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Transcriptional repression and developmental functions of the atypical vertebrate GATA protein TRPS1

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Known vertebrate GATA proteins contain two zinc fingers and are required in development, whereas invertebrates express a class of essential proteins containing one GATA-type zinc finger. We isolated the gene encoding TRPS1, a vertebrate protein with a single GATA-type zinc finger. TRPS1 is highly conserved between *Xenopus* and mammals, and the human gene is implicated in dominantly inherited tricho-rhino-phalangeal (TRP) syndromes. TRPS1 is a nuclear protein that binds GATA sequences but fails to transactivate a GATA-dependent reporter. Instead, TRPS1 potently and specifically represses transcriptional activation mediated by other GATA factors. Repression does not occur from competition for DNA binding and depends on a C-terminal region related to repressive domains found in Ikaros proteins. During mouse development, TRPS1 expression is prominent in sites showing pathology in TRP syndromes, which are thought to result from TRPS1 haploinsufficiency. We show instead that truncating mutations identified in patients encode dominant inhibitors of wild-type TRPS1 function, suggesting an alternative mechanism for the disease. TRPS1 is the first example of a GATA protein with intrinsic transcriptional repression activity and possibly a negative regulator of GATA-dependent processes in vertebrate development.

Keywords: GATA proteins/mammalian development/transcriptional repression/tricho-rhino-phalangeal syndromes/TRPS1

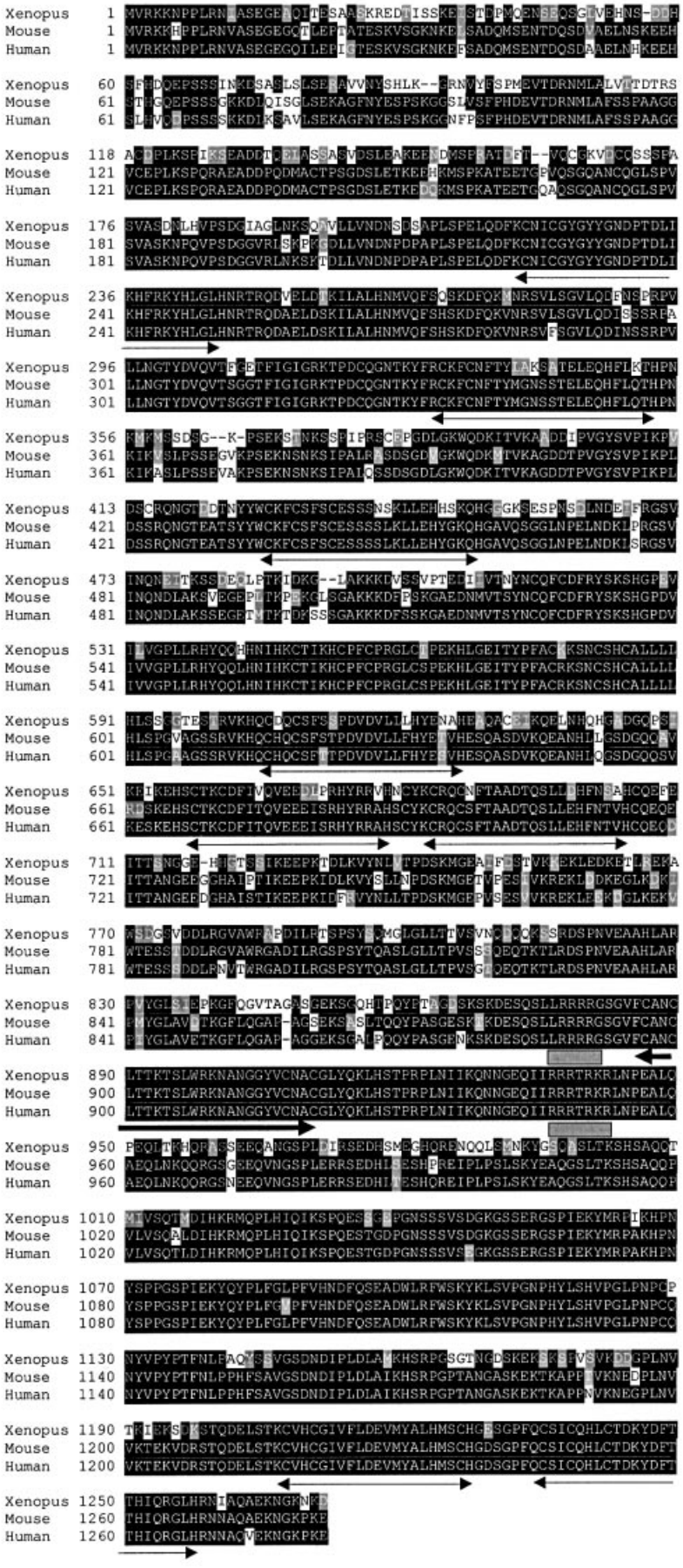
Introduction

Zinc finger transcription factors of the GATA family execute critical, non-redundant functions in vertebrate and invertebrate development. The defining feature of this family is the presence of one or two zinc fingers with the consensus sequence $CXNCX_{17}CNXC$. One of these motifs binds preferentially to the cognate DNA sequence WGATAR and related sequences found in the control regions of numerous lineage-restricted and developmentally regulated genes (Ko and Engel, 1993; Merika and Orkin, 1993). The six known vertebrate GATA-binding proteins are highly homologous and conserved, contain two GATA-type zinc finger domains, and fall into two subgroups. GATA-1, -2 and -3 are expressed primarily in blood cell lineages and regulate key aspects of hematopoiesis (Pevny *et al*., 1991; Tsai *et al*., 1994; Ting *et al*., 1996; Shivdasani *et al*., 1997). GATA-4, -5 and -6 are expressed principally in the heart and gut, and their germline absence results in early embryonic defects in heart formation and endoderm development (Laverriere *et al*., 1994; Kuo *et al*., 1997; Molkentin *et al*., 1997; Koutsourakis *et al*., 1999; Reiter *et al*., 1999). Phenotypes resulting from GATA factor deficiency in mice, flies and worms underscore the central role of this gene family in cell differentiation and development (Reuter, 1994; Shivdasani and Orkin, 1996; Zhu *et al*., 1997; Fukushige *et al*., 1998; Molkentin, 2000).

