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The co-chaperone XAP2 is required for activation of hypothalamic thyrotropin-releasing hormone transcription *in vivo*

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Transcriptional control of hypothalamic thyrotropin-releasing hormone (TRH) integrates central regulation of the hypothalamo-hypophyseal-thyroid axis and hence thyroid hormone (tri-iodothyronine (T_3)) homeostasis. The two β thyroid hormone receptors, TRβ1 and TRβ2, contribute to T_3 feedback on TRH, with TRβ1 having a more important role in the activation of TRH transcription. How TRβ1 fulfils its role in activating TRH gene transcription is unknown. By using a yeast two-hybrid screening of a mouse hypothalamic complementary DNA library, we identified a novel partner for TRβ1, hepatitis virus B X-associated protein 2 (XAP2), a protein first identified as a co-chaperone protein. TR–XAP2 interactions were TR isoform specific, being observed only with TRβ1, and were enhanced by T_3 both in yeast and mammalian cells. Furthermore, small inhibitory RNA-mediated knockdown of XAP2 *in vitro* affected the stability of TRβ1. *In vivo*, siXAP2 abrogated specifically TRβ1-mediated (but not TRβ2) activation of hypothalamic TRH transcription. This study provides the first *in vivo* demonstration of a regulatory, physiological role for XAP2.

Keywords: XAP2; hypothalamus; TRH; transcription; TRβ1 specificity

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INTRODUCTION

Hypothalamic thyrotropin-releasing hormone (TRH) is a central regulator of the hypothalamo-hypophyseal-thyroid axis, with the final output being the thyroid hormones (TH) tetraiodothyronine (T_4) and triiodothyronine (T_3), T_3 being the biologically active form. TH homeostasis is vital both during development and maturity, and is maintained by negative feedback exerted by T_3 on TRH and thyroid-stimulating hormone (TSH) production in the hypothalamus and pituitary, respectively.

As for other target genes, T_3 modulates TRH transcription through its nuclear TH receptors (TRs). Among the TR subtypes, TRβ1 and TRβ2 (as opposed to TRα subtypes) are the key regulators of TRH. Both TRβ1 and TRβ2 are equally implicated in T_3-dependent repression of TRH (Guissouma *et al*, 1998, 2002; Abel *et al*, 2001), whereas TRβ1 has a more marked role in activation (Dupre *et al*, 2004).

We addressed the mechanisms of TRβ1 activation of TRH by seeking TRβ1-specific partners. We used a yeast two-hybrid assay based on a library of adult male mice paraventricular nucleus (PVN) complementary DNA. One of the TRβ1 partners identified was the hepatitis virus B X-associated protein 2 (XAP2), also known as ARA9 or AIP (Carver & Bradfield, 1997; Ma & Whitlock, 1997; Meyer *et al*, 1998). XAP2 is known to modulate the transcriptional activity of the dioxin receptor (AhR) *in vitro* (Ma & Whitlock, 1997), associating with hsp90 and regulating the intracellular localization of AhR (Kazlauskas *et al*, 2001). However, no data have shown a physiological role for XAP2 or linked it to TRs.

Our experiments show that TRβ1 interacts specifically with XAP2 and that XAP2 exerts a functional role in modulating TRH transcription *in vivo*. By using a small inhibitory RNA (siRNA) knockdown approach, we show that XAP2 is necessary for T_3-independent activation of TRH transcription mediated by TRβ1. Thus, our results show for the first time a functional and receptor isoform-specific *in vivo* role for XAP2.

