and miR-16 families as novel E2F transcriptional targets that modulate E2F-dependent cyclin E expression and cell-cycle progression.

Discussion

We establish here that expression of miR-15 and miR-16 is regulated by E2F1. Specifically, we show that activation of ectopic E2F1 results in elevated levels of
miR-15a, miR-16-1 and miR-15b, miR-16-2 and of their respective precursors SMC4 and Dleu2. Moreover, we show that expression of these miRs is induced by E7, a viral protein that activates endogenous E2Fs, and ChIP analyses evidence that E2F1 is bound to the promoters regulating the expression of these miRNAs. In addition, having established E2F regulation of miRs-15 and miRs-16, we evidence the participation of this relationship in a larger regulatory loop, for these miRs target cyclin E, a pivotal E2F target gene. Accordingly, we show that inhibition of miR-15 expression enhances E2F1-induced upregulation of cyclin E1 mRNA and protein levels, and, conversely, ectopic expression of miR-15 reduces cyclin E protein levels and inhibits the G1/S transition, the latter a well-documented biological outcome of E2F activity. Furthermore, inhibition of both miR-15 and miR-16 expression augments E2F1-mediated G1/S transition. Summarily, our data reveal that miR-15 and miR-16 represent novel E2F targets that restrict E2F activity.

This is not the first example of E2F-dependent restriction of E2F activity. It was shown previously that ectopic expression of miR-15a, miR-16-1 and miR-15b, miR-16-2 downregulates cyclin E levels and reduced expression enhances E2F1-dependent cyclin E upregulation. Nevertheless, given that the seeds of miR-15 and miR-16 are highly similar and miR-16 was shown by others to negatively regulate cyclin E (40), we consider it reasonable to extrapolate that E2F1 upregulates the expression of 4 distinct miRs, namely, miR-15a, miR-16-1 and miR-15b, miR-16-2, all of which target cyclin E. Given this scenario, E2F1-regulated miRs likely exert a significant effect on cyclin E expression. Notably, data from other studies suggest that miR-15 and miR-16 downregulate not only cyclin E but also a number of other pivotal regulators of cell-cycle progression, such as cyclin D1, cyclin D3, and CDK6 (37–41). Thus, these 4 miRs can collaborate to restrict E2F1-induced cell-cycle progression by targeting multiple proliferation-related genes. A defect in the RB/E2F pathway that results in
deregulated and hyperactive E2F is a common event in many human tumors (5) and deletion of miR-15a and miR-16-1 is often detected in some human tumors (17). Our data suggest that these 2 molecular alterations may cooperate during tumor development to sustain enhanced proliferation of transformed cells.

In some settings, miR-15 and miR-16 can induce apoptosis by targeting the antiapoptotic genes Bcl-2 and Mcl-1 (36, 42). We did not find that elevated miR-15 and miR-16 expression was associated with increased apoptosis in the U2OS osteosarcoma cell line. This, notwithstanding, in other contexts, miR-15 and miR-16 may contribute to E2F1-induced apoptosis. A corollary of this premise is that the frequent deletion of the miR-15a, miR-16-1 locus in some human tumors could serve to limit E2F1-induced apoptosis, the latter a result of the deregulated E2F associated with common RB/E2F pathway mutations. Thus, these 2 molecular alterations bear the potential to cooperate during tumor development not only to sustain proliferation but also to suppress apoptosis of transformed cells.

Summarily, we show here that the cancer-related miR-15 and miR-16 families are transcriptionally regulated by E2F1 and can restrain E2F1 activity. We propose that these miRs serve to fine-tune E2F1 activity and thereby prevent excessive E2F1 activity that could harm normal cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 5. Inhibition of miR-15a and miR-16 expression enhances E2F1-induced G1 exit. U2OS cells expressing ER-E2F1 were transfected with anti-miRNA oligonucleotides complementary to miR-15a and miR-16, or a nonspecific miR (n.s.). Twelve hours posttransfection cells were serum starved (48 hours, 0.1% serum) and then treated with OHT (300 nmol/L) or left untreated for 24 hours before harvesting. A, cells were analyzed by FACS (fluorescence-activated cell sorting; PI). Percentages of cells exiting G1 after activation of E2F1 are depicted in the bar graph. The results show data from 3 independent experiments expressed as the mean ± SD. Statistical differences were calculated using unpaired 2-tailed Student’s t test. *, statistical significance P < 0.05. B, RNA levels of mature miR-15a, miR-15b and miR-16 were determined by real-time RT-PCR and normalized to RNU6B.
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