The C-terminal zinc finger of vertebrate GATA proteins binds DNA (Yang and Evans, 1992; Omichinski *et al*., 1993), whereas the N-terminal finger is implicated both in DNA binding and in mediating protein–protein interactions (Martin and Orkin, 1990; Yang and Evans, 1992; Tsang *et al*., 1997). In lower eukaryotes proteins of this family typically harbor a single GATA-type zinc finger, as exemplified by *Caenorhabditis elegans* END-1 and ELT-2 and *Drosophila* SERPENT/dGATAb, which are required in the earliest stages of endoderm development (Reuter, 1994; Zhu *et al*., 1997; Fukushige *et al*., 1998). Nearly complete genome sequences reveal 11 presumptive GATA proteins in *C.elegans* and four in *Drosophila*. In each case, the single GATA-type zinc finger is most homologous to the C-terminal finger of vertebrate GATA factors.

The N-terminal zinc fingers of vertebrate GATA proteins interact with a variety of nuclear proteins, including the multi-type zinc finger proteins FOG-1 and FOG-2 (Tsang *et al*., 1997; Holmes *et al*., 1999; Svensson *et al*., 1999; Tevosian *et al*., 1999). These proteins are co-expressed with various GATA factors and the phenotypes of knockout mice reveal essential, GATA-dependent roles in the development of blood and heart cell lineages (Tsang *et al*., 1998; Svensson *et al*., 2000b; Tevosian *et al*., 2000). FOG-1 and FOG-2 demonstrate activity as transcriptional co-activators in some settings and as co-repressors in others (Tsang *et al*., 1997; Holmes *et al*., 1999; Tevosian *et al*., 1999; Deconinck *et al*., 2000; Svensson *et al*., 2000a). Similarly, the FOG-related protein U-SHAPED functions in conjunction with the *Drosophila* GATA factor PANNIER/dGATAa in neural development (Cubadda *et al*., 1997; Haenlin *et al*., 1997). Thus, like many other transcription factors, GATA proteins appear to function as DNA-binding components of larger multiprotein complexes. The abundance of GATA sequences in *cis*-regulatory elements suggests a requirement for added complexity in regulating spatio-temporal expression of lineage-restricted genes.

The structural differences between invertebrate and the known vertebrate GATA proteins prompted us to search for atypical vertebrate GATA factors and to study their roles in transcriptional regulation and development. We have assembled full-length cDNAs encoding a vertebrate


Xenopus
Mouse
Human

protein, TRPS1, which contains a single GATA-type zinc finger. Besides this novel feature, TRPS1 differs from all other vertebrate GATA proteins in that it does not function as a transcriptional activator but rather as a potent, sequence-specific transcriptional repressor *in vitro* and *in vivo*. TRPS1 is associated with a dominantly inherited human disease of skeletal malformation (Momeni *et al*., 2000), attesting to its importance in development, and we show that its expression pattern accurately reflects the pathology in affected patients. We also demonstrate that mutations identified in independent kindreds encode truncated proteins that function as dominant antagonists of TRPS1 transcriptional repression activity. Our findings suggest that TRPS1 may act to restrict expression of GATA-regulated genes at selected sites and stages in vertebrate development.

Results

Isolation of a conserved vertebrate GATA factor representing a new class

To isolate members of a potential class of vertebrate proteins with a single GATA-type zinc finger, we performed degenerate RT–PCR on early-stage *Xenopus* embryos using primers corresponding to the highly conserved C-terminal zinc finger of all GATA proteins. All 24 sequenced PCR products encoded GATA-type zinc fingers, but only one of these encoded the peptide fragment (KNANGGYV) of a novel protein. We used this novel 24 bp PCR product to screen a *Xenopus* maternal cDNA library and recovered a single partial clone corresponding to a novel GATA protein. Subsequently, we used 5′ rapid amplification of cDNA ends (RACE) and additional library screening to assemble the full-length cDNA. We also compared the *Xenopus* sequence to identify one mouse expressed sequence tag (EST) clone, used the insert to isolate two partial clones from an embryonic gut cDNA library, and amplified these by 5′ RACE to assemble a full-length homologous murine cDNA.

In the course of this isolation, monoallelic mutations in the human homolog of this gene were reported to cause a rare inherited disorder of skeletal malformations, the tricho-rhino-phalangeal (Langer–Giedion) syndrome (TRPS) type I (Momeni *et al*., 2000). We therefore designate the novel gene products TRPS1. *Xenopus* (X) and mouse (m) TRPS1 encode proteins of 1272 and 1282 amino acids and show 73 and 93% sequence similarity to the human protein, respectively (Figure 1). TRPS1 includes nine putative zinc finger motifs, only one of which (#7) is of the GATA type. The sequence is 100% conserved in this and in the adjacent basic region, which are required for GATA proteins to bind DNA (Omichinski *et al*., 1993). The two most C-terminal zinc finger motifs (#8 and #9) and flanking residues constitute a conserved domain found within the Ikaros family of lymphoid transcription factors (Georgopoulos *et al*., 1997); the remaining sequence lacks homology with known vertebrate or invertebrate proteins.

The form of XTRPS1 expressed early in *Xenopus* embryos as a maternal transcript lacks amino acids 362–617 (data not shown). These residues are present both in mTRPS1 and in the later-expressed (zygotic) XTRPS1 isoform shown in Figure 1. In the following experiments, XTRPS1 constructs encode the early maternal product; notably, we detect no differences in function between mTRPS1 and this XTRPS1 isoform.

TRPS1 is a sequence-specific, DNA-binding nuclear protein

TRPS1 includes two conserved nuclear localization signals flanking the GATA-type zinc finger (Figure 1). To determine whether the protein can enter the cell nucleus, we transfected COS cells with plasmids encoding mTRPS1 or the maternally expressed form of XTRPS1. Immunostaining with specific antisera reveals predominantly nuclear accumulation of both proteins (Figure 2A). Although both constructs express well in COS cells, we consistently failed to detect the intact protein after overexpression of XTRPS1 in *Xenopus* embryos, which is important for subsequent experiments. Therefore, we constructed a truncated protein (XTRPS1ΔN) lacking the first 805 amino acids, which also localizes to the nucleus (Figure 2A). XTRPS1ΔN, which includes the solitary GATA-type zinc finger, binds specifically to the consensus GATA sequence in gel mobility shift assays (Figure 2B). This is confirmed by competition from oligonucleotides containing the sequence GATA but not from those containing the sequence GATC. Interaction with DNA is lost when the GATA-type zinc finger is disrupted, as in the mutant construct XTRPS1ΔNmut, or in the presence of a specific XTRPS1 antiserum. Thus, TRPS1 possesses defining properties of a GATA protein by virtue of its amino acid sequence and specific binding to DNA.