RESULTS AND DISCUSSION

XAP2 shows TRβ1-specific functional interactions

To identify new TRβ1 partners, we used a yeast two-hybrid system. Full-length mouse TRβ1, fused to the GAL4 DNA-binding domain in pGBKT7, was used to screen a mouse PVN cDNA library fused to the GAL4 activation domain; the library was screened with and without T_3 (1 μM). XAP2 was isolated in the presence of T_3. TRβ1 and XAP2 interactions in yeast were T_3 dependent and TR isoform specific (Fig 1A). No other TR isoform tested (TRβ2 and TRα1) interacted with XAP2 with or

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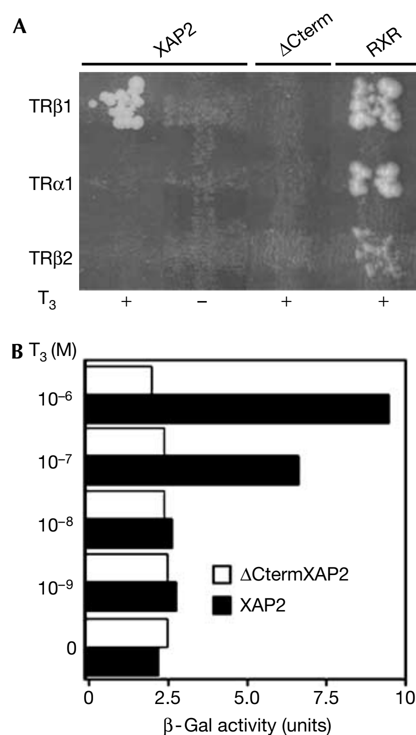

Fig 1 | Functional interactions between hepatitis virus B X-associated protein 2 and thyroid hormone receptor in yeast. (**A**) XAP2: two-hybrid experiments in yeast show T_3-dependent specific interactions of XAP2 with TRβ1 but not with TRα1 or TRβ2. ΔCterm: when the carboxy-terminal region of XAP2 is deleted, the interaction between TRβ1 and XAP2 is lost. RXR: positive control of interaction with each TR. (**B**) XAP2 and TRβ1 interactions in yeast depend on T_3 concentration. Plasmids expressing different Gal4 fusion proteins were used in mating. Diploids were grown with increasing doses of T_3 in selection medium. β-Galactosidase (β-Gal) activity was measured by densitometry. Each bar represents the average of two different transformants. T_3, triiodothyronine; TR, thyroid hormone receptor; TRα1, TRβ1, TRβ2, TR subtypes; XAP2, hepatitis virus B X-associated protein 2.

without T_3, but all TRs tested interacted with a known partner, RXRβ (Fig 1A).

To further analyse TRβ1 and XAP2 interactions, we deleted three conserved domains in the carboxy-terminal end of XAP2, the so-called tetratricopeptide repeats (TPR; ΔCtermXAP2 construct). These domains show high homologies with those of the steroid hormone receptor-interacting immunophilin FKBP52 (Carver & Bradfield, 1997; Ma & Whitlock, 1997; Meyer *et al*, 1998) implicated in protein–protein interactions (Lamb *et al*, 1995). These deletions caused loss of XAP2–TRβ1 interactions in yeast (Fig 1A). To further validate this finding, we tested the T_3 dose dependency of the TRβ1–XAP2 or the TRβ1–ΔCtermXAP2 interaction in yeast using the β-galactosidase assay. Fig 1B shows that the strength of the TRβ1–XAP2 interaction is T_3 dose dependent. Interaction between full-length XAP2 and TRβ1 was reduced by half as the T_3 concentration decreased from 10^{-6} to 10^{-7} M and no interaction was seen at 10^{-8} M T_3.

