Published in final edited form as:

Mol Cell Endocrinol. 2012 April 4; 351(2): 184–198. doi:10.1016/j.mce.2011.12.004.

Transcription of the human *EAP1* gene is regulated by upstream components of a puberty-controlling Tumor Suppressor Gene network

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Abstract

Mammalian puberty is initiated by an increased pulsatile release of gonadotropin-releasing hormone (GnRH) from specialized neurons located in the hypothalamus. GnRH secretion is controlled by neuronal and glial networks, whose activity appears to be coordinated via transcriptional regulation. One of the transcription factors involved in this process is thought to be the recently described gene Enhanced at Puberty 1 (*EAP1*), which encodes a protein with dual transcriptional activity. In this study we used gene reporter and chromatin immunoprecipitation (ChIP) assays to examine the hypothesis that *EAP1* expression is controlled by transcriptional regulators earlier postulated to serve as central nodes of a gene network involved in the neuroendocrine control of puberty. These regulators include Thyroid Transcription Factor 1 (TTF1), Yin Yang 1 (YY1) and CUX1, in addition to *EAP1* itself. While TTF1 has been shown to facilitate the advent of puberty, YY1 (a zinc finger protein component of the Polycomb silencing complex) may play a repressive role. The precise role of CUX1 in this context is not known, but like *EAP1*, CUX1 can either activate or repress gene transcription. We observed that DNA segments of two different lengths (998 and 2744 bp) derived from the 5'-flanking region of the human *EAP1* gene display similar transcriptional activity. TTF1 stimulates transcription from both DNA segments with equal potency, whereas YY1, CUX1, and *EAP1* itself, behave as transcriptional repressors. All four proteins are recruited *in vivo* to the *EAP1* 5'-flanking region. These observations suggest that *EAP1* gene expression is under dual transcriptional regulation imposed by a trans-activator (TTF1) and two repressors (YY1 and CUX1) previously postulated to be upstream components of a puberty-controlling gene network. In addition, *EAP1* itself appears to control its own expression via a negative auto-feedback loop mechanism. Further studies are needed to determine if the occupancy of the *EAP1* promoter by these regulatory factors changes at the time of puberty.

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Keywords

Puberty; EAP1; transcription; gene expression

1. Introduction

Several years ago, Rampazzo and colleagues described a DNA sequence in human chromosome 14q23-14q32 predicted to encode an intronless gene named *C14ORF4* (Rampazzo et al., 2000). The single exon of *C14ORF4* is predicted to originate a protein of 796 amino acids with a calculated molecular mass of 82.7 kDa. More recently, a DNA array screen of the female monkey hypothalamus followed by extensive molecular validation showed that *C14ORF4* encodes a transcription factor and that *C14ORF4* mRNA abundance increases in the hypothalamus at the time of puberty (Heger et al., 2007). This increase occurs in the absence of the ovaries indicating that it is centrally originated (Matagne et al., 2009). Based on these findings, *C14ORF4* was renamed *Enhanced at Puberty 1* (*EAP1*). We also showed that *EAP1* displays dual transcriptional activity, trans-activating the *GnRH* promoter, but repressing the *Proenkephalin* (Heger et al., 2007), and to a lesser extent, the *Kiss1* promoter (Mueller et al., 2011).

The importance of *EAP1* in controlling both the initiation of puberty and adult female reproductive function was demonstrated by the finding that RNA interference (RNAi)-mediated knock-down of *Eap1* expression in the anteroventral periventricular region of female rats delayed puberty and disrupted estrous cyclicity (Heger et al., 2007). Two recent reports have provided evidence that *EAP1* is not only required for normal adult reproductive function in rodents, but also in higher primates. RNAi targeted to the arcuate nucleus (ARC) of the nonhuman primate hypothalamus obliterated menstrual cyclicity (Dissen et al., 2011), and a single nucleotide polymorphism in the *EAP1* promoter region was found to be associated with loss/disruption of menstrual cyclicity in nonhuman primates (Lomniczi et al., 2011). Other recent findings have made clear that the biological importance of *EAP1* transcends its involvement in neuroendocrine reproductive function. These studies showed that *EAP1* is a critical component of a repressive complex that also includes DIF-1 (Death Domain-interacting factor; also known as Interferon Regulatory Factor-2 Binding protein 2, IRF-2BP2), and IRF2BP1 (Interferon Regulatory Factor-2 Binding Protein 1). The interaction of DIF-1, IRF2BP1, and *EAP1* occurs through the conserved C4 zinc-fingers of these proteins, and results in transcriptional repression of a proapoptotic gene in cancer cells (Yeung et al., 2011). These observations suggest that *EAP1* plays a fundamental role in the control of basic cellular processes, and that the contribution of *EAP1* to the control of neuroendocrine reproductive development and adult reproductive function depends on its ability to modify the transcriptional activity of downstream puberty-controlling genes expressed in the neuroendocrine brain. The recently described *EAP1* involvement in cancer biology (Yeung et al., 2011), and the potential involvement of a tumor suppressor gene (TSG) network in the neuroendocrine control of female puberty (Roth et al., 2007), highlight the importance of exploring the functional connections that may exist between upper-echelon TSGs and *EAP1* transcriptional activity.

Here, we report the location of the human *EAP1* gene Transcription Start Site (TSS), examine the transcriptional activity of 5'-flanking *EAP1* fragments using a neuronal and a non-neuronal cell line, and provide evidence that *EAP1* transcriptional activity is regulated by *EAP1* itself and by thyroid-transcription factor 1 (TTF1), CCAAT displacement protein (CDP, also known as CUTL1 and CUX1), and Yin-Yang1 (YY1), three major upstream components of the TSG network postulated to contribute to the neuroendocrine control of female puberty (Roth et al., 2007). TTF1 is a homeodomain-containing transcription factor

(Price et al., 1992). It activates the expression of different genes in the thyroid, lung, and restricted regions of the brain (Bingle, 1997), and is required for development of the hypothalamus (Kimura et al., 1996; Sussel et al., 1999). It is also involved in facilitating female puberty (Mastronardi et al., 2006). In several tissues, the transcriptional activity of TTF1 is increased by a co-factor named TAZ (transcriptional co-activator with a PDZ-binding motif) (Di Palma et al., 2009; Park et al., 2004; Kanai et al., 2000).

CUX1, another homeodomain gene, behaves both as a transcriptional repressor and activator, depending on the cellular context (Dufort and Nepveu, 1994; Superti-Furga et al., 1989; Valarche et al., 1993; Harada et al., 2008; Kim et al., 1997). Loss of *Cux1* results in reduced male fertility, deficiency in T and B cell function, retarded differentiation of the lung epithelia, and retarded growth (Ellis et al., 2001; Luong et al., 2002; Sinclair et al., 2001; Nepveu, 2001).

Finally, YY1 is a member of the Polycomb silencing complex (Schwartz and Pirrotta, 2007) that exerts its effects via its ability to initiate, active or repress gene transcription (Shrivastava and Calame, 1994; Gordon et al., 2006). YY1, plays a fundamental role in normal biological processes like embryogenesis, differentiation, replication, and cellular proliferation (reviewed in Gordon et al., 2006).

2. Materials and Methods

2.1 Identification of the *EAP1* Transcription Start Site using 5' RACE-PCR

To map the Transcription Start Site (TSS) of *EAP1*, total RNA from human cortex tissue (Ambion, Austin, USA) was used for 5' Rapid Amplification of cDNA Ends (RACE), performed with the GeneRacer kit (Invitrogen, Karlsruhe, Germany). Briefly, total RNA was treated with calf intestinal phosphatase (provided with the kit) to dephosphorylate truncated mRNAs and non-mRNAs, and with tobacco acid phosphatase to remove the 5' cap structure from intact, full-length mRNAs. The GenRacer RNA oligonucleotide was then ligated to the treated mRNA using T4 RNA ligase provided with the kit. Linked RNA was reverse transcribed with Omniscript Kit (Qiagen, Hilden, Germany) using either oligo-DT primers or random hexamer primers. The conditions for reverse transcription were: 1h at 37°C and 5 min at 93°C. PCR amplification utilizing HotStarTaq DNA Polymerase (Qiagen) and the primer pairs 1F/R (Table 1) were used for hot start, touchdown PCR performed with the following cycling conditions: 15 min at 95°C, 30 sec at 94°C and 4 min at 72°C for 5 cycles, 30 sec at 94°C and 70°C for 5 cycles, and 30 sec at 94°C and 4 min at 68°C for 20 cycles. Final extension was 10 min at 72°C. The resulting amplicon was used as template for nested PCR (primer pair 2F/R, Table 1). The PCR product was visualized on an agarose gel and cloned into pCR® II TOPO®-Vector (TOPO TA Cloning Kit, Invitrogen). XL1-Blue super competent cells (Stratagene, Heidelberg, Germany) were transformed with this plasmid and 24 clones were analyzed by sequencing.