TRPS1 functions as a transcriptional repressor

In many experiments with several variations, XTRPS1 and mTRPS1 failed to activate a luciferase reporter gene under the control of a GATA-dependent promoter (data not shown), whereas the representative vertebrate GATA protein XGATA4 consistently activated this reporter in mammalian cells (Figure 3A). We therefore tested the possibility that TRPS1 functions instead as a transcriptional repressor. Indeed, in co-transfection experiments, XTRPS1, XTRPS1ΔN and mTRPS1 potently repress the GATA-dependent activation induced by XGATA4 (Figure 3A). The degree of repression is proportional to the amount of co-transfected plasmid and, like the binding to DNA, also depends on integrity of the GATA-type zinc finger. The mutant proteins XTRPS1mut, XTRPS1ΔNmut and mTRPS1mut, in which two cysteine residues in this zinc finger are altered, fail to repress the transcriptional activation induced by XGATA4 (Figure 3A). Co-transfection of TRPS1 with TCF-1 and β-catenin does not repress the GATA-independent activation of a reporter gene regulated by the unrelated Tcf-family proteins (Figure 3B). Thus, repression by TRPS1 occurs specifically in the

Fig. 1. TRPS1 is a highly conserved and atypical vertebrate GATA protein. Deduced amino acid sequences of *Xenopus*, mouse and human TRPS1. *Xenopus* and mouse cDNAs were isolated as described; the human sequence is based in part on confirmed EST clones and in part on recently published data (Momeni *et al*., 2000). Identical amino acids are shaded in black, and conservative substitutions in gray. Positions of the nine putative zinc finger motifs are indicated by arrows under the sequence, the GATA-type zinc finger (residues 886–910 in XTRPS1) is marked by a thick arrow, and the two putative nuclear localization signals are marked by shaded boxes.

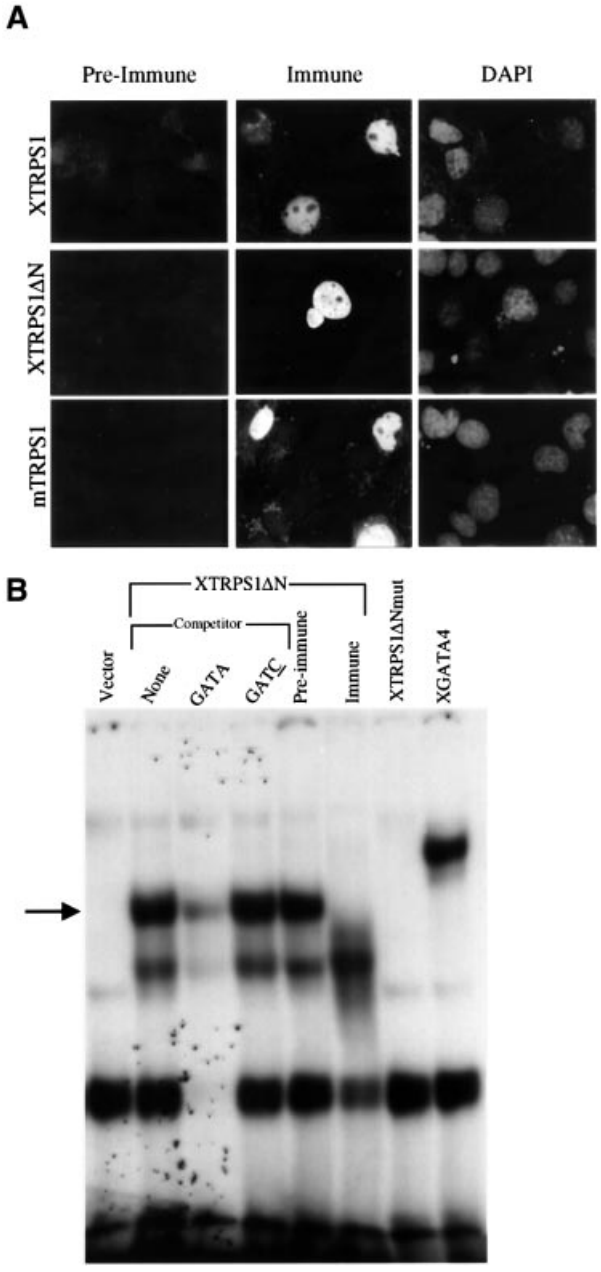


Fig. 2. TRPS1 is a sequence-specific, DNA-binding nuclear protein. (**A**) Subcellular localization by immunofluorescence of COS cells transfected with cDNA constructs encoding full-length XTRPS1 (top), an N-terminal truncated protein (XTRPS1ΔN, middle) or full-length mTRPS1 (bottom). Cells transfected with *Xenopus* or mouse proteins were stained with rabbit antisera directed against *Xenopus* and mouse peptides, respectively. Results with the appropriate pre-immune serum and with 4′-6-diamidine-2-phenylindole (DAPI) nuclear stain are also shown. (**B**) Electrophoretic mobility shift assay (EMSA) of *in vitro* translated XTRPS1ΔN using a double-stranded GATA oligonucleotide probe. Cold competitions are with either the same oligonucleotide or one in which the GATA sequence was altered to GATC. The complex formed with XTRPS1ΔN (arrow) is abrogated in the presence of appropriate antiserum. Full-length XGATA4, and a mutant protein in which two cysteine residues within the XTRPS1 GATA-type zinc finger are altered (XTRPS1ΔNmut), serve as additional controls.