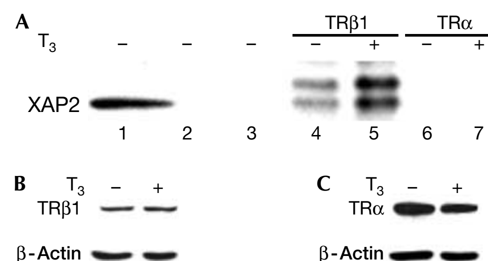

Fig 2 | Hepatitis virus B X-associated protein 2–TRβ1 interactions in mammalian cells. (**A**) XAP2 interacts with TRβ1 (but not with TRα) in mammalian cells. P19 cells were incubated in the absence or presence of 1 μM of T_3 for 2 h. Cells were then collected and whole-cell extracts (WCEs; lane 1) prepared. Extracts were used for immunoprecipitation using IgG (lane 2) or no antibody (lane 3) as controls, and TRβ1 (lanes 4,5) and TRα antibodies (lanes 6,7). The presence of XAP2 was examined by western blot analysis using XAP2 antibody. (**B**, **C**): Both TRβ1 (**B**) and TRα (**C**) are present in the P19 WCE. Samples (100 μg) of the WCE of the P19 cells used for the above experiment were fractionated on a 10% SDS–polyacrylamide gel electrophoresis gel. The levels of TRβ1 and TRα were detected by western blot analysis using TRβ1 and TRα antibodies, showing that both receptors were expressed in these cells. β-Actin was used as loading control. T_3, triiodothyronine; TRα, TRβ1, thyroid hormone receptor subtypes; XAP2, hepatitis virus B X-associated protein 2.

The TR–XAP2 interactions were tested in mammalian cell lines. The TRβ1 specificity was confirmed, but there was no interaction with TRα (Fig 2A, lanes 6,7). However, in contrast to the yeast data, XAP2 still interacted with TRβ1 in the absence of T_3, but less than with T_3. A similar discrepancy between ligand-dependent interactions in yeast and ligand-independent interactions *in vitro* in mammalian cells was described by Carver & Bradfield (1997) for XAP2 and AhR interactions.

XAP2 colocalizes with TRH and TRβ1 in the PVN

We next determined whether XAP2 was expressed in the PVN and whether it colocalized with TRH and TRβ1, as colocalization of TRH and TRβ1 had already been documented by Lechan *et al* (1994). Using *in situ* hybridization (supplementary information and supplementary Fig S1A online), we found that TRH and XAP2 transcripts were expressed in the same regions of the PVN, and XAP2 seemed to be more widely expressed than TRH. To assess the expression of XAP2 protein, we used immunocytochemistry with antibodies against XAP2 and TRβ1 on immediately successive PVN sections. XAP2 and TRβ1 were expressed in the same neurons (supplementary Fig S1B online). Thus, the localization of XAP2 is compatible with a functional interaction with TRβ1 in the hypothalamus, and with its role in regulating TRH transcription.

Extrapolating from the results reported on interactions of XAP2 and AhR (Kazlauskas *et al*, 2000), showing that XAP2 modified the subcellular localization of AhR, we next investigated whether TRβ1 localization was modified in the presence of XAP2. We used a green fluorescent protein (GFP)–TRβ fusion construct transfected into HeLa cells and followed GFP distribution by epifluorescence. The presence or absence of XAP2 did not modify the principally nuclear localization of TRβ1 (supplementary Fig S2 online). Moreover, addition of T_3 did not affect

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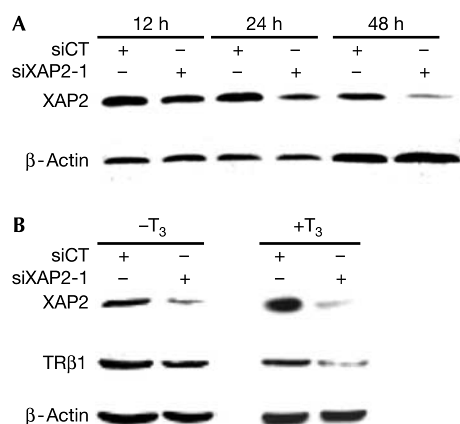