2.2 Identification of transcription factor binding sites

We identified putative binding sites for TTF1, CUX1, YY1, and SP1 within the human *EAP1* promoter and rat *Eap1* promoter using the Transcription Element Search System [TESS; <http://www.cbil.upenn.edu/cgi-bin/tess/tess>; (Schug, 2008)], TFSEARCH: Searching Transcription Factor Binding Sites (ver 1.3; <http://www.cbrc.jp/research/db/TFSEARCH.html>), and literature search (PubMed; <http://preview.ncbi.nlm.nih.gov/pubmed/>) combined with Vector NTI 9.0.0 (Invitrogen; Sep 02, 2003; InforMax, Frederick, USA).

2.3 Gene reporter constructs

To generate *EAP1* promoter constructs of different lengths, two DNA fragments were amplified from human genomic DNA (Promega, Mannheim, Germany) using the primer pair 3F/R, 4F/R, and 5F/R (Table 1). The shorter (998 bp) PCR product (−599/+399; +1 =TSS) was cloned into the pCR[®]II TOPO[®]-Vector utilizing the Rapid Ligation Kit (Fermentas, St. Leon-Rot, Germany). To generate a 2744 bp promoter fragment, a 2556 bp (−2345/+210) PCR-product, amplified with primer pair 4F/R, and a 1649 bp (−1250/+399) PCR-product, amplified with primers 5F/R, were cleaved with BsrGI and HindIII. Thereafter, a digested segment of the 1649 bp fragment was ligated to the 3'-end of the 2556 bp product (Rapid Ligation Kit, Fermentas) to generate a 2744 bp (−2345/+399) product. This product was then cloned into the EcoRI site of pCR[®]II TOPO[®]-vector as above. After verifying their sequence and orientation, the promoter fragments were subcloned into the KpnI and XhoI of the luciferase pGL4.10 basic vector (Promega).

2.4 Plasmid constructs

Human CUX1—We used two different CUX1 constructs cloned into pXJ42 (both kind gifts from A. Nepveu, McGill University, Montreal, Canada). The pXJ42/MCH construct contains the full length human *CUX1* (p200; NM_181552.2; nucleotides (nt) 39-4554), and pXJ42/MCH/878-1505 the *CUX1* isoform p110 (NM_181552.2; nt 2671-4554). Both isoforms differ in length and transcriptional activity. P200 has been reported to be inhibitory, while p110 exhibit either repressive or trans-activating activity, depending on the promoter context (Sansregret and Nepveu, 2008).

Human EAP1—We used an expression vector containing the coding region of human *EAP1* mRNA, as previously described (Heger et al., 2007). A construct lacking the RING finger domain of *EAP1* (Heger et al., 2007; Waterman et al., 1999) was used to determine the structural requirements for EAP1 action.

Human SP1—hSP1 (2891 bp) was purchased from the German Resource Center of Research on Genome (RZPD GmbH, Berlin, Germany), cleaved out from the pOTB7-hSP1 vector by EcoRI and XhoI and sub-cloned into pcDNA3.1/zeo.

Murine Taz—We used a pCMV-Taz construct (the kind gift of Mariastella Zannini, Istituto di Endocrinologia ed Oncologia Sperimentale, Italy). The *Taz* coding region (AJ299430) was removed by MluI and blunted using a Klenov Kit (Amersham USB, Freiburg, Germany). Thereafter, the DNA fragment was digested with EcoRI and subcloned into the EcoRI and EcoRV sites of the pcDNA3.1 vector (Invitrogen).

Rat Taz—To generate a pGEMT-rTaz construct, a partial rat *Taz* coding sequence (NM_001024869; nt 245–715) was amplified from GnV3 cells (the kind gift from Francois P. Pralong, Faculté de biologie et de médecine, Switzerland) using the primer pair 15F/R, (Table 1). The PCR product was cloned into the pGEM[®]T vector (Promega). After transformation of the pGEMT-rTaz into XL1-Blue super competent cells (Stratagene) and purification using QIAprep Spin Miniprep Kit, the orientation and sequence of the rTaz cDNA was verified by automatic sequencing.

Rat Ttf1—A pcDNA3.1⁺/zeo-rTtf1 construct (Lee et al., 2001) containing the complete open reading frame of rat *Ttf1* (X53858) was used.

Human YY1—A pcDNA3.1⁺/zeo-HAYY1 construct containing the full coding sequence of human *YY1* (NM_003403.3) was generated by excising with XbaI and ApaI a hemagglutinin

epitope (HA)-tagged YY1 from pCMV-HAYY1 (kind gift from Y. Shi, Harvard Medical School, Boston, USA).

2.5 Site directed mutagenesis

Putative binding sites for TTF1 and the forward oriented ATNNAT CUX1 binding sites were deleted by site directed mutagenesis using the pCR[®]II TOPO[®]-*EAP1*p998 as the template and the QuikChange XL Site-directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). Primer pairs resulting in deletion of 4–6 bp within the TTF1 or CUX1 recognition site core sequences were used (6F/R to 12F/R, Table 1). Each mutation was verified by sequencing. Thereafter, the mutated fragments were subcloned into pGL4.10 basic vector (Promega).

2.6 Cell culture

GripTite[™] 293 MSR cells (Invitrogen) were maintained in Dulbecco's modified Eagles medium containing high glucose (4.5 g/l, PAA Laboratories, Pasching, Germany), supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 0.1 mM non-essential amino acids (PAA), and 600 µg/ml G-418 Sulphate (PAA). The *GripTite*[™] 293 MSR cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

GT1-7 cells (kindly provided by Pamela Mellon, Department of Reproductive Medicine, University of California, San Diego, La Jolla, Ca, USA), were cultured in Dulbecco's modified Eagles medium containing high glucose (4.5 g/l; PAA Laboratories), supplemented with 10% FBS (Biochrom), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Hela cells (LGC Standards GmbH, Wesel, Germany), were grown in a humidified atmosphere containing 5% CO₂ and 37°C. The culture medium was Dulbecco's modified Eagles medium containing high glucose (4.5 g/l; PAA Laboratories), supplemented with 10% fetal bovine serum, 2mM Glutamine (PAA Laboratories), 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.7 Transient transfection and Dual-Luciferase promoter assays

To determine *EAP1* promoter activity, two constructs of different lengths (998 and 2744 bp) cloned into the luciferase reporter plasmid pGL4.1 basic (Promega) were used.

The transregulatory effects of *EAP1*, YY1, or CUX1 on *EAP1* gene activity were examined in the human embryonic kidney cell line *GripTite*[™] 293 MSR and in the mouse immortalized gonadotropin-releasing hormone neuron cell line GT1-7. *GripTite*[™] 293 MSR cells were seeded in antibiotic-free medium at a density of 2×10⁵ cells per well in 24-well dishes (Greiner bio-one, Frickenhausen, Germany) 18 h before transfection. Each of the reporter plasmids (pGL4.1h*EAP1*p998; pGL4.1h*EAP1*p2744 at 500 ng/well) was transiently transfected for 4 h using Nanofectin (PAA) for *GripTite*[™] 293 MSR cells or TurboFect[™] *in vitro* Transfection Reagent (Fermentas) for GT1-7 cells, in conjunction with an expression vector pcDNA3.1zeo (Invitrogen at 500 ng/well) containing or not the coding region of *EAP1*, YY1, or *Ttf1*, or the expression vector pXJ42 containing *CUX1*-p200 or *CUX1*-p110. To determine if the co-activator TAZ interacts with TTF1 to affect *EAP1* transcriptional activity, transfections were performed using 400 ng reporter plasmids in conjunction with 400 ng *TTF1* expression vector and/or 200 ng of TAZ expression vector. The same concentrations were used studying the interaction of SP1 and YY1. Transfection experiments were done in quadruplicates and the luciferase values obtained were normalized by cotransfecting the renilla plasmid pGL4.70 [hRluc] (Promega) at 20 ng/well.