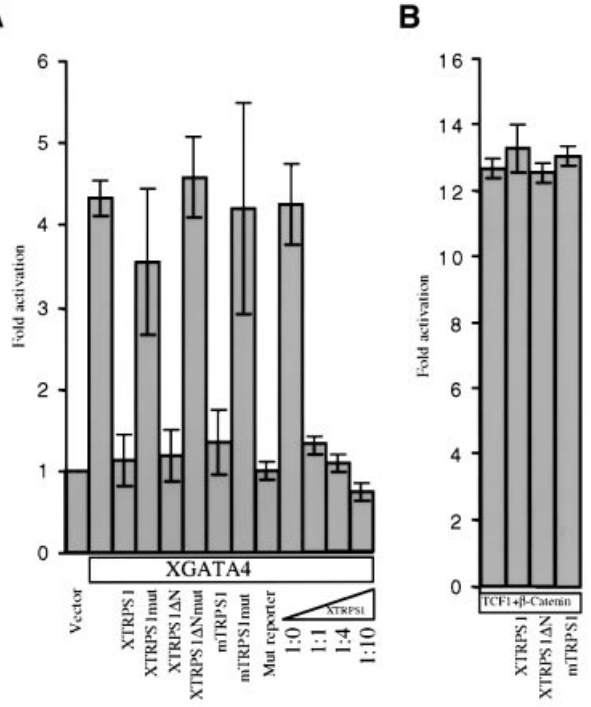


Fig. 3. TRPS1 functions as a sequence-specific transcriptional repressor. Results of transient transfection of COS cells with a GATA-dependent luciferase reporter (**A**) and expression constructs encoding the transcriptional activator XGATA4 either alone or in combination with constructs expressing full-length XTRPS1, the truncated protein XTRPS1ΔN, full-length mTRPS1 and the corresponding mutant proteins XTRPS1mut, XTRPS1ΔNmut and mTRPS1mut, in which the GATA-type zinc finger is disrupted. Mut reporter, a reporter carrying mutated GATA sites. Dose dependence of XTRPS1-mediated transcriptional repression was established by co-transfection of XGATA4 and XTRPS1 plasmids in the indicated ratios. (**B**) TRPS1 fails to repress a GATA-independent reporter that is activated by the combination of a TCF-family protein and β-catenin.

context of GATA *cis*-elements and represents a novel intrinsic activity for a vertebrate GATA factor.

We and others have shown that some of the known GATA proteins induce endoderm differentiation in ectodermal explants of early *Xenopus* embryos (Shoichet *et al*., 2000; Weber *et al*., 2000). This activity is likely to reside downstream of the primitive endoderm inducer Mixer/Mix.3 (Henry and Melton, 1998; Mead *et al*., 1998) and provides an independent assay to verify the transcriptional repression function of TRPS1. We therefore co-expressed TRPS1 and XGATA4 mRNAs in *Xenopus* embryos and assayed ectodermal explants for ectopic expression of the endodermal markers intestinal (IFABP) and liver-specific (LFABP) fatty acid binding proteins and XGATA5 (Shi and Hayes, 1994; Henry and Melton, 1998; Mead *et al*., 1998). XTRPS1ΔN and mTRPS1 potently repress XGATA4-induced expression of each of these lineage markers (Figure 4A and B). This repression relies on an intact GATA-type zinc finger (data not shown) and is also seen when endoderm is induced by Mixer (Figure 4B). Results on representative samples from lanes 3 and 4 in Figure 4A confirm that the PCRs were performed in the linear range of amplification (Figure 4C). Thus, TRPS1 functions as a sequence-specific repressor of GATA-dependent processes in two independent biological assays.

The repressive function of TRPS1 maps to the two C-terminal Ikaros-related zinc fingers

Transcriptional repression by TRPS1 may occur through competition for binding to GATA sites in DNA or,

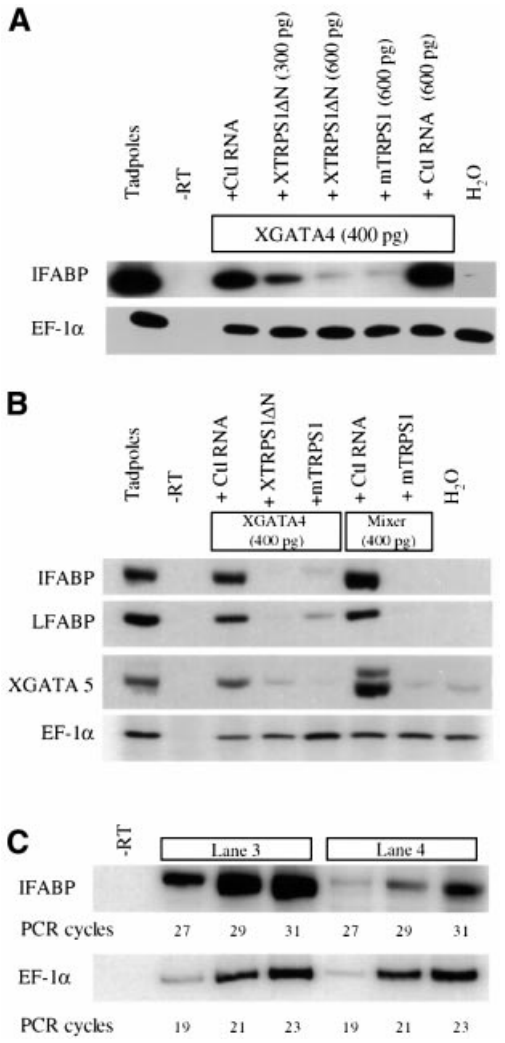


Fig. 4. TRPS1 represses GATA-mediated induction of endoderm in *Xenopus* embryos. RT–PCR analysis of the endodermal marker genes IFABP, LFABP and XGATA5 on explanted *Xenopus* animal caps co-injected with 400 pg of XGATA4 (**A** and **B**) or Mixer (B) mRNA and 300–600 pg of XTRPS1ΔN or 600 pg of mTRPS1 mRNA. Controls include embryos injected with H_2O or with a neutral filler (Ctl) RNA, and RT–PCR analysis for EF-1α and on samples not treated with reverse transcriptase (–RT). Results are representative of four independent experiments. (**C**) PCR on representative samples [lanes 3 and 4 from (A)] confirms that reactions were performed in the linear range of amplification.

alternatively, through an active mechanism that requires additional motifs. Repression by mTRPS1 and XTRPS1ΔN are comparable (Figures 3 and 4), which indicates that the first ~800 amino acids of TRPS1 may be dispensable for this function. To map the repressive domain more precisely, we constructed two C-terminal deletions of 320 (XTRPS1ΔNΔC320) or 119 (XTRPS1ΔNΔC119) amino acids (Figure 5A, constructs 2 and 3). As expected, each resulting protein retains specific binding to the GATA consensus sequence (Figure 5B) but does not itself activate a GATA-dependent reporter (data not shown). Neither protein, including the shorter one which can bind DNA efficiently (construct 2), represses XGATA4-induced activation (Figure 5C). These results point to the requirement for a region contained within the terminal 119 residues.

