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The Krüppel-like factor 4 controls biosynthesis of thyrotropin-releasing hormone during hypothalamus development

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ABSTRACT

Embryonic neurogenesis is controlled by the activation of specific genetic programs. In the hypothalamus, neuronal thyrotropin-releasing hormone (TRH) populations control important physiological process, including energy homeostasis and autonomic function; however, the genetic program leading to the TRH expression is poorly understood. Here, we show that the *Klf4* gene, encoding the transcription factor Krüppel-like factor 4 (Klf4), was expressed in the rat hypothalamus during development and regulated *Trh* expression. In rat fetal hypothalamic cells Klf4 regulated *Trh* promoter activity through CACCC and GC motifs present on the *Trh* gene promoter. Accordingly, hypothalamic *Trh* expression was down-regulated at embryonic day 15 in the $Klf4^{-/-}$ mice resulting in diminished bioactive peptide levels. Although at the neonatal stage the *Trh* transcript levels of the $Klf4^{-/-}$ mice were normal, the reduction in peptide levels persisted. Thus, our data indicate that Klf4 plays a key role in the maturation of TRH expression in hypothalamic neurons.

1. Introduction

Discrete populations of hypothalamic neurons play important roles in homeostasis by regulating hormone secretion from the pituitary gland. These include neurons producing oxytocin, vasopressin, corticotropin-releasing hormone, thyrotropin-releasing hormone (TRH), somatostatin, growth hormone releasing hormone, gonadotropin releasing hormone, and dopamine. Despite extensive studies on the physiological functions of these neurohormones, the molecular pathways regulating their expression during development are less well known.

In the hypothalamus, development of the hypophysiotropic TRH neurons of the paraventricular nucleus (PVN) depends on a partially characterized cascade of transcription factors. The transcription factor Otp, along with the Sim1/Arnt2 heterodimer, controls proliferation and differentiation of neuronal hypothalamic phenotypes. Inactivation of these transcription factors by homologous recombination leads to the absence of the PVN with the resulting loss of *Trh* expression (Burbach, 2000). However, the transcriptional cascade that directly regulates *Trh* expression is still unknown.

To gain insight into the mechanism governing *Trh* expression during embryonic development, we have characterized the transcriptome of rat fetal hypothalamic TRH neurons. This analysis showed that the mRNA encoding the transcription factor Krüppel-like factor 4 (Klf4) was enriched in TRH neurons during development (Guerra-Crespo et al., submitted for publication). Klf4 regulates cell cycle progression (Chen et al., 2003), and cell differentiation by controlling target gene expression in various cell types (Shields et al., 1996; Segre, 2003; Suske et al., 2005). Klf4 knockout mice die shortly after birth, as a consequence of altered barrier skin function and marked feeding difficulties (Segre et al., 1999). Nonetheless, the molecular role of Klf4 during CNS development is unknown.

In this report we show that Klf4 regulates *Trh* gene expression during hypothalamic development. Klf4 up-regulates *Trh* promoter activity in an additive manner with Sp1. This effect resulted from binding of Klf4 and Sp1 to specific CACCC and GC motifs within

Abbreviations: TRH, thyrotropin releasing hormone; Klf4, Krüppel-like factor 4; Sp1, transcription factor specific protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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the *Trh* gene promoter. Accordingly, Klf4 deficient mice showed reduced hypothalamic *Trh* expression at the embryonic day 15 that resulted in diminished bioactive peptide levels. Thus, our data indicate that Klf4 is part of the differentiation program leading to maturation of TRH expression within the hypothalamus.

2. Materials and methods

2.1. Cell culture and transfection

Hypothalamic primary cultures were prepared as described (Perez-Martinez et al., 2001). Cells ($1.4 \times 10^4/mm^2$) were transfected as reported (Guerra-Crespo et al., 2003) with a vector containing the *Trh* promoter sequence located between −776 and +84 bp (TRH-Luc) (Balkan et al., 1998) or a mutated version, alone or together with the pcDNA-GKLF and/or pN3-Sp1FL expression vectors. An SV40 promoter-driven β-galactosidase expression vector was used for normalization. Luciferase and β-galactosidase activities were determined 72 h later.

2.2. Site-directed mutagenesis

The KEM1 and GC-A motifs in the rat *Trh* promoter were mutated using the TRH-Luc vector as the template and the GeneTailor Site-Directed mutagenesis kit (Invitrogen). The KEM1 motif (CACCC) at −92/−88 bp was mutated using forward (5′-TCCCCCGGCTCTGCCGTCAGGTCCCCTGTCTTC-3′) and reverse (5′-CTGACGGC AGAGCCGGGGGACCCGCCCGCT-3′) primers with the base substitutions underlined. For the GC-A motif (GGGCGGG) at 119/113 bp the primers with the base substitutions underlined were: forward, 5′-CACCAGGGTTTCCGGAAAGCTGCCTAGTCCCCCGGC-3′; and reverse, 5′-GCTTTCCGGAAACCCTGGTGGGGGCTGAGC-3′. The resulting constructs were sequenced to verify mutagenesis.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from hypothalamic tissue as described (Schreiber et al., 1989). Labeled oligonucleotides (Supplementary Table 1) were incubated with 5 μg of nuclear extract for 20 min at 4 °C. For super shift assays, nuclear extracts were incubated with 4 μg of anti-Klf4 antibody or anti-Sp1 antibody (sc-14027) for 1 h at 4 °C, before the addition of the labeled probe. Then the reaction was carried out as described (Pedraza-Alva et al., 2009).