Cells were harvested 48 h after transfection (or 24 h in the case of TAZ experiments) and lysed in passive lysis buffer (Promega). Luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) in a luminometer according to the manufacturer's protocol. Relative firefly luciferase activities were calculated by correction of transfection efficiency using the Renilla luciferase activities as the normalizing unit.

2.8 Chromatin Immunoprecipitation (ChIP) Assay

These assays were carried out essentially as described (Mueller et al., 2011). To determine the association of EAP1 and TTF1 to the *EAP1* promoter, we transfected HeLa cells with either an expression vector carrying the *EAP1* coding region tagged with an influenza hemagglutinin (HA) epitope (kindly provided by H. Samuels, New York University School of Medicine, New York) or the rat *Ttf1* construct described in section 2.4. In brief, 25×10^6 cells were seeded in 15 cm diameter plates, and 24 h later 45 μ g of each expression vectors were transiently transfected using 45 μ l Plus reagent and 67.5 μ l Lipofectamine LTX (Invitrogen). Forty-eight hours after transfection, the cells were harvested for chromatin immunoprecipitation. To provide experimental evidence for interactions of TTF1, CUX1, and YY1 with the promoter region of *Eap1* in an *in vivo* context, we also performed ChIP assays of these transcription factors using chromatin extracted from the medial basal hypothalamus (MBH) of prepubertal female rats. After completion of the chromatin isolation procedure, the sonicated chromatin was precleared with Protein A/G beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) for 1 h at 4°C. One aliquot (15 μ l of 1000 μ l) of the cleared chromatin was stored at -80°C as input DNA. Aliquots (150 μ l) of the remaining chromatin were then incubated with 5 μ g of a mouse monoclonal antibody against HA (Covance, Berkeley, CA) to detect EAP1, rabbit polyclonal antibodies against TTF1 (kindly provided by C. Mendelson, Southwestern Medical Center, Dallas, TX, USA), a mouse monoclonal antibody (sc-7341 X, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect YY1, or rabbit polyclonal antibodies (sc-13024, Santa Cruz Biotechnology) to detect CUX1. One chromatin aliquot from HeLa cells was incubated with normal mouse IgG (sc-2025, Santa Cruz Biotechnology) as a negative control for immunoprecipitation. Likewise, aliquots (100 μ l) of rat MBH chromatin were incubated with 10 μ g of antibody against TTF1 and YY1 as indicated above, or a mouse monoclonal antibody (ab54583, Abcam Inc, Cambridge, USA) to detect CUX1. Two additional aliquots of chromatin from the rat MBH were incubated with either a monoclonal antibody against *Drosophila melanogaster* Ecdysone Receptor (ECR) (kindly provided by C. Thummel, Department of Human Genetics, University of Utah, USA) or a rabbit polyclonal antibody against β -Galactosidase (β -Gal)(Cortex Biochem, Inc, San Leandro, USA) and used as negative controls for the immunoprecipitation. Each reaction was carried out in final volume of 1 ml ChIP dilution buffer. Additional details of the immunoprecipitation procedure have been reported (Mueller et al., 2011).

2.9 PCR detection of Chromatin Immunoprecipitated DNA

To detect CUX1, EAP1, YY1, and TTF1 binding to the 5'-flanking region of the human *EAP1* gene in HeLa cells we PCR-amplified two DNA fragments using two different sets of primers. For **region I** (nt -1731 to -1300), primer pair 13F/R amplifies an amplicon of 432 bp. For **region II** (nt -307 to -83), primer pair 14F/R amplifies an amplicon of 225 bp. We also PCR-amplified two DNA segments to detect CUX1, YY1 and TTF1 binding to the promoter region of the *Eap1* gene in rat MBH samples. For **region I** (nt -1794 to -1706), primer pair 18F/R amplifies an amplicon of 87 bp. For **region II** (nt -1177 to -866), primer pair 19F/R amplifies an amplicon of 311 bp. PCR reactions were carried out using Hot Star Taq polymerase (Qiagen); the thermocycling conditions used were 95°C for 5 min, followed by 40 (13, 14 and 19F/R) or 35 (18F/R) cycles of 95°C for 30 sec, 59°C (13 and 14F/R),

55°C (18F/R) or 50°C (19F/R) for 30 sec, and 72°C for 1 min. All primers were purchased from Eurofins MWG Operon (Huntsville, AL) and are shown in Table 1.

2.10 Collection of brain tissues

Cerebral cortex (CTX), MBH, and preoptic area (POA) were collected from prepubertal (postnatal day 21, PND21) female Sprague-Dawley rats at the Hannover Medical School, Hanover, Germany for qPCR. The MBH of 28-day-old female rats was collected at the Oregon National Primate Research Center, Beaverton, USA, for ChIP assays. The rats were anesthetized using 5% isoflurane. After decapitation and removal of the brain, the MBH and POA were rapidly dissected. The boundaries used to dissect the MBH were the caudal aspect of the optic chiasm, the rostral margin of the mammillary bodies, and the lateral hypothalamic sulci. To dissect the POA, the meninges and optic chiasm were discarded and the POA region was isolated under a binocular magnifying glass with Vannas spring scissors (Fine Science Tools GmbH, Heidelberg, Germany), using three cuts: one along the caudal aspect of the optic chiasm, and two converging cuts each initiated at the lateral edge of the optic chiasm and ending in front of the anterior commissure. Tissues were immediately frozen in nitric oxygen. Animal experiments were duly approved by the local Ethics Committee for animal research, at Hanover, Germany and Oregon, USA performance sites.

2.11 RNA isolation and cDNA synthesis

RNA was isolated from brain tissue using QIAzol Lysis Reagent (Qiagen) and the RNeasy[®] Mini Kit (Qiagen). Tissue samples were homogenized in 1 mL QIAzol Lysis Reagent. After addition of chloroform, the homogenates were separated into aqueous and organic phases by centrifugation. The upper aqueous RNA phase was collected, and 70% ethanol was added. Then, the sample was applied to an RNeasy spin column (Qiagen) and processed according to the manufacturer's instructions. cDNA synthesis was performed as previously described (Mueller et al., 2011).

2.12 Quantitative PCR (qPCR)

To detect *Ttf1* and *Taz* mRNA in rat brain tissue, we employed the QuantiTect SYBR Green[™] technology (Qiagen). Reactions were performed using the DNA Engine Opticon[™] Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). A volume of 25 µl was used for the PCR reactions; the samples were first denatured at 94°C for 2 min followed by up to 40 PCR cycles. Each PCR cycle consisted of a denaturing step at 94°C for 30 sec, annealing at 58°C or 62°C for 30 sec, and an extension at 72°C for 30 sec. Each PCR reaction was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined and the purity of PCR products was verified by melting curves. The primer pair 16F/R was used to amplify rat *beta-Actin* mRNA and 17F/R for *Taz* mRNA (Table 1); for *Ttf1* mRNA we used the Rn_Nkx2-1_3_SG QuantiTect Primer Assay (Qiagen). The mRNA values obtained were corrected using *beta-Actin* mRNA values as the normalizing unit.

2.13 Double in situ Hybridization

This procedure was performed using riboprobes transcribed from *Ttf1* and *Taz* cDNA templates. Both cDNAs were generated by RT-PCR of total RNA extracted from rat brain, and were cloned into the plasmid pGEM[®]T (Promega, Madison, WI) for subsequent in vitro transcription. The *Ttf1* cDNA used was described previously (Lee et al., 2001). The *Taz* cDNA is a 471 bp fragment corresponding to nt 245–715 in rat *Taz* mRNA (NM_001024869.1). To perform the *in situ* hybridization procedure, the brains of immature (28–30-day-old) female rats were fixed by transcardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5 (Simmons et al., 1989). Thereafter, the brains were postfixed for 16 to

20 h at 4°C in the same fixative containing 10% sucrose, blocked coronally, frozen on dry ice, and stored at -85°C until use. The blocks were sectioned at 30 µm intervals using a freezing sliding microtome and the sections were processed for hybridization as reported (Berg-von der Emde et al., 1995; Simmons et al., 1989). A double label *in situ* hybridization procedure (Berg-von der Emde et al., 1995) combining a digoxigenin-labeled *Taz* cRNA with ³⁵S-UTP-labeled *Ttfl* cRNA probes was used to determine if *Ttfl* mRNA-containing neurons also express *Taz* mRNA. The hybridization procedure employed was that recommended by Simmons et al. (Simmons et al., 1989), with the modifications previously reported (Berg-von der Emde et al., 1995). The digoxigenin-labeled *Taz* probe was prepared exactly as described (Berg-von der Emde et al., 1995) using 500 ng of linearized plasmid template and SP6 RNA polymerase (Promega, Madison, WI) in a 10 µl reaction volume. The ³⁵S-UTP-labeled *Ttfl* probe was prepared by transcribing a linearized *Ttfl* cDNA template with SP6 RNA polymerase in the presence of ³⁵S-UTP (Amersham Biosciences, Buckinghamshire, England). Following labelling, the probes were purified using a Nick column (Amersham Biosciences). Sense probes were prepared using the same templates, but transcribing them from the opposite direction. Following an overnight hybridization at 55–56°C, the slides were washed and processed for digoxigenin detection of *Taz* mRNA (Berg-von der Emde et al., 1995). The sections were then dehydrated and the slides were dipped in Ilford K5 emulsion (without defatting) instead of the NTB-2 emulsion used for isotopic hybridization, and were exposed to the emulsion for three weeks at 4°C in the dark. At this time the slides were developed, quickly dehydrated, dried, and coverslipped (without prior counterstaining) for microscopic examination.