Fig 3 | *In vitro* hepatitis virus B X-associated protein 2 small inhibitory RNA experiments. (**A**) Inhibition of XAP2 by siRNA: HC11 cells were transiently transfected with siRNA against mouse XAP2 (siXAP2-1). The cells were then collected after 12, 24 or 48 h and whole-cell extracts were prepared and fractionated on a 10% SDS–polyacrylamide gel electrophoresis gel. Levels of protein expression were examined by western blot analysis using XAP2 or β-actin antibodies. (**B**) The stability of TRβ1 is affected by the absence of XAP2: P19 cells were transiently transfected with scrambled siRNA (siCT) or siRNA against mouse XAP2 (siXAP2-1) and grown for 48 h. The cells were then incubated ($+T_3$) or not ($-T_3$) with 1 μM of T_3 for 2 h. WCEs were prepared and fractionated on a 10% SDS–polyacrylamide gel electrophoresis gel. The stability of TRβ1 was detected by western blot analysis using TRβ1 antibody. XAP2 and β-actin antibodies were used as controls. siRNA, small inhibitory RNA; T_3, triiodothyronine; TRβ1, thyroid hormone receptor subtype; XAP2, hepatitis virus B X-associated protein 2.

the distribution of interactions, with or without XAP2 (supplementary Fig S2 online).

siXAP2 abrogates TRH activation *in vivo*

The central question that remained was whether XAP2 influences the effects of TRβ1 on activation of TRH transcription. To address this question, we used siRNA against XAP2 (siXAP2-1) and first tested their capacity to abrogate XAP2 protein production in HC11 cells *in vitro*. As shown in Fig 3A, siRNA against XAP2 diminished XAP2 protein expression after 24 h and even more at 48 h. The scrambled sequence (siCT) did not affect XAP2 protein production. To test whether XAP2 was implicated in the stability of TRβ1, we knocked down XAP2 protein and followed TRβ1 protein levels. XAP2 knockdown was associated with lowered TRβ1 protein levels (Fig 3B), especially in the presence of T_3 (Fig 3B, right panel).

Next, we examined the effects of XAP2 knockdown *in vivo* at the site of TRH production, the hypothalamus, using a TRH-specific transcriptional assay and a recently developed, novel *in vivo* siRNA delivery technique (Hassani *et al*, 2005). siRNAs against XAP2 were complexed with JetSI lipids and injected into the hypothalamus of newborn mice along with a TRH reporter gene (TRH-luciferase (TRH-Luc); Guissouma *et al*, 1998; Dupre *et al*, 2004) and a TRβ1 expression vector. In this experimental paradigm, TRH-Luc expression is activated in the absence of T_3, and repressed by more than 50% in the presence of T_3 (Fig 4B, left-hand columns). When co-injecting with TRH-Luc and TRβ1 into the hypothalamus of hypothyroid newborn mice, siXAP2 (20 and 200 nM) abolished the T_3-independent TRH-Luc activation (Fig 4A, middle columns). This effect was sequence specific, as no significant change in TRH transcription was seen with the control siRNA (Fig 4A, siCT). However, the effect at 20 nM was not statistically different from the effect seen at 200 nM, suggesting that the maximal level of inhibition was already attained with 20 nM. However, at 2 nM, no significant effect on TRH transcription was observed (data not shown). This inhibition of TRH activation was confirmed by the use of two different siRNAs (siXAP2-1 and siXAP2-2; Fig 4B). Given that siRNA against XAP2 abolished activation of TRH-Luc transcription in the short term (18 h post-transfection), we explored whether XAP2 knockdown affected TH production through modified endogenous TRH signalling. We found that, in the longer term (48 h post-transfection), siXAP2 increased total T_4 levels slightly, but significantly (supplementary Fig S3 online), probably reflecting a rebound after the initial inhibition. This result confirms that XAP2 contributes to endogenous TRH control and that interference with XAP2 expression affects hypothalamo-hypophyseal-thyroid axis output.