2.4. Luciferase and β-galactosidase assays

Reporter gene activity was measured using the Dual-Light® system according to manufacturer's instructions (Applied Biosystems). Data were represented as the ratio of luciferase activity/β-galactosidase activity.

2.5. Immunoprecipitation and Western blot

Immunoprecipitation was carried out with 100 μg of nuclear extracts and 1 μg of anti-Klf4 antibody, anti-Sp1 antibody or anti-TrkB antibody (sc-012, Santa Cruz, Biotechnology). Immunocomplexes were harvested with protein A/G sepharose (Santa Cruz, Biotechnology), washed as described (Perez-Martinez and Jaworski, 2005) separated by SDS-PAGE, transferred to a PVDF membrane, and detected as described (Perez-Martinez and Jaworski, 2005).

2.6. Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed essentially as described (De La Rosa-Velazquez et al., 2007). The chromatin (fragmented to 300–600 bp long) was subjected to immunoprecipitation using 4 μg of anti-Klf4 antibody or, anti-Sp1 antibody. Immunoprecipitated DNA was analyzed by PCR using specific primers for the region encompassing the CACCC and GC sequences of the *Trh* gene promoter (Supplementary Table 1). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene primers were used as negative control (Perez-Martinez et al., 2001).

2.7. Animal genotyping and skin permeability assay

Procedures that involved mice and rats were performed according to Institutional guidelines. Klf4$^{-/-}$ mice (Segre et al., 1999) were maintained on a C57BL/6 background. Genotyping and the skin barrier assay were performed as described (Hardman et al., 1998).

2.8. RT-PCR

RNA extraction and cDNA synthesis were done as described (Girard et al., 2002). Semiquantitative PCR was carried out as reported (Perez-Martinez et al., 1998) using 50 pmol each of either the *Klf4*-, *Sp1*- or *cyclophilin*-specific forward and reverse primers (Supplementary Table 1). PCR amplification products were analyzed by densitometry using the Fluor-S MultiImager acquisition system (Bio-Rad) and the Quantity One software (Bio-Rad, v 4.2.0). Data were normalized to the non-developmentally regulated gene *cyclophilin* (Al-Bader and Al-Sarraf, 2005).

2.9. Real time quantitative PCR

Total RNA was treated with the Turbo DNA-free kit (Ambion), reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), and amplified with Power SYBR Green PCR Master Mix (Applied Biosystems). *Actin* mRNA level was used as internal normalization control. The real time quantitative PCR (qPCR) was performed on a Rotor-Gene RG-3000 detector (Corbett Research). Relative gene expression was determined by the ΔCt method, using the wild-type hypothalamic cDNA as reference.

2.10. Radioimmunoassay

E15 or P0 fresh rat hypothalamic tissue was sonicated in 20% acetic acid. The extracts were dried and TRH peptide levels were determined by radioimmunoassay as previously described using 1:10,000 anti-TRH antibody (Joseph-Bravo et al., 1979).

2.11. Statistical analysis

Results were calculated as percentage of control. Data shown are the mean ± SEM; they were analyzed by ANOVA, considered significant at $p < 0.05$, followed by Fisher's PLSD test. In general, data correspond to at least two independent experiments.

3. Results

3.1. Klf4 is expressed in the rat hypothalamus during the establishment of the TRH neurons in vivo

Since the *Klf4* transcript is enriched in fetal hypothalamic TRH neurons (Guerra-Crespo et al., submitted for publication), we determined the developmental expression profile of hypothalamic Klf4. Compared to adult stage, *Klf4* expression was higher at the earliest developmental stages examined [embryonic day (E) 15] peaking at neonate (P0) and postnatal (P) day 7; expression then decreased gradually until adult stage (Fig. 1A). Klf4 protein was detected at the earliest time point examined (E15) and increased gradually with similar levels maintained between E20 and P21. By adult stage,

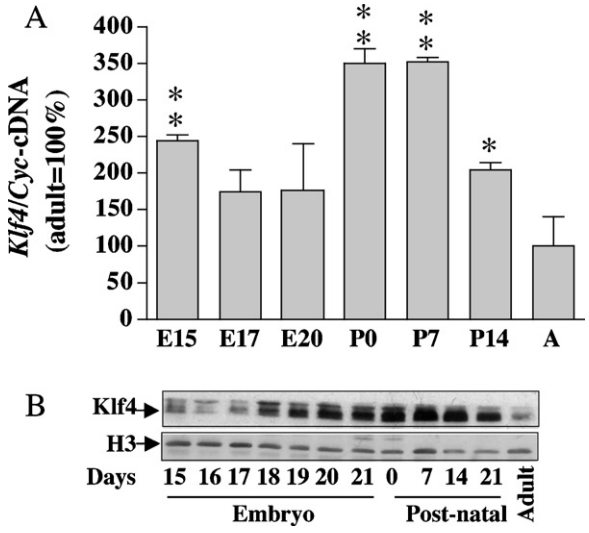

Fig. 1. Klf4 is expressed during hypothalamic development. (A) Developmental profile of Klf4 mRNA. At the indicated developmental stages, hypothalami were processed for RT-PCR assays. Data are the ratio of *Klf4* cDNA to *cyclophilin* cDNA (*Cyc*; internal control) signals in percentage of the adult signal. Results represent the mean ± SEM of three independent experiments each in triplicate. (B) Klf4 protein levels during hypothalamus ontogeny. At the indicated developmental stages, nuclear extracts were used for Western-blot assays. Histone H3 (H3) was used as a loading control. $^*p < 0.05$ and $^{**}p < 0.01$ *vs.* adult stage by post hoc Fisher analysis.