To verify these results independently, we co-expressed XGATA4 and truncated XTRPS1 mRNAs in *Xenopus* animal caps and examined ectopic endoderm differentiation. Again, neither XTRPS1ΔNΔC119 nor the mutant protein XTRPS1ΔNmut (in which the GATA-type zinc finger is disrupted) represses XGATA4-induced expression of IFABP (Figure 5D). These findings confirm that the repressive function of TRPS1 requires a C-terminal region of, at most, 119 amino acids. This region encompasses a double zinc finger motif found in the Ikaros family of transcriptional regulators (Georgopoulos *et al*., 1997). mTRPS1 shows 45–50% sequence similarity with other Ikaros-type zinc fingers in this domain, over which all the known Ikaros family proteins share >80% similarity with each other (Figure 5E). TRPS1 is the only vertebrate or invertebrate protein known to possess both GATA- and Ikaros-type zinc fingers.

To determine whether this region functions as an autonomous repression module, we created two fusion constructs between the activator XGATA4 and the C-terminal 119 residues of XTRPS1 (Figure 5A, constructs 4 and 5). XGATA4+C119 retains almost the complete sequence of XGATA4, while the control (XGATA4tr+C119) is truncated in the basic region adjacent to the GATA-type zinc finger and so does not bind DNA (Figure 5B). The fusion protein XGATA4+C119 represses GATA4-induced activation to basal levels, whereas XGATA4tr+C119 does not (Figure 5C). Thus, the transcriptional repression activity of TRPS1 is imparted by a conserved C-terminal domain that can override the intrinsic activation capacity of a typical GATA protein such as GATA4.

Developmental expression of mTRPS1

Patients with TRPS types I and III have short stature, hip and phalangeal malformations, characteristic facial anomalies and sparse hair (Langer *et al*., 1984). However, it is not known whether the disease results from TRPS1 expression in the affected tissues or in other sites. Skeletal malformations are manifested much after birth and are progressive, but it is not known whether TRPS1 expression is important principally during development or throughout life. In mouse embryos, TRPS1 mRNA is detected prior to E7.5, with peak levels at around E11.5, and expression in mid-gestation is detected in both visceral and skeletal tissues (data not shown). Although these findings raise the possibility that transient TRPS1 activity may modulate many developmental processes, the highest TRPS1 expression in mid-gestation correlates precisely with sites of documented involvement in the TRP syndromes. mRNA *in situ* hybridization analysis reveals intense TRPS1 expression in the maxilla, mandible and snout at E12.5 (Figure 6A); lateral expression is higher than in the midline (Figure 6B). Expression in visceral organs, detected readily by northern analysis (data not shown), is not revealed by *in situ* hybridization, suggesting that mRNA expression in the developing face is especially high. At E12.5 and E13.5, prominent expression is also observed in the prospective phalanges (Figure 6C) and in the femoral head within the developing hip (Figure 6D). The only other site of high TRPS1 mRNA expression is in the hair follicles (Figure 6E). Northern analysis reveals that TRPS1 mRNA levels in the developing limbs and face