2.14 Detection of CUX1, TTF1, and YY1 in EAP1-containing neurons

Because CUX1, TTF1, and YY1 modify *EAP1* promoter activity in cell lines, we sought to determine if these transcription factors are present in hypothalamic neurons containing EAP1. We performed double immunohistofluorescence on 30 µm frozen sections obtained from the brains of prepubertal 28-day-old female rats fixed by trans-cardiac perfusion with 4% paraformaldehyde- PBS, pH 7.4. The sections were mounted on Superfrost glass slides, dried for 2 h under an air stream, and subjected to an antigen retrieval protocol (Shi et al., 1991). Thereafter, the sections were incubated for 48 h at 4°C with EAP1 rabbit polyclonal antibodies (diluted 1:3,000) (Heger et al., 2007) and each of three monoclonal antibodies: CUX1 (Abcam No. 54583, Cambridge, MA, USA; 1:1000), YY1 (Santa Cruz SC-7341; 1:3000), and TTF1 (Santa Cruz, SC-.53136). At the end of the 48 h incubation period, EAP1 staining was visualized using Alexa 594 donkey antirabbit IgG (Invitrogen, 1:500). TTF1, CUX1, and YY1 staining was developed using biotinylated donkey antimouse IgG (1:250; Invitrogen) followed by incubation with Streptavidin-Alexa 488 (1:500; Invitrogen).

2.15 Statistics

Differences between groups were analyzed using one-way Anova or Student's t-test for individual means. Cell culture experiments were performed at least 3 times in triplicates or quadruplicates. Data are expressed as mean ± SEM. n= is the number of independent experiments. The level of significance was p<0.05.

3. Results

3.1 Transcription of the human EAP1 gene is initiated at a preferential TSS

EAP1 is an intronless gene (NM_0244961) with a predicted TSS 900 bp upstream of the ATG translational start site (Rampazzo et al., 2000). To experimentally determine the TSS of *hEAP1*, we analyzed 24 clones derived from RACE-PCR of human cortex mRNA. All 24 clones showed transcription initiation at a region located between -37 bp and +75 bp from the computer-predicted TSS (NM_0244961) (Fig. 1A). In twelve of them, the TSS was

found 45 bp downstream of the predicted TSS, indicating that cells of the human cerebral cortex utilize a preferred TSS to transcribe *EAP1*. However, we also found that in 6 clones the TSS was located in neighboring nucleotides (Fig. 1A, grey shaded area in Table). In another 6 clones, the TSS was located more than 7 nucleotides from the preferred +45 site. Similar findings have been reported for rat *Eap1* (Matagne et al., 2009).

3.2 The human *EAP1* promoter is active in both neuronal and non-neuronal cell-lines

To examine the transcriptional activity of DNA fragments derived from the experimentally defined 5'-flanking region of *EAP1*, we performed luciferase assays utilizing fragments of two different lengths (998 bp to 2744 bp, Fig. 1B). The activity of both fragments was much higher in non-neuronal GripTite™ 293 MSR cells than in neuronal GT1-7 cells (Fig. 1C). Furthermore, the *EAP1*p2744 fragment showed a slightly higher activity than *EAP1*p998 in both cell lines.

3.3 CUX1, YY1, *EAP1*, and TTF1 are recruited to the human *EAP1* promoter in Hela cells

A Web-based search for transcriptional regulatory motifs upstream the *EAP1* TSS revealed the existence of several putative TTF1 recognition sites within 2500 bp upstream of the TSS (Fig. 2). All sites contain the consensus sequences 5'-GCNCTNNAG-3' (Bohinski et al., 1994; Guazzi et al., 1990), 5'-CAAG-3', or 5'-CTTG-3' (Son et al., 2003). Several predicted CUX1 [5'-TATNG-3', 5'-CCAAT-3', and 5'-ATNNAT-3' (Harada et al., 1995)] and YY1 binding sites (Shi et al., 1997; Shrivastava and Calame, 1994) were also detected. These sites have been shown to mediate activating and repressive effects of CUX1 and YY1 on other genes (Sansregret and Nepveu, 2008; Shrivastava and Calame, 1994) (Fig. 2). We did not search for *EAP1* binding sites, because it is unclear if *EAP1* binds directly to DNA.

ChIP assays analyzing the *EAP1* promoter region were performed using Hela cells and PCR primers (13F/R and 14F/R) targeting two different segments of the *EAP1* 5'-flanking region (Table 1). The segment most distal from the TSS was targeted by primers that amplify the fragment nt -1731 to -1300 (Fig. 2; amplicon I); the proximal region was targeted by primers amplifying a segment comprised between nt -307 and -83 (Fig. 2; amplicon II). We observed that endogenous CUX1 is recruited to both the distal and the proximal segments (Fig. 2; amplicons I and II). In contrast, endogenous YY1 is recruited only to the distal fragment (Fig. 2; amplicon I). ChIP assays using Hela cells transfected with a construct expressing the rat *Ttf1* coding region showed that TTF1 only binds to the proximal region (Fig. 2; amplicon II). Other ChIP assays using Hela cells transfected with an HA-tagged construct expressing the *EAP1* coding region demonstrated that, like TTF1, *EAP1* also associates only to the proximal *EAP1* promoter (Fig. 2).

3.4 CUX1 modifies *EAP1* transcription in an isoform- and cell- dependent manner

The *EAP1* promoter contains multiple putative CUX1 recognition sites (Fig. 2). We performed reporter gene assays to examine the effect of the full length CUX1 protein (p200) and the shorter p110 isoform on *EAP1* transcription. When tested in GripTite™ 293 MSR cells, we observed an inhibitory effect of CUX1-p110 on both the *EAP1*p998 and *EAP1*p2744 promoter constructs, while CUX1-p200 trans-activated both *EAP1* constructs (Fig. 3A). The CUX1 consensus binding site ATNNAT was present twice in forward orientations in *EAP1*p998 at nt -490 and -501. To determine if these binding sites were necessary for CUX1 to repress *EAP1* transcriptional activity, we deleted both sites from *EAP1*p998. Under these conditions, CUX1-p110 failed to suppress and CUX1-p200 failed to stimulate *EAP1* transcription (Fig. 3B). In GT1-7 cells, CUX1-p110 behaved as in GripTite™ 293 MSR cells repressing the shorter (*EAP1*p998) promoter fragment (Fig. 3C). In contrast, CUX1-p200 had either a marginally suppressive effect on *EAP1*p998 or no effect on *EAP1*p2744 (Fig. 3C). Like in GripTite™ 293 MSR cells, deletion of the CUX1 nt

–490 to –501 recognition sites also abolished the CUX1 repressive effect in GT1-7 cells (Fig. 3D). These results indicate that p110, a proteolytically derived CUX1 isoform, consistently represses *EAP1* transcription in two different cell types, but that the full-length CUX1 isoform p200 can only trans-activate the *EAP1* promoter in a cell context-specific manner.