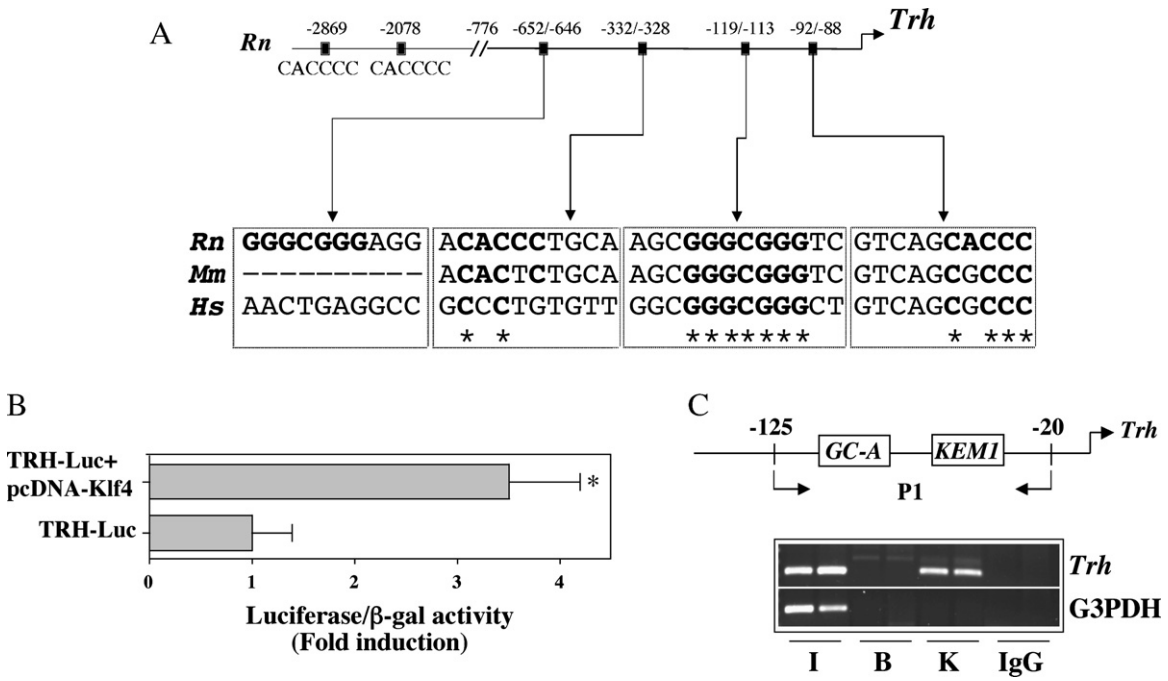

Fig. 2. Klf4 regulates *Trh* gene promoter activity. (A) Schematic representation of the *Trh* gene promoter region of the *Rattus norvegicus* (Rn) indicating relative positions of the identified CACCC and GC motifs. Note that some of the depicted elements are conserved (*) in the *Mus musculus* (Mm) and *Homo sapiens* (Hs) promoter. The position of each motif is indicated relative to the transcription start site. (B) Klf4 enhances *Trh* promoter activity *in vitro*. Primary cultures of fetal (E17) hypothalamic cells were transfected with the minimal *Trh* promoter fused to the luciferase reporter gene (TRH-Luc) alone or together with the pcDNAKlf4 expression vector. TRH-Luc transfected cells included the equivalent concentration of empty pcDNA vector. The luciferase activity was normalized to β-galactosidase activity. Fold induction was calculated relative to TRH-Luc transfected cells. Bars represent the mean ± SEM of three replicates in three independent experiments. $^{*}p < 0.05$ *vs.* TRH-Luc by post hoc Fisher analysis. (C) Klf4 is recruited to the rat *Trh* gene promoter *in vivo*. *Upper panel*: diagram indicating the relative positions (left and right arrows) of the PCR primers (P1) on the rat *Trh* promoter region. *Lower panel*: PCR product derived from ChIP-enriched genomic DNA from E15 hypothalamus. This experiment is a representative of 2–3 replicates in three independent experiments. I: input; B: beads; K: α-Klf4 antibody; IgG immunoprecipitation.

Klf4 protein levels were comparable to E15 levels (Fig. 1B). In rat the amount of *Trh* mRNA per hypothalamus increases between E17 and P14 (Perez-Martinez et al., 2001); thus, Klf4 expression occurs during a period in which the biosynthesis of TRH is up-regulated.

3.2. Klf4 regulates rat Trh gene promoter activity

Klf4 promotes cell differentiation through binding to CACCC or GC motifs present on the regulatory region of target genes (Shields et al., 1996; Philipsen and Suske, 1999). Using the MatInspector program (Cartharius et al., 2005) we identified four potential binding sites for Krüppel-like transcription factors within the minimal promoter region of the rat *Trh* gene: two CACCC (−92/−88 and −332/−328 bp) and two GC motifs (−119/−113 and −652/−646 bp) (Fig. 2A). Interestingly, among these elements the −92/−88 and −119/−113 are conserved in the rat, mouse and human *Trh* promoter (Fig. 2A).