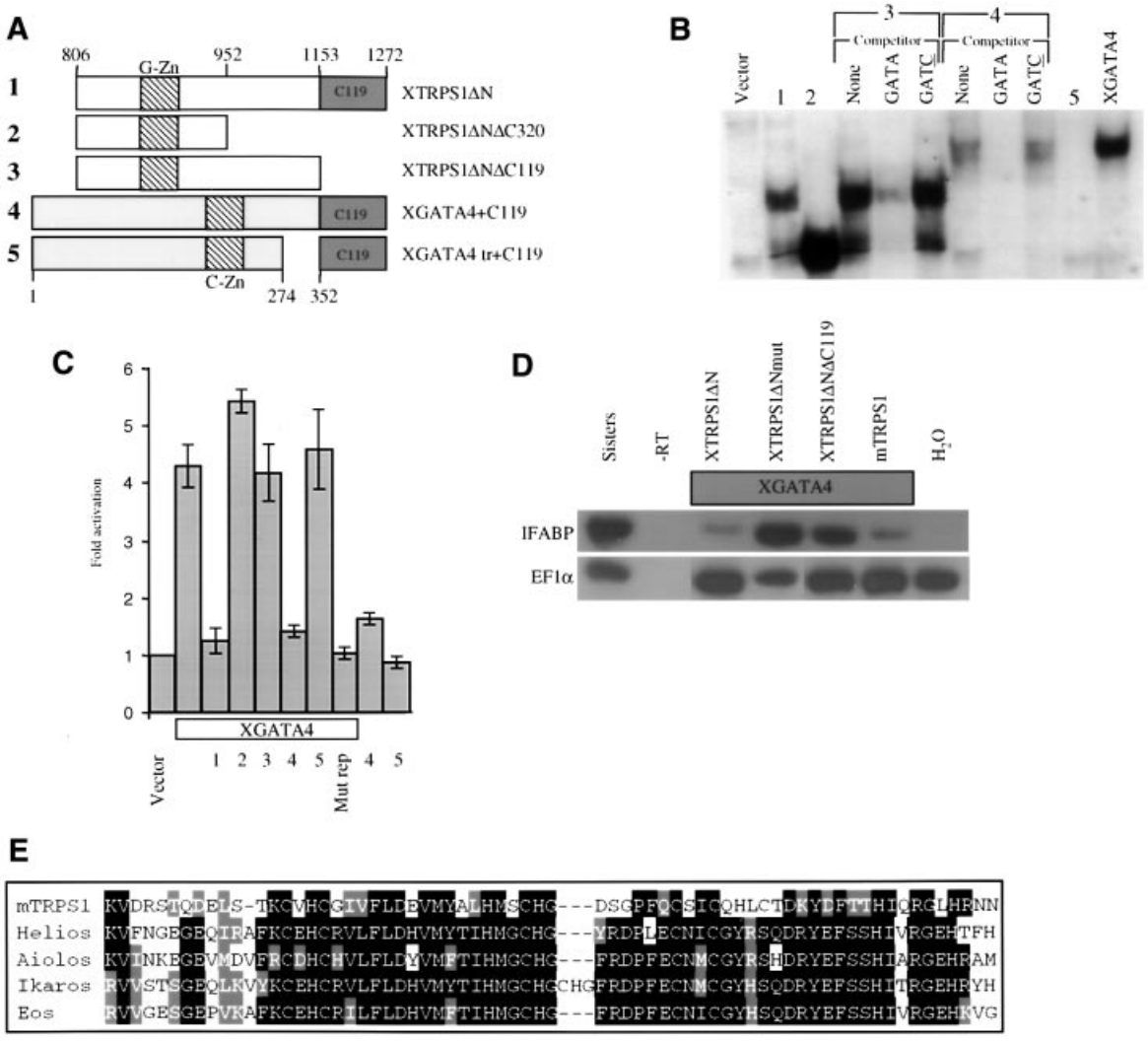


Fig. 5. The repressor domain of TRPS1 maps to a C-terminal region encompassing two Ikaros-type zinc fingers. (**A**) Schematic of expression constructs 1–5 used in these experiments. 1–3 are truncation mutants of XTRPS1; 4 and 5 are fusion constructs between portions of the transcriptional activator XGATA4 and the C-terminal 119 residues of XTRPS1. G-Zn and C-Zn designate the TRPS1 GATA-type zinc finger and the C-terminal zinc finger of XGATA4, respectively. (**B**) EMSA analysis of the proteins encoded by constructs 1–5 using a GATA probe and competitor oligonucleotides as shown in Figure 2. (**C**) Results of transient transfection of COS cells with a GATA-dependent luciferase reporter and either constructs 4 or 5 alone, or constructs encoding XGATA4 either alone or in combination with constructs 1–5. Mut rep, a reporter carrying mutated GATA sites. (**D**) RT–PCR analysis of IFABP expression in explanted *Xenopus* animal caps injected with H_2O or co-injected with XGATA4 (400 pg) and the indicated TRPS1 (600 pg) mRNAs. (**E**) Comparison of the amino acid sequence of mTRPS1 residues 1204–1270 with the C-terminus of each murine Ikaros-family protein. Identical amino acid residues are shaded in black and conservative substitutions in gray.

are highest at E13.5 and decline dramatically thereafter (Figure 6F). These observations strongly suggest that the cardinal features of TRPS result from defects that occur during formative stages in the fetal development of tissues with high TRPS1 expression.

We mapped *mTrps1* by Southern analysis using an interspecific backcross panel (Rowe *et al*., 1994). *mTrps1* is non-recombinant with *D15Mit143*, *D15Wsu126* and *Atox1*, placing it on mouse chromosome 15, 28 cM distal to the centromere (Figure 6G). This region shows extensive conservation with human chromosome 8q, and also a small region of conservation with human 5q (http://www.informatics.jax.org). We confirmed the chromosomal location of the human TRPS1 locus by fluorescent *in situ* hybridization (FISH). The specific probe hybridized to chromosome 8q23–8q24 on metaphase spreads of normal human lymphocytes (Figure 6H, red signal); an alpha-satellite probe specific for chromosome 8 (green signal) is shown as a control. These results are consistent with those from positional cloning of *hTRPS1* (Momeni *et al*., 2000) and with the high conservation between the murine and human *TRPS1* genes (Figure 1).

Mutations in TRPS kindreds encode truncated proteins that antagonize TRPS1 function

Among the six mutations identified originally in families with type I TRPS, five result in truncation of the protein N-terminal to the GATA-type zinc finger and one leads to truncation between the GATA- and Ikaros-type zinc fingers (Momeni *et al*., 2000). All patients retain one wild-type allele, which has been interpreted to suggest that TRPS is a haploinsufficiency syndrome. Our findings raise