3.5 YY1 modifies *EAP1* promoter activity in a cell-dependent manner

YY1, tested in GripTite™ 293 MSR cells inhibited the transcriptional activity of both *EAP1* promoter constructs (Fig. 3E), but surprisingly it had a trans-activating effect in GT1-7 cells (Fig. 3F). SP1 has been shown to physically interact with YY1 to facilitate YY1 trans-regulatory activity (Lee et al., 1993) and the *EAP1* 5'-flanking region contains several putative SP1 binding sites. When we tested the effect of SP1 on *EAP1* promoter activity, utilizing *EAP1*p998 as the target, SP1 alone did not alter *EAP1*p998 activity in neuronal and non-neuronal cells, and did not enhance either the repressive or enhancing effect of YY1 on *EAP1* transcription (Fig. 3E,F).

3.6 The *EAP1* promoter is trans-activated by TTF1

TTF1 trans-activates both *EAP1* promoter constructs in GripTite™ 293 MSR cells (Fig. 4A, b and f). Deletion of all 7 TTF1 recognition sites ablates the stimulatory effect of TTF1 (Fig. 4A, e). Crucial for this effect is the TTF1 binding site located at –382 bp upstream of the putative TSS (Fig. 4A, d). Deletions of all other putative TTF1 binding sites shown in Fig. 2 do not abrogate the TTF1 effect (data not shown). In GT1-7 cells, TTF1 had no effect (Fig. 4B). These results suggest that TTF1 mainly utilizes a binding site (–382) near the TSS to trans-activate the *EAP1* promoter and that, as seen with CUX1 and YY1, the effect of TTF1 is also cell context-dependent.

3.7 *Taz* mRNA co-localizes with *Ttf1* mRNA in the hypothalamus and enhances the trans-activational effect of TTF1 on *EAP1* transcription

TAZ facilitates TTF1 transcriptional activity in several tissues including the thyroid (Di Palma et al., 2009), lung, and kidney (Park et al., 2004). To determine if the *Taz* gene is expressed in brain, we measured *Taz* mRNA levels by qPCR in the MBH, POA and CTX of immature (PND21) female rats. *Taz* mRNA was detected in all three brain regions (Fig. 5A). In contrast, *Ttf1* mRNA was detected in the MBH and to a lesser extent in the POA, but not in the CTX (Fig. 5B).

Double *in situ* hybridization analysis revealed that *Ttf1* mRNA-containing cells also express *Taz* mRNA, a distribution illustrated by the presence of both transcripts in the lateral ventromedial nucleus of the MBH (Fig. 5C). Promoter assays showed that TAZ enhances the trans-activational effect of TTF1 on *EAP1* transcription, but that TAZ alone has no effect on *EAP1* expression in GripTite™ 293 MSR (Fig. 5D). Changing the cell context and utilizing GT1-7 cells did not unveil an effect of TAZ alone on *EAP1* expression (Fig. 5E). These results suggest that TAZ cooperates with TTF1 in the hypothalamus to enhance *EAP1* gene transcription.

3.8 *EAP1* represses its own transcriptional activity

EAP1 suppressed the transcriptional activity of both *EAP1*p998 and *EAP1*p2744, and this effect was abolished by deletion of the *EAP1* RING finger in both GripTite™ 293 MSR cells (Fig. 6A) and GT1-7 cells (Fig. 6B). These results indicate that *EAP1* represses its own transcriptional activity, and that the RING finger domain is required for this effect.

3.9 Transcription factors regulating EAP1 transcription are expressed in EAP1 containing neurons of the rat hypothalamus

Because the above described transcriptional activities were observed in cell lines, we sought to determine if CUX1, TTF1, and/or YY1 are expressed in hypothalamic EAP1 neurons *in situ*. Double immunohistofluorescence staining of these proteins in sections of the immature female rat hypothalamus revealed that, although CUX1, TTF1, and YY1 are also present in nonEAP1 neurons, most if not all EAP1 neurons contain these proteins (Fig. 7). In all cases, CUX1, TTF1, and YY1 localize to the cell nucleus. Omitting the primary antibodies eliminated all specific staining (not shown).

3.10 CUX1 and TTF1 are recruited in vivo to the rat Eap1 promoter in MBH

Utilizing the same on-line tools, and the same criteria to predict CUX1, TTF1, and YY1 binding sites in the 5'-flanking region of the rat *Eap1* gene, we found the presence of several sites within 2 kb from the TSS. We also observed a tendency for the sites to be less abundant in the 500 bp region located immediately upstream from the TSS (Fig. 8). Next, we performed ChIP assays using chromatin derived from the MBH of prepubertal female rats and the PCR primers 18F/R and 19F/R (Table 1) that target two different segments of the rat *Eap1* 5'-flanking region. The segment most distal from the TSS was targeted by primers that amplify the fragment nt -1794 to -1706 (Fig. 8; amplicon I); the proximal region was targeted by primers amplifying a segment comprised between nt -1177 to -866 (Fig. 8; amplicon II). We observed that, as in human *EAP1*, CUX1 is recruited to both the distal and the proximal segments (Fig. 8; amplicons I and II), whereas TTF1 only binds to the proximal region (Fig. 8; amplicon II). Surprisingly, no association of YY1 to either region was detected.

4. Discussion

Utilizing high throughput approaches, combined with computational and systems biology methods, a gene network was identified and predicted to operate within the female hypothalamus to control the initiation of puberty (Roth et al., 2007). The network was termed TSG (for Tumor Suppressor Gene) network, because it is composed of genes earlier identified as being involved in tumor suppression/tumor formation. Computational analysis predicted the existence of major hubs controlling the network at the transcriptional level (Roth et al., 2007). In addition, it predicted that these hubs are not only connected to a plethora of subordinate genes, but also to other upper-echelon genes found in separate studies to be involved in the transcriptional regulation of the pubertal process (Heger et al., 2007; Mastronardi et al., 2006; Ojeda et al., 1999).

EAP1 has been postulated to be one of those upper-echelon genes (Heger et al., 2007; Mastronardi et al., 2006). *EAP1* is a member of a family of genes known as interferon regulating factor-2 binding proteins (IRF2BPs) (Teng et al., 2010). The protein products of these genes share similar domains, including an N-terminal C4 zinc-finger and C-terminal RING-finger (Childs and Goodbourn, 2003). In our earlier study, we showed that EAP1 is a transcriptional regulator that displays both repressive and trans-activating effects (Heger et al., 2007), depending on the cellular context. We also showed that RNA interference (RNAi)-mediated knock-down of *Eap1* targeted to the preoptic region of prepubertal female rats results in delayed pubertal onset and disruption of estrous cyclicity. More recently, using a lentiviral construct and MRI-guided stereotaxic surgery to deliver RNAi to the ARC of adult female rhesus monkey undergoing regular menstrual cycles, we showed that knocking down *EAP1* expression in this brain region results in prompt interruption of menstrual cyclicity (Dissen et al, 2011). We further observed that a single nucleotide polymorphism in the promoter region of *EAP1* is associated with amenorrhea/

oligomenorrhea in rhesus monkeys (Lomniczi et al., 2011). These results indicate that hypothalamic *EAP1* is not only important for maintaining reproductive cyclicity in rodents, but is also required for menstrual cyclicity in higher primates.

The present results show that, consistent with the organizational features of gene networks, *EAP1* transcription is under the regulatory control of at least two genes predicted to be upstream nodes of the TSG network (YY1 and CUX1) and one presumed non-TSG component (TTF1). In addition, *EAP1* transcription is self-regulated by *EAP1*. TTF1 is a non-TSG previously shown to be involved in the transcriptional control of puberty (Mastronardi et al., 2006). Experimental verification of the TSS of *EAP1*, indicate that it is located 40–50 bp downstream from the TSS predicted earlier by computational methods (Rampazzo et al., 2000). Using this information for the design of gene reporter constructs to analyze the transcriptional activity of the *EAP1* 5'-flanking region, we obtained results compatible with the concept that *EAP1* is under dual transcriptional regulation. While *EAP1* transcription is repressed by CUX1-p110, YY1, and *EAP1* itself, it is trans-activated by TTF1. These results were obtained using a non-neuronal (GripTite™ 293 MSR) cell line. CUX1-p110 and *EAP1* also suppressed *EAP1* promoter activity in GT1-7 cells, but surprisingly, YY1 enhanced *EAP1* transcription and TTF1 had no effect in these cells. The non-neuronal definition of GripTite™ 293 MSR cells is not, however, absolute because these cells share attributes with certain neuronal cell lines (Shaw et al., 2002; Geschwind et al., 2001; Terskikh et al., 2001; Phillips et al., 2000).