To assess whether Klf4 can regulate *Trh* promoter activity, primary fetal hypothalamic cells were co-transfected with the minimal *Trh* promoter region (−776/+84 bp) driving the luciferase reporter gene (TRH-Luc) together with a Klf4 expression vector. This minimal promoter drives *in vitro* reporter expression preferentially in TRH neurons (Balkan et al., 1998). We observed an about 3.5-fold induction of *Trh* promoter activity by Klf4 compared to control cells (Fig. 2B). Consistently with this, chromatin immunoprecipitation (ChIP) assays showed that Klf4 binds to the *Trh* gene promoter *in vivo*. As shown in Fig. 2C, using chromatin derived from E15 rat hypothalamus and specific primers spanning the initial CACCC (KEM1) and GC (GC-A) motifs, we observed the enrichment of the PCR amplification product using the Klf4 immunoprecipitated fraction; in addition, no amplification was detected using an IgG control antibody. This result was specific for the *Trh* promoter since no PCR products were detected using primers from the G3PDH coding region (Fig. 2C). These data suggest that Klf4 can trans-activate the *Trh* promoter by binding to the two proximal putative Klf sites.

3.3. Klf4 binds to specific sequences on the rat Trh gene promoter region

To confirm that the two proximal Klf elements within the minimal *Trh* promoter region are responsible for recruiting Klf4, we performed electrophoretic mobility shift assays (EMSA) using synthetic oligonucleotides containing either the first CACCC sequence (KEM1) or the GC-A motifs (Fig. 3A) together with nuclear extracts from rat hypothalamus. The KEM1 oligonucleotide formed two major DNA–protein complexes (* and **, Fig. 3B). The formation of these complexes was developmentally regulated; however, only the complex (*) could be competed by the addition of a 20–100-fold molar excess of non-labeled KEM1 oligonucleotide (data not shown). The DNA–protein complex (*) was detected at E14, diminished at E15, and was barely detected at E17. It reappeared at E20, decreased gradually at P0 and P7, and disappeared at the adult stage (Fig. 3B).

Experiments using an oligonucleotide spanning the GC motif located at −119/−113 bp (GC-A) showed the formation of a major DNA–protein complex at E14, which then decreased from E15 to E17 (Fig. 3C, *). Complex formation increased at E20, gradually decreased between P0 and P7, and was absent in the adult stage (Fig. 3C, *). Additionally, two minor DNA–protein complexes were also observed (Fig. 3C, **); however, only the major complex (*) could be competed by the addition of a 20- to 100-fold molar excess of non-labeled GC-A oligonucleotide (data not shown).