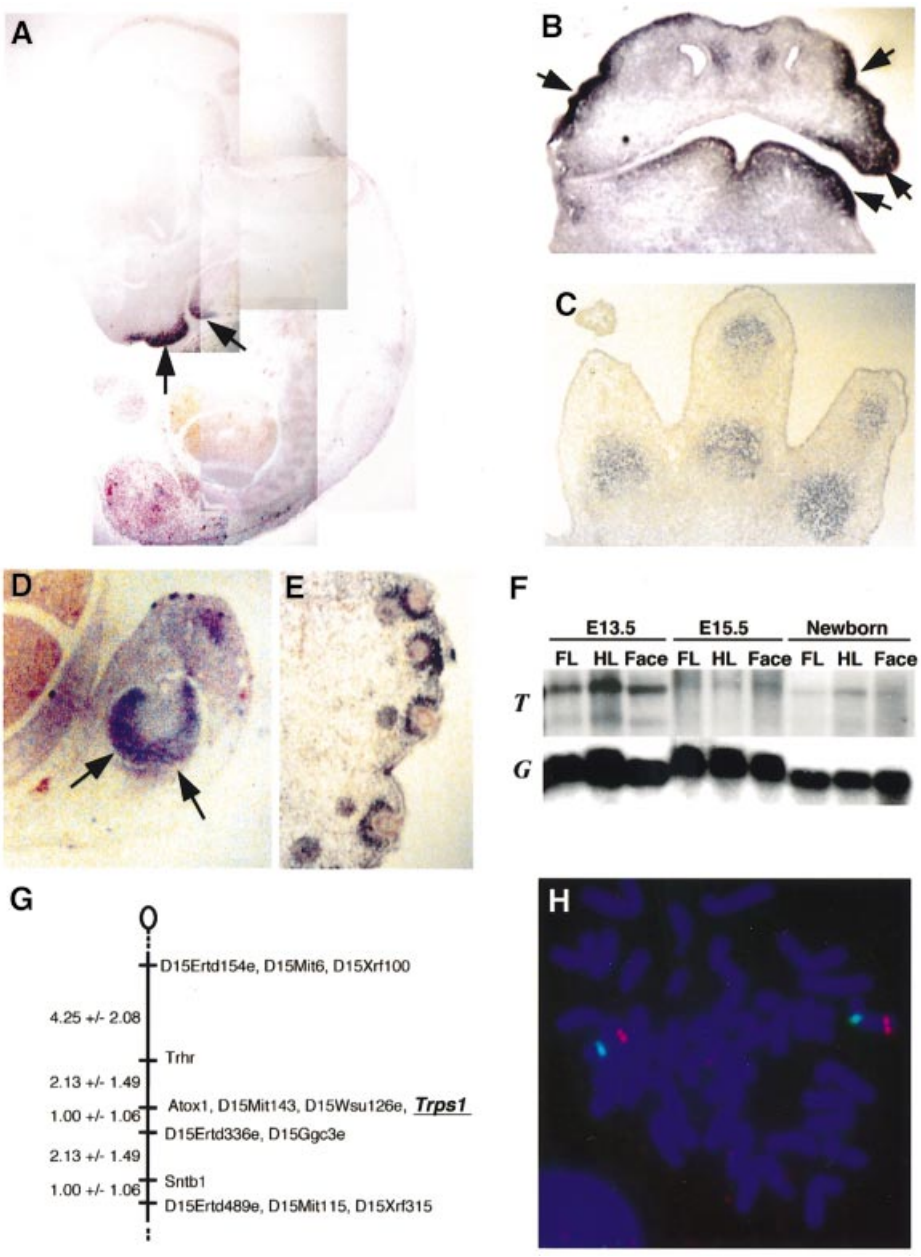


Fig. 6. Correlations between TRPS1 in development and disease. (A–E) mRNA *in situ* hybridization analysis of mTRPS1 expression in whole mouse embryos (**A**), the jaw (**B**, coronal section), digits (**C**), femoral head (**D**) and scalp hair follicles (**E**) at E12.5–13.5. Hybridization with a sense probe yielded no detectable signal (data not shown). (**F**) Northern analysis of mTRPS1 (*T*) or GAPDH (*G*) expression in tissues isolated from mouse fetuses at the indicated gestational age. FL, forelimbs; HL, hind limbs. (**G**) Map from the Jackson Laboratory BSS backcross showing a portion of chromosome 15 with loci linked to *Trps1*, depicted with the centromere at the top. Percentage recombination between adjacent loci (±SE) are indicated to the left and gene symbols to the right. Loci mapping to the same position are listed on the same line. Missing typings were inferred from surrounding data where assignment was unambiguous. Panel data and references for mapping other depicted loci are available at http://www.jax.org/resources/documents/cmdata. (**H**) FISH analysis of hTRPS1 showing localization to chromosome 8q23–24.

the alternative possibility that truncated proteins lacking the GATA- and/or Ikaros-type zinc fingers function as dominant inhibitors of the wild-type protein. To test this possibility, we co-expressed four disease forms of mTRPS1 (P1–P4) together with the wild-type cDNA in each of the available assays for its transcriptional repression function. TRPS1-mediated repression in transfected cells is partially relieved upon co-expression of each of the four truncation mutants we examined (Figure 7A, compare lanes 2, 3 and 4–7); none of these mutants shows independent transactivation (lanes 8–11) or repression (data not shown). We also co-expressed XGATA4 and mTRPS1 in *Xenopus* animal caps together with mRNAs encoding two of the truncated forms. The repressive activity of mTRPS1 is completely abrogated by both P1 and P3, as shown by rescued expression of all three endodermal markers tested (Figure 7B, compare lanes 6 and 4–5). P1 and P3 lack intrinsic repressive activity (lanes 1–3), as predicted. This result does not reflect a trivial effect on the level of injected mTRPS1 mRNA, which is

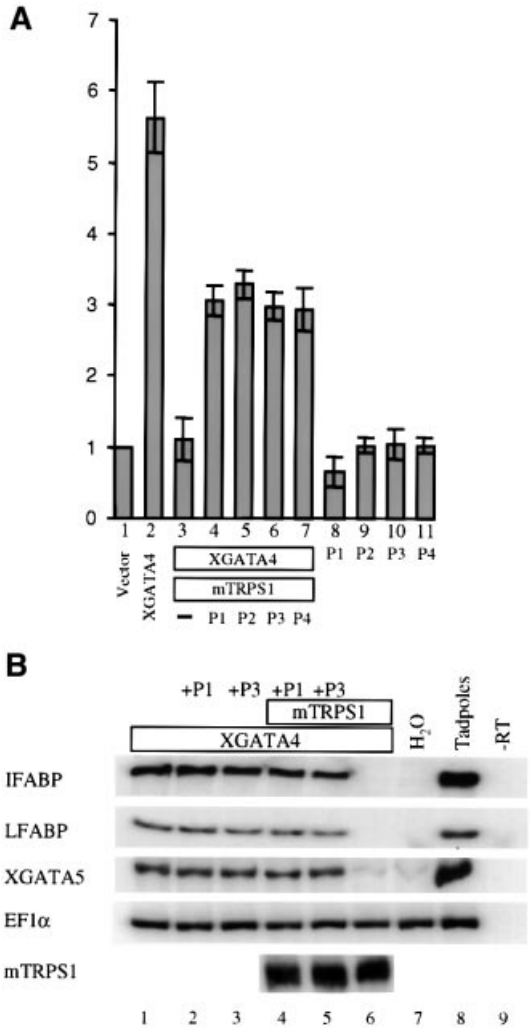


Fig. 7. TRPS1 truncation mutations associated with type I tricho-rhino-phalangeal syndrome function as dominant inhibitors of the wild-type protein. (**A**) Results of transient transfection of COS cells with a GATA-dependent luciferase reporter and XGATA4 plasmid, either alone (lane 2) or in combination with mTRPS1 and the disease isoforms P1–P4 (lanes 3–7). P1–P4 are mutants of mTRPS1 designed to mimic the following truncation mutations identified in TRPS patients (Momeni *et al*., 2000), respectively: C338X, R611X, R840X and a frameshift from codon 1121 that results in premature termination. Lanes 8–11 show that P1–P4 lack intrinsic transactivation capacity. (**B**) RT–PCR analysis of *Xenopus* IFABP, LFABP and GATA5 expression, or the controls EF-1α and mTRPS1 on *Xenopus* animal caps injected with H_2O or co-injected with 300 pg of XGATA4 (lanes 1–6), 500 pg of mTRPS1 (lanes 4–6) and 500 pg of P1 or P3 (lanes 2–5) mRNAs.

detected at the same level in the presence or absence of the disease isoforms P1 and P3 (Figure 7B, bottom panel; the PCR primers used here detect full-length mTRPS1 but not P1 or P3). Our findings hence suggest that the reason for autosomal dominant inheritance of type I TRPS may be that the function of the wild-type allele is effectively antagonized by the truncated proteins.

Discussion

Experimental evidence supports a model in which GATA proteins regulate gene expression by targeting multi-protein complexes to GATA sequences in the *cis*-elements of selected lineage-restricted genes. The previously characterized vertebrate GATA factors contain two zinc fingers of the type that defines this family. The C-terminal zinc finger binds the DNA sequence WGATAR, whereas the N-terminal motif appears principally to mediate protein–protein interactions. It is therefore intriguing that most GATA proteins identified in lower eukaryotes harbor only a single canonical GATA-type zinc finger, yet mediate critical aspects of gene regulation and development (Reuter, 1994; Haenlin *et al*., 1997; Zhu *et al*., 1997; Fukushige *et al*., 1998). This suggests that evolution has exploited several alternative strategies for GATA proteins to interact with other regulators. TRPS1 is the only known vertebrate protein with a single GATA-type zinc finger and has no orthologs in *Drosophila* or *C.elegans*. Phylogenetic analysis of the GATA-type zinc finger (data not shown) further indicates early divergence of TRPS1 from a presumptive ancestral vertebrate GATA protein. Nevertheless, TRPS1 is remarkably conserved between amphibians and mammals, which diverged >350 million years ago, attesting to its likely importance in development and homeostasis.