The repressive effect of CUX1 is consistent with earlier observations showing the ability of CUX1 to inhibit gene transcription (Nepveu, 2001; Sansregret and Nepveu, 2008; Mueller et al., 2011). Interestingly, this inhibitory effect of CUX1 on *EAP1* transcription was only exerted by the CUX1-p110 isoform. In contrast, the full-length CUX1 protein, CUX1-p200, which when proteolytically processed yields the smaller p110 isoform (Sansregret and Nepveu, 2008), trans-activated *EAP1* transcription. Previous reports have shown that CUX1-p110 has either repressive or trans-activational capabilities depending on the cellular context (Dufort and Nepveu, 1994; Superti-Furga et al., 1989; Valarche et al., 1993; Harada et al., 2008; Kim et al., 1997; Coqueret et al., 1998; Goulet et al., 2002; Sansregret and Nepveu, 2008). In our studies, CUX1-p110 suppressed transcription of both a short (*EAP1*p998) and a long (*EAP1*p2744) *EAP1* DNA segment, an effect consistent with ChIP assays results showing that CUX1 binds to both a proximal and a distal segment of the *EAP1* 5'-flanking region. Deletion of the two CUX1 binding sites present in *EAP1*p998 obliterated both CUX1-p110 mediated repression and CUX1-p200 trans-activation indicating that CUX1 regulates *EAP1* transcription by binding to specific recognition sites in the *EAP1* 5'-flanking region.

EAP1 transcription was also repressed by YY1 in GripTite™ 293 MSR cells, a protein that either represses or enhances gene transcription depending upon cell type, biological relevant stimuli and promoter context (Shi et al., 1997; He and Casaccia-Bonnel, 2008). The repressive effect of YY1 was modestly increased by the ubiquitous transcription factor SP1, which was previously reported to function as a YY1 partner in repressing gene expression (Lee et al., 1993). As a member of the Polycomb (PcG) silencing complex (Schwartz and Pirrotta, 2007; Simon and Kingston, 2009; Kohler and Villar, 2008; Simon and Kingston, 2009), YY1 can repress transcription by virtue of its ability to recruit other PcG components (PRC2 and PRC1 proteins), in addition to histone 3 trimethylated in lysine 27H3K27me3, to gene promoters to silence transcription (Wilkinson et al., 2006; Woo et al., 2010). Although YY1 was very effective in repressing the transcriptional activity of *EAP1*p998, our ChIP assays performed in Hela cells showed that endogenous YY1 is not recruited to this DNA segment. An explanation for this discrepancy is not clear, but it may be due to that in reporter assays the high levels of YY1 produced by the transfected expression vector are

recognized by binding sites with both high and low affinity. In contrast, in ChIP assays endogenous YY1 may bind to only sites displaying high affinity. This idea is supported by the ChIP analysis of the rat *Eap1* 5'-flanking region. In this case, no YY1 binding was observed despite the presence of many predicted binding sites throughout 2kb of 5'-flanking DNA.

As previously observed for other central nodes of gene networks (Roth et al., 2007), we observed that EAP1 represses its own transcription. EAP1 inhibits the transcriptional activity of both *EAP1*p998 and *EAP1*p2744, but only associates to the proximal region. This suggests that EAP1 exerts its inhibitory effect on *EAP1* transcription by associating, directly or indirectly, to DNA sequences near the *EAP1* TSS. Because EAP1 was recently shown (Yeung et al., 2011) to be a key component of a repressive complex that includes two other related proteins (IRF-2BP2; interferon regulatory factor1 binding protein 2, and IRF2BP1; interferon regulatory factor2 binding protein1), it is likely that EAP1 is recruited to its own promoter as part of a protein complex, and not as an individual entity.

EAP1 expression increases rather abruptly in the hypothalamus at the time of female puberty, in both rodents (Matagne et al., 2009; Heger et al., 2007) and nonhuman primates (Heger et al., 2007). A more detailed analysis of the changes in *Eap1* mRNA abundance that occur in the hypothalamus during prepubertal and pubertal development of female rats revealed that *Eap1* expression decreases between the first week of postnatal life and the initiation of puberty, to increase transiently in an ovary-independent and hypothalamic-specific manner at the time of puberty (Matagne et al., 2009). When analyzed in the context of the present findings, the possibility arises that the infantile-juvenile decline in hypothalamic *Eap1* may be due to an increased inhibitory influence exerted by transcriptional repressors, such as CUX1 p110, YY1, and EAP1 itself. The pubertal increase in *Eap1* expression can be interpreted as due to both a loss of transcriptional repression, and increased trans-activating inputs. These changes are likely to occur in a region-specific manner, because the pubertal increase in *Eap1* mRNA levels is only observed in the MBH and not the POA or cerebral cortex of the rat (Matagne et al., 2009). Perhaps this increase is due to loss of a repressive influence (exerted at least in part by CUX1, YY1, and EAP1) occurring concomitantly with an increase in transactivation exerted by other transcriptional factors. The identity of these factors is unknown, but the present results suggest that TTF1, a transcription factor involved in the facilitatory control of female puberty (Matagne et al., 2009) may be one of them. The colocalization of EAP1 with CUX1, TTF1, and YY1 in the nucleus of hypothalamic neurons further supports the notion that these proteins are functionally linked in hypothalamic EAP1-containing cells.

Our results indicate that despite the existence of many theoretical TTF1 binding sites in the *EAP1* 5'-flanking region, TTF1 utilizes only one of these sites, located at nt -382 from the TSS (NM_024496) to stimulate *EAP1* transcription. Consistent with this result is the outcome of ChIP assays showing that TTF1 binds to a DNA segment proximal to the TSS and not to a distal region. We observed a similar pattern when analyzing via ChIP assays the binding of endogenous TTF1 to the rat *Eap1* 5'-flanking sequence in the hypothalamus *in situ*. Our results also indicate that the non-DNA binding co-activator TAZ – shown by others to interact with TTF1 in the trans-activation of target genes (Di Palma et al., 2009; Park et al., 2004), facilitates the trans-activating effect of TTF1 without having an activity on its own. A physiological interaction between TAZ and TTF1 in the hypothalamus function is suggested by the presence of *Taz* mRNA in *Ttf1*-containing hypothalamic cells. We have previously shown that TTF1 is expressed in *Kiss1* mRNA-containing cells of the MBH, and that TTF1 transactivates the *Kiss1* promoter (Mastronardi et al., 2006; Mueller et al., 2011). It is thus, plausible that the facilitatory effect of TTF1 on the advent of puberty (Mastronardi

et al., 2006) is exerted at least in part via its trans-activating effects on both the *Kiss1* and *EAP1* genes.

Tempering these interpretations are two findings. One of them shows that YY1 trans-activates, instead of repressing, the *EAP1* promoter in GT1-7 cells. The other demonstrates that TTF1 does not affect *EAP1* transcription in these cells. The trans-activating effect of YY1 may be related to the ability of YY1 to promote the transcription of anti-apoptotic genes, such as c-Myc and IAP (Shrivastava and Calame, 1994). Because *EAP1* is an anti-apoptotic gene (Yeung et al., 2011), it may be inferred that in GT1-7 cells YY1 would also enhance *EAP1* transcription. Another explanation comes from findings showing that the repressive effects of YY1 on gene transcription require interactions with co-repressors able to induce chromatin modifications (Gordon et al., 2006). It may be speculated that in particular cell contexts lacking such co-repressors YY1 may become unable to exert its silencing effects. An explanation for the ineffectiveness of TTF1 to modify *EAP1* expression in GT1-7 cells is less clear, although one may speculate that it is related to a TTF1-dependent activation of repressive pathways able to modulate *EAP1* transcription. Why are these differences in *EAP1* transcriptional regulation seen only in GT1-7 cells is also unclear. Further studies using different hypothalamic cell lines are warranted to resolve this issue.

In summary, the present results offer direct evidence for the concept, derived from the *in silico* analysis of protein-DNA interactions, that proteins predicted to be central nodes of a TSG network regulate the transcription of *EAP1*, another upstream regulator of the pubertal process (Fig. 9). Although until now *EAP1* and TTF1 have been considered as no-TSGs molecules (Ojeda et al., 2010), recent evidence implicating *EAP1* in the biology of cancer (Yeung et al., 2011), and loss of *TTF1* expression in lung adenocarcinoma progression (Winslow et al., 2011), suggest that these genes – by virtue of their involvement in cancer – are additional high-hierarchy components of the TSG network postulated to control the pubertal process.