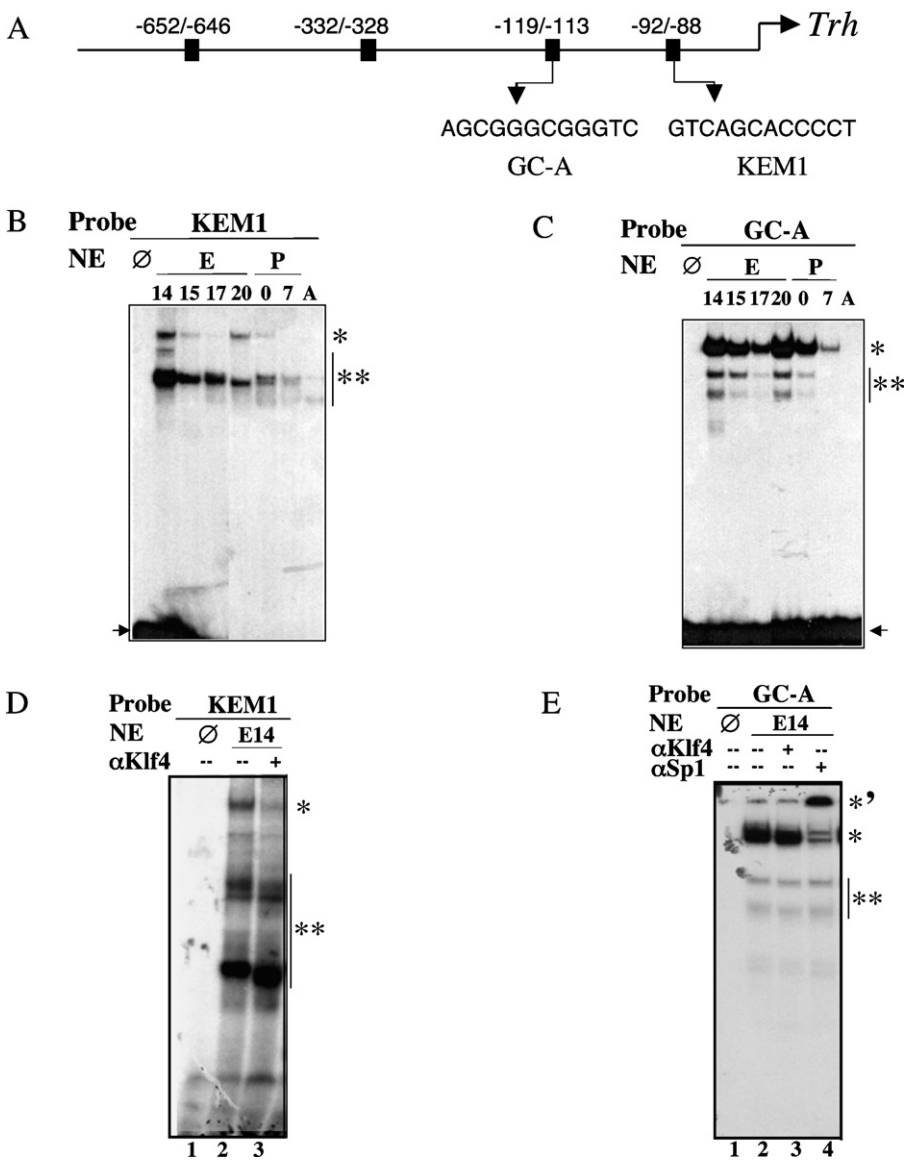

Fig. 3. The Klf4 and Sp1 transcription factors bind to the response elements of the rat *Trh* gene promoter region *in vitro*. (A) Diagram showing the relative position of the KEM1 (CACCC box) and GC-A (GC-rich) motifs. (B and C) EMSA assays were performed with nuclear extracts (NE) from the indicated developmental hypothalamic stage, incubated with the [γ^{32}P] ATP-labeled KEM1 (B) or GC-A (C) oligonucleotides and DNA–protein complexes resolved in non-denaturing conditions. (*) Complex of interest and (**) unspecific complexes. (D and E) Super-shift assays using 14-day-old embryonic nuclear extracts (E14) incubated with KEM1 (D) or GC-A (E) oligonucleotides in the absence (D and E, lane 2) or presence (D and E, lane 3; (*)) of the anti-Klf4 antibody or anti-Sp1 antibody (E, lane 4, (*')). (**): Unspecific complexes; (arrow): free probe; (∅): without NE. In the super-shift assays the probe ran out of the gel. Data representative of three independent experiments are shown.

To determine whether Klf4 binds to the KEM1 and GC-A motifs, super-shift experiments were performed using an anti-Klf4 antibody previously used for EMSA (Zeng et al., 2006) and nuclear extracts from E14 hypothalamus, a stage at which DNA–protein complex formation was more prominent (Fig. 3B and C). The anti-Klf4 antibody abrogated low mobility complex formation with the KEM1 oligonucleotide, indicating Klf4 binding to this sequence (Fig. 3D, compare lanes 2 and 3, *). However, the anti-Klf4 antibody did not alter the complexes formed by incubation of the E14 nuclear extracts with the GC-A oligonucleotide, strongly suggesting that Klf4 was not recruited to the GC motif (Fig. 3E, compare lanes 2 and 3, *). Since Klf4 belongs to the transcription factors of the Sp family, and because Sp1 binds preferentially GC boxes and synergizes with Klf4 to control transcription of target genes (Higaki et al., 2002), we investigated whether Sp1 is present in the DNA–protein complexes formed with the GC-A motif. The addition of anti-Sp1 antibody super-shifted the major complex formed with the GC-A oligonucleotide, indicating that Sp1 binds to this sequence (Fig. 3E, compare lanes 2 and 4, *'). Consistent with this, RT-PCR and Western-blot analyses demonstrated Sp1 expression during hypothalamus ontogeny from the earliest developmental stage examined (E14) (data not shown). These results indicate that *in vitro* Klf4 is bound to the CACCC motif (KEM1) while Sp1 was able to bind to the GC-A box of the rat *Trh* promoter region.

Since Klf4 and Sp1 are expressed during hypothalamic development and bind to a CACCC motif and a GC box on the rat *Trh* gene promoter, we determined whether both transcription factors regulate *Trh* transcription. Primary fetal hypothalamic cultures were transfected with TRH-Luc alone or together with Klf4 and/or Sp1 expression vectors. As shown in Fig. 2B, over-expression of *Klf4* augmented significantly the reporter activity compared to control cells (Fig. 4A). In contrast, *Sp1* over-expression did not change transcriptional rate compared to control cells; however, *Klf4* and *Sp1* co-expression increased additively luciferase activity (Fig. 4A). These results indicate a trans-acting potential of Klf4 and Sp1 over *Trh* gene expression in fetal hypothalamic cells *in vitro*.