The main result from these knockdown experiments is that although siRNA against XAP2 abrogated T_3-independent activation of TRH transcription, it had no effect on TRH repression by T_3 (compare means of white bars in Fig 4B). Thus, XAP2 is necessary for T_3-independent TRH activation but not for T_3-dependent repression. Interestingly, TRβ1 has a more pronounced role than TRβ2 in T_3-independent activation of TRH (Dupre *et al*, 2004). We thus tested whether XAP2 knockdown differentially affected TRβ1 and TRβ2 effects on TRH transcription. As shown in Fig 4C, siXAP2 effects were specific to TRβ1-dependent regulation of TRH and had no effect if TRβ2 was overexpressed. Thus, the *in vivo* finding that siRNA against XAP2 specifically abrogated the TRβ1-dependent TRH activation provides a strong physiologic correlate for the TRβ1-specific XAP2 interaction seen in the different *in vitro* situations. To further confirm the role of TRβ1–XAP2 in T_3-independent activation of TRH transcription, we looked for the presence of TRβ1 on the site 4 thyroid hormone response element (Satoh *et al*, 1996) of the TRH promoter. As expected, chromatin immunoprecipitation experiments on hypothalamus from hypothyroid newborn mice showed the presence of TRβ1 on the TRH promoter (representing about 3% of the input DNA; see supplementary Fig S4 online).

In conclusion, this report shows for the first time a central, regulatory role for XAP2, with XAP2 contributing specifically to TRβ1 activation of hypothalamic TRH transcription in a physiologically relevant context.

METHODS

Plasmids. TRH-Luc and pSG5-rTRβ1 were as described by Guissouma *et al* (2002), pSG5-rTRβ2 as described by Dupre *et al* (2004) and pSG5-hXAP2 as described by Kazlauskas *et al* (2000).

Two-hybrid experiments. *Plasmid construction.* A Gal4 DBD-mTRβ1 (full-length) expression vector was constructed by inserting an *Eco*R1–*Pst*1 fragment encoding full-length mTRβ1 into the pGBKT7 vector (Clontech, Palo Alto, CA, USA). This fragment was generated by PCR using the following primers to create,