Acknowledgments

This work was supported by German Research Foundation Grant HE 3151/3-1, 3151/4-1 (SH), and NIH grants HD25123, the Eunice Kennedy Shriver NICHD/NIH through cooperative agreement U54HD18185 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research, and RR000163 for the operation of the Oregon National Primate Research Center (SRO). We thank Ms. Maria Costa for performing the *in situ* hybridization studies. We thank Prof. Dr. Sigurd Lenzen (Institute of Clinical Biochemistry, Hannover Medical School) for his excellent methodological support. We thank Dr. Katrin Tefs for her support with qPCR experiments.

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Highlights

- EAP1 is an upper-echelon transcriptional regulator of female puberty and adult reproductive function
- Transcription of *EAP1* mRNA is initiated at a preferred transcription start site
- The *EAP1* promoter is activated by two transcription factors, TTF1 and CUX1-p200
- The *EAP1* promoter is repressed by EAP1 itself, in addition to two transcriptional repressors, YY1 and CUX1-p110
- TAZ co-localizes with TTF1 in neurons of the hypothalamus and cooperates with TTF1 in trans-activating the *EAP1* promoter
- All four transcription factors are recruited *in vivo* to the *EAP1* promoter

Figure 1. Identification of the EAP1 transcription start site (TSS), and basal activity of EAP1 promoter constructs
A, The *EAP1* gene is predominantly transcribed from a TSS (light gray) located between nt 40–52 downstream from the *in silico* predicted TSS (dark grey; NM_0244961), as determined by 5' RACE PCR. The table below the sequence illustrates the TSSs found by RACE-PCR. The thick arrow denotes the nucleotide most frequently used as a TSS. **B**, The two segments (*EAP1*p998 and *EAP1*p2744) from the *EAP1* 5'-flanking region used to assess *EAP1* transcriptional activity. The numbering corresponds to the *in silico* predicted transcription start site. **C**, *EAP1* promoter constructs have a higher basal activity in a non-neuronal than a neuronal cell line. Promoter activity was determined using luciferase reporter assays; each constructs (*EAP1*p998 and *EAP1*p2744) was transfected at 500 ng/well. Bars are mean and vertical lines are SEM, n=3 experiments/group, *** = P< 0.001 vs. pGL4.1 basic.

Figure 2. CUX1, YY1, EAP1, and TTF1 are recruited to different regions of the human *EAP1* promoter in Hela cells, as assessed by ChIP assays

Putative binding sites for three transcription factors (TTF1, CUX1, and YY1) implicated in the control of puberty were identified *in silico* (TESS, Vector NTI) and are shown by horizontal wide arrows. The direction of each arrow indicates the presence of each binding site on either the sense or antisense DNA strand. Potential EAP1 binding sites are not depicted, because it is currently unknown if EAP1 binds directly to DNA. Endogenous CUX1, as well as transfected HA-tagged EAP1 and untagged TTF1, strongly associates with the proximal promoter region of the *EAP1* gene (Amplicon II). No association of YY1 to this region was detected. In contrast to the proximal promoter, an upstream region (Amplicon I) showed CUX1 and YY1 binding, but no EAP1 or TTF1 association. The endogenous proteins (CUX1 and YY1) were immunoprecipitated using specific monoclonal antibodies (see Material and Methods) and mouse IgGs as a negative control for the immunoprecipitation. To immunoprecipitate proteins produced by transfected expression vectors (EAP1-HA and TTF1), we used a mouse monoclonal antibody against HA (for EAP) and rabbit polyclonal antibodies (against TTF1). Chromatin prepared from cells transfected with the empty expression vector was used as the negative control. Inp: input DNA, chromatin precleared with protein A beads before immunoprecipitation. Transf = transfected.

Figure 3. CUX1-p110 represses EAP1 transcriptional activity in both a non-neuronal and a neuronal cell line, while YY1 inhibits EAP1 transcriptional activity in a non-neuronal but not in a neuronal cell line

A, CUX1-p110 inhibits the transcriptional activity of two *EAP1* promoter constructs of different lengths (*EAP1*p998 and *EAP1*p2744) in GripTite™ 293 MSR cells, while CUX-p200 is trans-activating (n=5 experiments/group). **B**, Deletion of two CUX1 binding sites located between nt -490 and -501 upstream of the TSS (NM_0244961) prevents both the repressive effect of CUX1-p110 and the trans-activating effect of CUX-p200 in GripTite™ 293 MSR cells (n=4 experiments/group). **C**, Both CUX1 isoforms repress *EAP1*p998 transcriptional activity in GT1-7 cells, but CUX1-p200 has no effect on *EAP1*p2744 transcription (n=4 experiments/group). **D**, Deletion of two CUX1 binding sites located between nt -490 and -501 upstream of the TSS (NM_0244961) prevents the effects of

CUX1-p110 and CUX1-p200 in GT1-7 cells (n=4 experiments/group). **E**, YY1 represses *EAP1* transcription in GripTite™ 293 MSR; SP1 does not enhance this effect (n=3 experiments/group). **F**, In contrast, YY1 trans-activates the *EAP1* gene in GT1-7 cells, SP1 does not alter this effect (n=3 experiments/group). Bars are mean and vertical lines are SEM, ** = $P < 0.01$, *** = $P < 0.001$ vs. *EAP1*p998 or *EAP1*p2744 respectively.

Figure 4. TTF1 trans-activates EAP1 transcription in a non-neuronal cell line preferentially using a binding site located near the TSS

A, TTF1 trans-activates transcription of both *EAP1* promoter constructs (*EAP1p998* and *EAP1p2744*) in GripTite™ 293 MSR cells. Deletion of the TTF1 binding site located at nt -382 upstream of the TSS (NM_0244961) abolishes the stimulatory effect of TTF1, whereas deletion of other binding sites does not abolish the TTF1 effect (n=3 experiments/group). **B**, In GT1-7 cells TTF1 has no effect (n=3 experiments/group). Bars are mean and vertical lines are SEM, * = P< 0.05, ** = P< 0.01 vs. *EAP1p998* or *EAP1p2744* respectively.

Figure 5. Taz mRNA is expressed in the hypothalamus, colocalizes with Ttf1 mRNA in cells of the lateral ventromedial nucleus (LVMH), and TAZ enhances TTF1-dependent trans-activation of the EAP1 promoter

A, *Taz* mRNA detected by qPCR in selected brain regions of 21-day-old female rats. CTX = cerebral cortex; MBH = medial basal hypothalamus; POA = preoptic area. **B**, *Ttf1* mRNA detected by qPCR in the brain of 21-day-old female rats is expressed in the POA and MBH, but not the CTX. **C**, Double *in situ* hybridization shows that *Taz* mRNA positive cells of the LVMH (**C1**, purple color) also contain *Ttf1* mRNA (**C2**, silver grains); **C3**, Merged image; **C4**, Higher magnification image showing the colocalization of *Taz* and *Ttf1* mRNAs. In C1–C3 bars = 50 μ m; in C4 bar = 20 μ m. **D**, TTF1 trans-activates *EAP1* transcription and TAZ enhances this effect, without having an effect on its own in GripTite™ 293 MSR (n=3 experiments/group). **E**, In GT1-7 cells TAZ does not alter the lack of TTF1 effect on *EAP1* expression (n=3 experiments/group). Bars are mean and vertical lines are SEM, * = P < 0.05, ** = P < 0.01, *** = P < 0.001 vs. *EAP1*p998 or *EAP1*p2744 respectively.

Figure 6. EAP1 represses EAP1 transcription in non-neuronal and neuronal cell lines
A, EAP1 represses *EAP1p998* and *EAP1p2744* directed transcription in GripTite™ 293 MSR cells. This effect is prevented by deletion of the EAP1 RING finger domain (n=3 experiments/group). **B**, A similar repressive effect is observed in GT1-7 cells (n=3 experiments/group). Bars are mean and vertical lines are SEM, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ vs. *EAP1p998* or *EAP1p2744* respectively.

bar = 25um

Figure 7. EAP1 neurons of the prepubertal female rat hypothalamus contain CUX1, TTF1, and YY1 immunoreactive material as detected by double immunohistochemistry
A–D, CUX1; E–H, TTF1. I–L, YY1. Arrows point to examples of colocalization. Bar = 25 μ m.