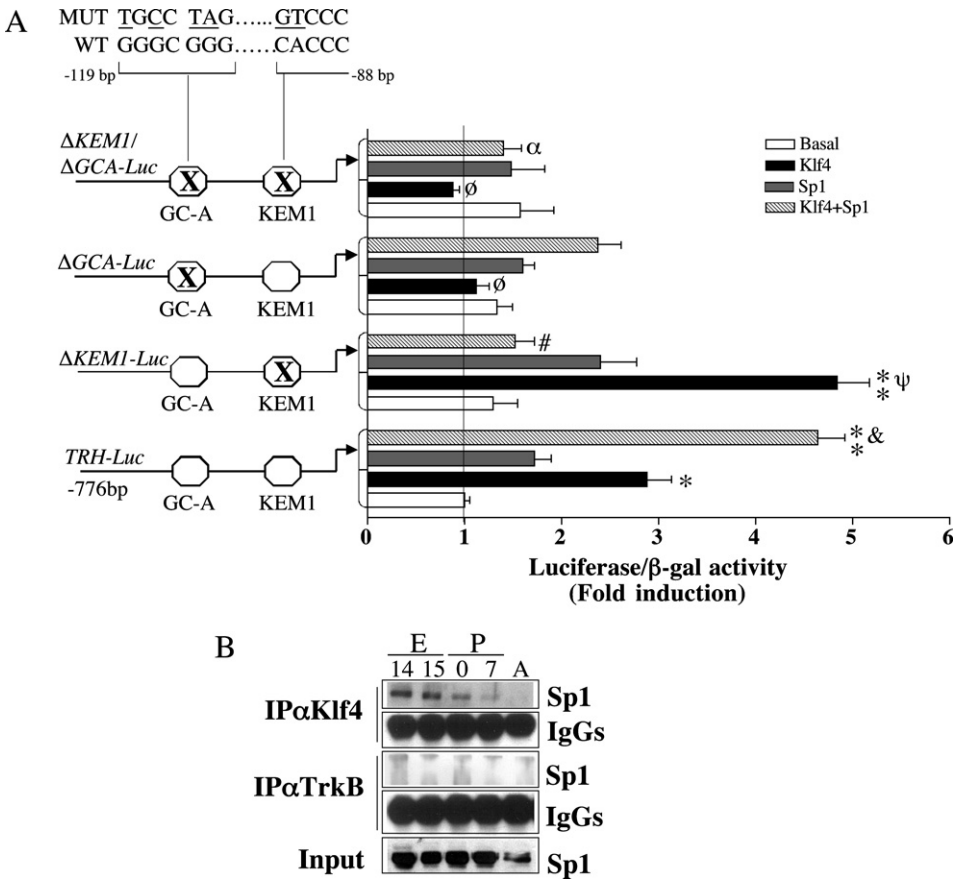

Fig. 4. Klf4 and Sp1 regulate the transcriptional rate of *Trh* gene *in vitro*. (A) Primary cultures of fetal (E17) hypothalamic cells were transfected with either the wild-type *Trh* promoter fused to the luciferase reporter gene (TRH-Luc), or the KEM1 (ΔKEM1-Luc), GC-A (ΔGCA-Luc), or KEM1/GC-A (ΔKEM1/ΔGCA-Luc) mutated versions of TRH-Luc, alone or together with the pcDNAKlf4 and/or pN3-Sp1FL expression vectors. Wild-type sites are clear white and the presence of the X indicates a mutation. The luciferase activity was normalized to β-galactosidase activity. Fold induction was calculated relative to TRH-Luc transfected cells. Bars represent the mean ± SEM of three replicates in three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.001$ *vs.* TRH-Luc. $^{\&}p < 0.05$ *vs.* TRH-Luc/Sp1. $^{\Psi}p < 0.05$ *vs.* ΔKEM1-Luc. $^{\#}p < 0.05$ *vs.* TRH-Luc/Klf4/Sp1. $^{\varnothing}p < 0.05$ *vs.* TRH-Luc/Klf4. $^{\alpha}p < 0.05$ *vs.* TRH-Luc/Klf4/Sp1. Basal denotes cells transfected with either the wild-type or mutated version of the TRH promoter alone or together with the equivalent concentration of pcDNA and/or pN3 empty vectors. (B) Klf4–Sp1 interaction *in vivo* at different hypothalamic developmental stages. Nuclear extracts were immunoprecipitated with anti-Klf4 antibody or anti-TrkB antibody used as negative control. Immunocomplexes were subjected to Western-blot analysis using anti-Sp1 antibody. Proteins were revealed with HRP-coupled secondary antibody. E: embryonic day; P: post-natal day; A: adult.

To investigate the role of the KEM1 and GC-A motifs on the Klf4 and Sp1 mediated *Trh* promoter activity, we mutated the KEM1 (*gt*CCC; ΔKEM1) and/or GC-A (*tGcCtaG*; ΔGCA) elements to abolish binding of Krüppel-like transcription factors (Higaki et al., 2002), and/or Sp1 (Li and Kellems, 2003), respectively. Primary hypothalamic cultures were co-transfected with the wild-type promoter construct (TRH-Luc), or with ΔKEM1-Luc and/or ΔGCA-Luc constructs, together with Klf4 and/or Sp1 expression vectors. Mutation of the KEM1 site did not alter *Trh* promoter basal activity (Fig. 4A) but, contrary to what we expected, it enhanced Klf4 effect on promoter activity (Fig. 4A). Nevertheless, mutation of the KEM1 site abolished the Klf4–Sp1 mediated *Trh* promoter activity (Fig. 4A). These results indicate that the wild-type KEM1 site exerts a negative effect on the *Trh* promoter activity in response to Klf4; however, Klf4–KEM1 interaction is necessary for Klf4–Sp1 transactivation when Sp1 is bound to the GC-A motif (Fig. 3E, compare lanes 2 and 4, *'). Accordingly, mutation of the GC-A site diminished Klf4, and Klf4–Sp1 mediated *Trh* promoter activity (Fig. 4A). These results point out an important regulatory role for the GC-A motif on *Trh* expression. Thus, the fact that the GC-A site is critical for Klf4 and Klf4–Sp1 transcriptional properties, in spite of the absence of binding of Klf4 to the GC-A element (Fig. 3E, compare lanes 2 and 3, *), suggests that Klf4 mediates its positive transcriptional activity on the *Trh* promoter independently of DNA binding. Previous studies have shown that Klf4–Sp1 protein–protein interactions regulate transcription (Zhang et al., 1998). To test this possibility we performed co-immunoprecipitation experiments using embryonic and neonatal hypothalamic nuclear extracts. Klf4–Sp1 interaction was detected at E14 and E15, decreased by P0 and absent in the adult (Fig. 4B). Thus, we propose that the GC-A element cooperates with the KEM1 motif to control Klf4–Sp1 mediated transcriptional activity. Consistently, mutation of both sites had the same effect as the GC-A mutant (Fig. 4A). Therefore, in rat fetal hypothalamic cell cultures the GC-A motif is necessary for positive regulation of *Trh* promoter activity mediated by Klf4 or Klf4–Sp1 but not for basal activity.