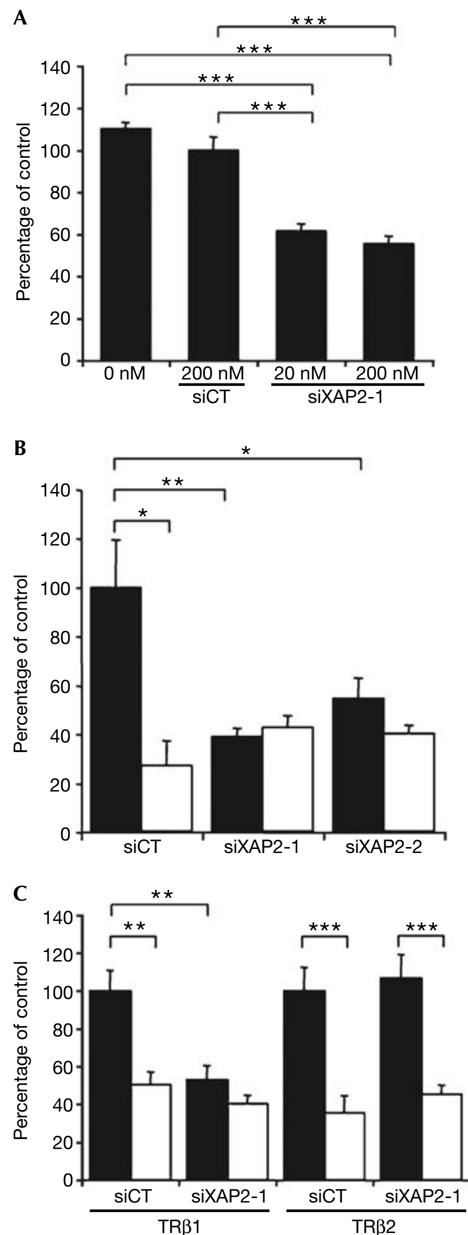

Fig 4 | In the mouse hypothalamus, hepatitis virus B X-associated protein 2 small inhibitory RNA affects only TRβ1-mediated activation of thyrotropin-releasing hormone transcription. (**A**) Dose-dependent effect of XAP2 siRNA on basal TRH transcription. TRH-Luc transcription was followed in hypothyroid 2-day-old mice, 20 h after hypothalamic injection of 1 μg TRH-Luc, 100 ng TRβ1 expression vector and 0, 20 or 200 nM of siXAP2-1 or 200 nM of scrambled siRNA (siCT) complexed with JetSI. Values are means ± s.e.m., $n \geqslant 30$; $^{***}P < 0.001$. (**B**) Two different XAP2 siRNAs affect the activation but not the T_3-dependent repression of TRH transcription. TRH-Luc transcription was measured in hypothyroid 2-day-old mice, 20 h after hypothalamic injection of 1 μg reporter construct, 100 ng of TRβ1 expression vector and 200 nM of siXAP2-1, siXAP2-2 or scrambled siRNA (siCT) complexed with JetSI. After transfection, mice were treated with T_3 (2.5 μg/g of body weight, filled bars) or saline (open bars). Values are means ± s.e.m., $n \geqslant 6$; ns: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$. (**C**) XAP2 siRNA affects activation of TRH transcription mediated by TRβ1 but not by TRβ2. TRH-Luc transcription was measured in hypothyroid 2-day-old mice, 20 h after hypothalamic injection of 1 μg reporter construct, 100 ng of either TRβ1 or TRβ2 expression vectors and 200 nM of siXAP2-1 or scrambled siRNA (siCT) complexed with JetSI. After transfection, mice were treated with T_3 (2.5 μg/g of body weight, filled bars) or saline (open bars). Values are means ± s.e.m., $n \geqslant 14$; ns: not significant; $^{**}P < 0.01$; $^{***}P < 0.001$. Each experiment was repeated at least three times. siRNA, small inhibitory RNA; T_3, triiodothyronine; TRβ1, TRβ2, thyroid hormone receptor subtypes; TRH, thyrotropin-releasing hormone; XAP2, hepatitis virus B X-associated protein 2.

respectively, the *Eco*R1 and *Pst*1 sites (in bold): 5′-CCG**GAATTCA**TGACTCCTAACAGTATGACA-3′ and 5′-AA**CTGCAG**TTAGTCCTCAAATACTTCTAA-3′. To obtain Gal4 DBD-mTRβ2 and Gal4 DBD-mTRα1 constructs, full-length mTRβ2 and mTRα1 were cloned by the same method using the following primers to create *Eco*R1 and *Bam*H1 sites (in bold), respectively: for mTRβ2, 5′-CC**GGAATTC**ATGAACTACTGTATGCCAGAG-3′ and 5′-CG**GGATCC**TTAGTCCTCAAATACTTCTAA-3′; for mTRα1, 5′-CCG**GAATTC**ATGGAACAGAAGCCAAGCAAG-3′ and 5′-CG**GGATCC**TTAGACTTCCTGATCCTC-3′. The pGADT7-ΔCtermXAP2 plasmid was made by digesting pGADT7-XAP2 plasmid with *Age*I and *Xho*I, thus deleting the region corresponding to the C terminus of XAP2, from base 420 to base 1,032, including the three TPR motifs and the GIFSH motif (Carver *et al*, 1998). The pGADT7-RXR plasmid was isolated in the two-hybrid screen.

Library construction, screening and analysis. The MATCHMAKER Library Construction and Screening Kit (Clontech) was used to construct a cDNA library using PVN of adult male mice. Briefly, after generating double-stranded cDNA, haploid *Mata* yeast strain (AH109) was co-transformed with double-stranded cDNA and pGADT7-Rec vector, whereas the pGBKT7-TRβ1 vector served to transform haploid *Matα* yeast strain (Y187). Two-hybrid screening was carried out by mating the two transformed haploid yeast strains, according to the manufacturer's protocol. Diploids expressing the interacting proteins were identified by nutritional selection using three reporters (*ADE2*, *HIS3* and *MEL1*) ±1 μM T_3. After analysis, positive clones were rescued in *Escherichia coli*, sequenced and compared with GenBank.