Figure 8. CUX1 and TTF1 are recruited to different regions of the *Eap1* 5'-flanking region in the MBH of prepubertal rats, as assessed by ChIP assays

Putative binding sites for the transcription factors TTF1, CUX1, and YY1 were identified *in silico* (TESS, Vector NTI) and are shown by horizontal wide arrows. Location of the TSS is that described by Matagne et al., 2009. The direction of each arrow indicates the presence of each binding site on either the sense or antisense DNA strand. Potential EAP1 binding sites are not depicted, because it is currently unknown if EAP1 binds directly to DNA. CUX1 associates with the two regions examined (Amplicon I and II), TTF1 only interacts with region nearest to the TSS (Amplicon II), and YY1 fails to associate to either region. CUX1 and YY1 were immunoprecipitated using specific mouse monoclonal antibodies, and TTF1 was immunoprecipitated using a rabbit polyclonal antibody (see Material and Methods). Monoclonal antibodies to *Drosophila melanogaster* Ecdysone Receptor (ECR) or β -Galactosidase (β -Gal), two proteins not expressed in mammalian systems, were used as negative controls. Inp: input DNA, chromatin precleared with protein A beads before immunoprecipitation.

Figure 9. Schematic representation depicting the transcriptional regulation of the EAP1 gene by transcription factors in relation to pubertal onset

This simplified model predicts that escape of EAP1 from repressive control leads to enhanced EAP1-mediate suppression of genes that are inhibitory to puberty. **Regulatory Level I** represents the dual control of *EAP1* gene activity by transcriptional repressors (EAP1, YY1, CUX1-p110), and activators (TTF1, CUX1-p200). The PcG silencing complex, recently reported to repress directly the *Kiss1* gene (Lomniczi et al., 2011) is shown as an additional component of this regulatory level. **Regulatory Level II** represents neurotransmitters/neuromodulators that either inhibit (NPY, PENK, GABA, GnIH) or facilitate (KiSS1, GLUT) the pubertal process. Whether the transcription of genes involved in the generation of these molecules is regulated by components of Regulatory Level I is not known. *Kiss1* gene transcription is directly regulated by the repressors and trans-activators of Regulatory Level I, including the PcG silencing complex. An increase in kisspeptin secretion is depicted as necessary for GnRH secretion to increase at puberty, and for puberty to occur. TTF1: Thyroid Transcription Factor 1; EAP1: Enhanced at puberty 1; YY1: Ying Yang 1; CUX1: CCAAT displacement protein 1; GnRH: Gonadotrophin releasing hormone; PcG: Polycomb genes; PENK: Preproenkephalin; GABA: γ-Aminobutyric acid; NPY: Neuropeptide Y; GnIH: Gonadotrophin inhibiting hormone; GLUT: Glutamate; Red: repression; Black: trans-activation. Dark grey box: inhibitors; Light grey box: stimulators

Table 1

Primers used in this study

Name	Sequence	Position according to Acc. no.: NT_026437.12 (human EAP1)
1F/R	5'-CGA CTG GAG CAC GAG GAC ACT GA-3'	nt 5234288–5234304
	5'-CCA GGG CAT GCG GGG CAG GTC G-3'	nt 58494064–58494085
2F/R	5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3'	nt 5845573–5845586
	5'-CTT CCT GGT GCT GCA GAC TT-3'	nt 58494417–58494436
3F/R	5'-GGT TCC TAG ATC AGG GCA CAA-3'	nt 58495635–58495615
	5'-CCC GGT GCC CAG GTC CAA TCT T-3'	nt 58494637–58494658
4F/R	5'-ACT GTA ATA GGC CGG TCA CG-3'	nt 58497381–58497362
	5'-CGC CGT GTG TGT ACA ACA G-3'	nt 58495245–58495263
5F/R	5'-CTT GGC CGG GCG TGG TGG TTC AT-3'	nt 58496286–58496264
	5'-CCC GGT GCC CAG GTC CAA TCT T-3'	nt 58494637–58494658
6F/R	5'-CGG GGA AGC AGC GGC TTC AG/ /// ATT GGA CCC GGG CAC CGG G-3'	nt 58494679–58494634
	5'-CCC GGT GCC CGG GTC CAA T/ /// C TGA AGC CGC TGC TTC CCC G-3'	nt 58494634–58494679
7F/R	5'-CGC CGC GCG AGC G/ /// A GCC AAC GTG AG-3'	nt 58494922–58494894
	5'-CTC AGC TTG GCT CGC T/ /// CGC GCG GCG-3'	nt 58494894–58494922
8F/R	5'-GCG GCA TCC CGC GCG GC/ /// CCG GGG ACA GCG CGA GC-3'	nt 58495074–58495038
	5'-GCT CGC GCT GTC CCC GG/ /// GCC GCG CGG GAT GCC GC-3'	nt 58495038–58495074
9F/R	5'-GCG TCC TGC CGC GCA TGC GC/ /// CCT GAG CAA /// TG GGC TCC ACA GAG GAA GTG-3'	nt 58495190–58495134
	5'-CAC TTC CTC TGT GGA GCC CA/ /// TTC GTC AGG /// GC GCA TGC GCG GCA GGA CGC-3'	nt 58495134–58495190
10F/R	5'-CTT AAA TGC GTT TTT A/ /// G GAA TGC CCG GCA GGG AAA G-3'	nt 58495372–58495332

Name	Sequence	Position according to Acc. no.: NT_026437.12 (human <i>EAP1</i>)
	5'-CTT TCC CTG CCG GGC ATT CC/ /// TAA AAA CGC ATT TAA G-3'	nt 58495332–58495372
11F/R	5'-CAT TTA AAT TCA CTG CCG AGG /// /GG GGT GGG GTG GGC AGC AGG G-3'	nt 58495440–58495395
	5'-CCC TGC TCC CCA CCC CAC CCC /// /CC TCG GCA GTG AAT TTA AAT G-3'	nt 58495395–58495440
12 F/R	5'-CTT CGG CAT CAG TGG TGG CG/ /// /TT AAC /// /GCC GGG GGG GAT AAG GTT CA-3'	nt 58495558–58495522
	5'-TGA ACC TTA TCC CCC CCG GC/ /// /G TTA A/ /// /CG CCA CCA CTG ATG CCG AAG-3'	nt 58495522–58495558
		Position according to Acc. no.: NM_024496 (human <i>EAP1</i>)
13F/R	5'-GGA GGC TGA GGT GGG AGG ATT ACT-3'	nt –1731–1708
	5'-ATG GCA CGA TGG ATA TGT TGG TCA-3'	nt –1323–1300
14F/R	5'-GCA GGG AAA GGA AAG GTC ATC GTC-3'	nt –307–284
	5'-TTC CCC CTC CCC TTT ACA CTT CCT-3'	nt –106–83
15 F/R	5'-CGA AGC CCT CTT CAA CTC TG -3'	Position according to Acc. no.: NM_001024869 (rat <i>Taz</i>): nt 245–264
	5'-GAC ACT GCC ATG GAC CTC TG -3'	nt 696–715
17F/R	5'- GGG TCT TGC CAT GTG GTG -3'	nt 551–568
	5'- GGA GAT GAC CTT CAC GGC -3'	nt 608–625
16F/R	5'- GAA CAC GGC ATT GTA ACC AAC TGG - 3'	Acc. No.: V01217.1 (rat β -Actin): nt 1544–1567
	5'- GGC CAC ACG CAG CTC ATT GTA - 3'	nt 1601–1621
18F/R	5'- AGC CCC CTG CAC CAT CTC TTG - 3'	TSS rat <i>EAP1</i> : (Matagne et al., 2009): nt –1794–1774
	5'- AGC TGG CCT GGG GTC TGA ATC - 3'	nt –1706–1726
19F/R	5'- GCA AAC CCA GCC TTA TCA AA - 3'	nt –1177–1158
	5'- GAC CCA GGC TCA GAA CAC TAT C- 3'	nt –866–887

Primer pairs 1F/R and 2F/R were used for RACE-PCR, 3F/R, 4F/R, and 5 F/R for promoter cloning, 6F/R to 11F/R for mutagenesis of TTF1 binding sites, 12F/R for mutagenesis of CUX1 binding sites, 13F/R, 14F/R, 18F/R, 19F/R and 19F/R for ChIP assays, 15F/R TAZ construct, and 16F/R to 17F/R for qPCR. Slashes represent deleted bases. nt = nucleotide.