3.4. *Klf4 is necessary for the early expression of Trh in the developing mouse hypothalamus*

To further characterize the role of Klf4 *in vivo*, we evaluated *Trh* expression during hypothalamus development in the $Klf4^{-/-}$ mouse. The null mutation was confirmed as previously described (Hardman et al., 1998). A survey of the brain of $Klf4^{-/-}$ mice did not reveal any apparent gross histological alteration in the hypothalamic area at E15 (data not shown). However, a severe reduction in *Trh* mRNA levels was detected in the $Klf4^{-/-}$ (KO) mice hypothalamus at E15 compared to $Klf4^{+/+}$ (WT) or $Klf4^{-/-}$ compared to

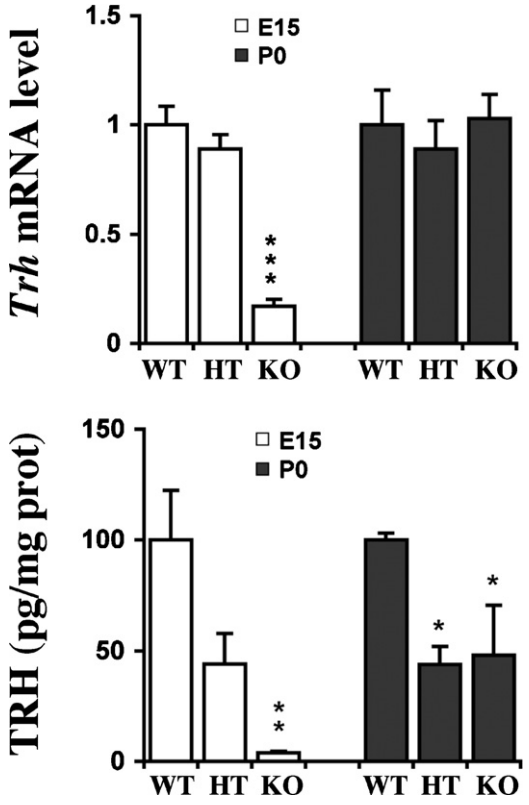

Fig. 5. The Klf4 transcription factor is necessary for proper *Trh* gene expression during fetal development of the mouse hypothalamus. *Trh* mRNA and peptide levels were determined by qRT-PCR (upper panel) and radioimmunoassay (lower panel). Total RNA or peptide extracts were prepared from 15 day-old embryo (E15) or neonate (P0) hypothalamus derived from wild-type (WT), heterozygous (HT) or knockout (KO) mice. For qRT-PCR, relative expression levels were determined using actin as a constitutive expression gene and the WT values for normalization. Data are in percentage of the signal in WT animals taken as one (upper panel) or 100% (lower panel). Results represent the mean ± SEM of three (qRT-PCR) or two (radioimmunoassay) independent experiments in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* WT by post hoc Fisher analysis.

Klf4$^{+/-}$ (HT). No statistical difference was observed between the *Klf4*$^{+/-}$ and *Klf4*$^{+/+}$ littermates (Fig. 5, upper panel). *Klf4* expression is therefore critical for proper *Trh* gene expression during mouse fetal hypothalamic development and mono-allelic expression of *Klf4* gene is sufficient to maintain *Trh* gene expression.

At the neonate stage (P0) no differences in *Trh* transcripts levels were detected among *Klf4*$^{-/-}$, *Klf4*$^{+/-}$ and *Klf4*$^{+/+}$ mice (Fig. 5, upper panel). Therefore, at P0 alternative signaling pathways that do not rely on Klf4, e.g. hormones (Farkas et al., 2008), neurotrophins (Guerra-Crespo et al., 2001), or neuropeptides (Lechan and Fekete, 2006) control *Trh* gene transcription, or other members of the Krüppel-like family (Klf2, Klf5 or Klf9) (Jiang et al., 2008; Scobie et al., 2009) compensate the loss of *Klf4*.

Reduction in *Trh* mRNA level at E15 was reflected at the peptide level. Hypothalamic TRH content decreased significantly in the E15 *Klf4*$^{-/-}$ mice compared to *Klf4*$^{+/+}$ mice (Fig. 5, lower panel). A trend for reduction in TRH levels was also observed in the haploinsufficient Klf4 mice with respect to *Klf4*$^{+/+}$ mice (Fig. 5, lower panel). In contrast to *Trh* mRNA levels, TRH peptide levels at P0 were significantly reduced in both *Klf4*$^{+/-}$ and *Klf4*$^{-/-}$ compared to Klf4$^{+/+}$ littermates (Fig. 5, lower panel). No difference was observed in the *Klf4*$^{-/-}$ compared to *Klf4*$^{+/-}$ mice (Fig. 5, lower panel). These results suggest that although compensatory mechanisms for *Trh* transcript expression lead to TRH synthesis recovery in the P0 *Klf4*$^{-/-}$ mice, there was an apparent delay in the accumulation of bioactive peptide.