Strength of interaction analysis by β-galactosidase assay. A total of about 2×10^8 cells were grown in synthetic medium lacking leucine and tryptophan, in the presence of T_3 (0, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M), and then incubated at 30 °C for 3–4 h until cells reached the mid-log phase. The A_{600} was measured. An aliquot of each sample was centrifuged and resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$). Cells were permeabilized by two snap-freezing cycles in liquid nitrogen. β-Galactosidase activity was assayed according to Lee *et al* (1995).

Cell cultures. HeLa cells were propagated as described by Kazlauskas *et al* (2001). HC11 cells were grown according to Olsen *et al* (2002). Before transfection, the HC11 culture medium was changed to filtered RPMI without phenol red, supplemented with 5% charcoal-treated fetal bovine serum (FBS), L-glutamine (2 mM), gentamycin (1%), insulin (5 μg/ml) and epidermal growth factor (10 ng/ml). P19 cells were grown on Nunc plates coated

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with 0.1% gelatin in Dulbecco's minimum essential medium supplemented with 10% FBS, L-glutamine (2 mM) and 1% non-essential amino acids at 37 °C and 9% CO_2.

Cell extracts and immunoblot assay. Immunoprecipitation and western blots were carried out according to Kazlauskas *et al* (2001). HC11 and P19 cells were grown in T_3-depleted medium $\pm T_3$ (1 μM). Cells were collected and whole-cell extracts were prepared. For immunoprecipitation, 250 μg of cellular proteins were incubated with anti-TRβ1 or anti-TRα antibodies (Affinity BioReagents, Ozyme, St Quentin en Yvelines, France). Anti-XAP2 antibody (1:1,000 dilution; Novus Biologica, Littleton, CO, USA) was used for western blot (1:1,000 dilution). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse immunoglobulins (Dako Cytomation, Glostrup, Denmark).

siRNA experiments. *Generation of siRNAs*. siRNAs against mouse XAP2 were designed using the HiPerformance Design Algorithm licensed from Novartis AG (Qiagen, Courtaboeuf, France). Duplexes targeted against mXAP2 nucleotides 489–509 were synthesized (siXAP2-1, 5′-CACGTAGTCCTGTATCCTCTA-3′). A second siRNA (SI00058562, siXAP2-2) was from Qiagen. Either a scrambled sequence (5′-CGTCACGAGTACTCCGAAGTT-3′) or an siRNA against an irrelevant GFP sequence served as the control (siCT).

***In vitro* siRNA transfection.** HC11 and p19 cells were transfected with a final concentration of 20 nM of siRNA (siXAP2-1 or siCT) using Lipofectamin Plus or Lipofectamin 2000, according to the manufacturer's instructions (Invitrogen, Cergy, Pontoise, France). The transfection medium was changed after 4 h (see the Cell cultures section). Cells were collected and used for western blotting using mouse anti-XAP2 antibody (1:1,000 dilution), mouse anti-TRβ1 antibody (1:1,000 dilution; Affinity BioReagents) or mouse β-actin antibody (1:5,000; Sigma, St Quentin Fallavier, France). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse immunoglobulins.

***In vivo* siRNA transfection–*in vivo* gene transfer.** Animal studies were conducted according to the principles and procedures of Guidelines for Care and Use of Experimental Animals. *In vivo* siRNA experiments were performed, as described previously (Hassani *et al*, 2005), using JetSI™ (Polyplus Transfection, France). XAP2 or control siRNA (0, 20 or 200 nM) was mixed with TRH-Luc (0.25 μg/μl; Plasmid Factory, Germany) and pSG5-TRβ1 or pSG5-TRβ2 (25 ng/μl). Complexes (2 μl) were injected bilaterally into the hypothalamus of hypothyroid 1-day-old mice, brains were dissected and luciferase assays were carried out 20 h after transfection (Guissouma *et al*, 1998).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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