4. Discussion

Some of the peptidergic neurons of the hypothalamus mediate homeostasis by regulating hormone secretion from the pituitary gland. Among these, TRH cells of the PVN play an important role coordinating the hypothalamus–pituitary–thyroid axis function. Other TRH neurons of the hypothalamus have yet poorly understood roles; they may control behavioral arousal, core body temperature and feeding (Gotoh et al., 2007; Lechan and Fekete, 2006; Hara et al., 2009). However, little is known about the mechanisms regulating *Trh* gene expression during hypothalamic development. To gain insight into the molecular mechanisms underlying TRH neuron development, we recently characterized the transcriptome of embryonic hypothalamic TRH neurons. This study demonstrated that the transcription factor Krüppel-like 4 (Klf4) is enriched in the TRH neurons (Guerra-Crespo et al., submitted for publication). Evidence showing that Klf4 contributes to neuronal physiology within the CNS is scarce. Treatment of embryonic cortical neurons cultures with NMDA induces *Klf4* gene expression, which facilitates apoptotic cell death (Zhu et al., 2009). Klf4 is one of the early genes induced by NGF during PC12 cell differentiation; however, the final Klf4 target genes are unknown (Dijkmans et al., 2009). These findings suggest that in response to different signaling pathways Klf4 may play a role in the development of some neuronal cells. Consistently, here we report that *Klf4* is expressed in the developing rat hypothalamus; overexpression of Klf4 induced *Trh* promoter activity in rat fetal hypothalamic cultures; mutation of a Klf element (GC-A) in the minimal *Trh* promoter significantly inhibited the ability of Klf4 to transactivate the promoter; and gel shift as well as ChIP studies both verified that Klf4 can bind to the minimal *Trh* promoter region. Furthermore, we show that Klf4 and the transcription factor Sp1 co-regulate *Trh* gene transcription in cultures of rat fetal hypothalamus. As for Klf4, mutation of the GC-A site significantly diminished Klf4–Sp1 mediated *Trh* promoter activity. Based on these observations, we propose that the GC-A motif is necessary for positive regulation of *Trh* promoter activity mediated by Klf4 or Klf4–Sp1. Previous studies in our laboratory have shown that this GC-A motif is bound by nuclear extracts from hypothalamic cells stimulated by cAMP or glucocorticoids (Diaz-Gallardo et al., 2010); it probably serves multiple roles in the transcriptional control of *Trh* expression.

The GC-A element may be involved in the interactions needed for higher-order complex formation to control *Trh* transcription. As previously shown (Zhang et al., 1998), here we demonstrated that Klf4 interacts with Sp1. Most notably, this physical association occurs early in development but it is absent in the adult hypothalamus. Protein–protein interactions among sequence-specific DNA binding transcription factors suggest specific modes of regulation. Thus, it is conceivable that Sp1 bound to the GC-A motif (our EMSA data) may increase the affinity of Klf4 to a weak recognition site (Fig. 2A) via protein–protein interactions. Alternatively, introduction of a mutation in the GC-A element hinders Klf4 or Klf4–Sp1 activity by impeding the recruitment of co-activators to the site. This might be achieved through specific chromatin modifications resulting from Klf4 interaction with either P300 or CBP as previously reported (Evans et al., 2007). Nevertheless, it is safe to suggest that we have identified a sequence motif that could mediate interactions between Klf4 and Sp1 on the minimal *Trh* promoter. Additional studies are therefore necessary to delineate the exact effect of Klf4–Sp1 interaction on regulating *Trh* promoter activity.

In accordance with our data showing regulation of *Trh* transcription by Klf4 *in vitro*, *Trh* gene expression was down-regulated in *Klf4* null mice embryos. Although it is necessary to corroborate the direct binding of Klf4 to the mouse *Trh* promoter, the *Klf4*$^{-/-}$ mice phenotype suggests that as for the rat, the mouse *Trh* gene is a target of Klf4. This is further supported by the high degree of identity in the

GC-A motif in the rat, mouse and human *Trh* promoter. Preliminary data in our laboratory suggest that the effect of Klf4 is specific for TRH, since the hypothalamic CRH mRNA levels were not changed in the *Klf4*$^{-/-}$ compared to *Klf4*$^{+/+}$ mice (data not shown). Further experiments are required to elucidate whether the reduction of *Trh* mRNA and peptide levels observed in the *Klf4*$^{-/-}$ mice occurs in non-hypophysiotropic and/or hypophysiotropic TRH neurons.

In summary, we show that *Klf4* is expressed in the developing hypothalamus, and together with Sp1 co-regulates *Trh* gene transcription during embryonic development. In accordance, *Trh* gene expression is down-regulated in *Klf4* null mice embryos in a narrow temporal window during hypothalamic development. Further experiments are required to elucidate whether this reduction occurs in the entire hypothalamic TRH cell population or in a specific subtype. We conclude that Klf4 is part of the developmental program that up-regulates *Trh* gene expression in hypothalamic neurons during the terminal phase of differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2010.12.020.

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