Although TRPS1 is a nuclear protein that binds DNA with the predicted specificity, it behaves as a transcriptional repressor, an intrinsic function new for any GATA protein. In the one other example of a vertebrate GATA factor that can behave as a context-dependent repressor (Zhang *et al*., 1999), this effect occurs through protein interactions, whereas repression by TRPS1 is critically dependent on an intact DNA-binding domain. Nevertheless, a truncated TRPS1 protein that retains only the GATA-type zinc finger fails to repress transactivation (Figure 5); thus, the repression does not occur through a passive mechanism of competing for binding to GATA sites in DNA. Rather, it requires a C-terminal region that encompasses two zinc fingers closely related to a domain found only in the Ikaros family of lymphoid transcriptional regulators. Within this family, this conserved motif is known to mediate transcriptional repression through protein–protein interactions and to be required for Ikaros to form protein complexes at centromeric heterochromatin, associated with transcriptionally silent genes (Kim *et al*., 1999; Koipally *et al*., 1999). Phylogenetic analysis of Ikaros-type zinc fingers (data not shown) suggests that they also diverged early in vertebrate phylogeny, and implies that TRPS1 is an evolutionarily primitive protein.

TRPS1 is a large, multi-type zinc finger protein that harbors the potential to interact with diverse protein families. However, TRPS1 is not similar to GATA co-factors characterized to date: FOG-1 and FOG-2 (Holmes *et al*., 1999; Deconinck *et al*., 2000; Svensson *et al*., 2000a). Future studies will help define the scope of the relevant protein–protein interactions. Meanwhile, we have shown that several truncating mutations associated with type I TRPS can inhibit TRPS1 repressive activity in a dominant fashion, which suggests that truncated proteins might inactivate other limiting components. Interestingly, even the smallest known disease isoform of TRPS1 (338 amino acids, here designated P1) displays dominant inhibitory activity. This suggests that limiting components of a transcriptional regulatory pathway may interact

physically with the N-terminus of TRPS1. However, our studies also indicate that this region is not essential, because a shorter C-terminal fragment, exemplified by XTRPS1ΔN, is sufficient to repress GATA-mediated transcriptional activation *in vitro* and in *Xenopus* embryos.

The identification of a developmentally regulated GATA protein with the intrinsic ability to repress transcription has interesting implications for understanding networks of developmental gene regulation. Functional GATA sites are abundantly represented in *cis*-elements of many genes whose expression is restricted in space and time, and there appear to be diverse mechanisms to accomplish this restriction. In some cases, the GATA co-factors FOG-1 and FOG-2 function as context-dependent transcriptional repressors (Holmes *et al*., 1999; Deconinck *et al*., 2000; Svensson *et al*., 2000a). We propose that for some GATA-dependent genes, silencing may be achieved in part through mechanisms that involve TRPS1.

The presence of mutations in a single copy of *hTRPS1* in families with dominantly inherited TRPS provides direct evidence for its importance in vertebrate development. TRP syndromes are highly penetrant disorders of short stature, hip and phalangeal malformations, sparse hair and characteristic facial anomalies. Each of the six nonsense mutations identified originally results in deletion of the Ikaros-type zinc fingers and five of these also delete the GATA-type zinc finger; all patients retain one wild-type allele (Momeni *et al*., 2000). A more recent study identified a total of five missense *TRPS1* mutations among 51 unrelated patients; each of these mutations targets the solitary GATA-type zinc finger and is associated with the more severe skeletal anomalies classified as type III TRPS (Ludecke *et al*., 2001). These clinical findings provide an important dimension to the present study, in which we show that the GATA- and Ikaros-type zinc fingers are essential for the activity of TRPS1 as a sequence-specific transcriptional repressor. The sum of the observations indicates that aspects of skeletal, craniofacial and hair development are critically dependent on cell-autonomous fetal expression of an atypical GATA protein that functions as a transcriptional repressor.

Indeed, TRPS1 is expressed most prominently during fetal life and specifically in those structures where patients with TRPS develop pathology. High expression in the developing snout and maxilla correlates perfectly with the uniform clinical findings of a bulbous nose, long and flat philtrum and thin lips. Likewise, prominent expression in the developing phalanges and femoral head indicates that the defects in TRPS patients result from developmental errors that occur selectively in these sites. Although skeletal defects in TRPS progress throughout life (Ludecke *et al*., 2001), we show that TRPS1 expression in the involved locations is limited to a brief developmental window. Hence, clinical manifestations of TRPS1 are likely to reflect the consequences of irreversible defects established during development. Interestingly, TRPS is reportedly not associated with abnormalities of visceral organs, where TRPS1 is expressed in both fetuses and adults. This may reflect either non-essential roles in these sites or clinically silent manifestations of abnormal development and function. The complete spectrum of essential requirements for TRPS1 in mammalian development will be more clear from analysis of mice with homozygous deficiency.

Materials and methods

Molecular cloning of XTRPS1 and mTRPS1

Total RNA from stages 6, 8 and 12 of *Xenopus laevis* embryos was reverse transcribed and amplified by PCR using degenerate primers corresponding to highly conserved GATA-type zinc finger sequences: 5′-CCGGAATTCRMIACIACNYTNTGGMG-3′ and 5′-TGYAAYGCN-TGYGGIYTITAYGGATCCGCG-3′. An ~75 bp amplified fragment was cloned, eight clones were sequenced per stage, and a single novel GATA fragment was identified. Radiolabeled insert from this clone was used to screen 10^6 plaques from a *Xenopus* maternal cDNA library and one λ bacteriophage clone encoding a 3′ fragment of XTRPS1 was isolated. 5′ RACE was carried out using the SMART RACE kit (Clontech, Palo Alto, CA) according to the supplier's protocols. We then used a radiolabeled 1 kb fragment from the RACE product to screen 10^6 plaques from a random hexamer-primed *Xenopus* stage 18 library as above, and isolated a single phage clone corresponding to the 5′ terminus of XTRPS1 cDNA. To assemble a full-length cDNA encoding the maternally expressed form of XTRPS1, these three fragments were cloned into the expression vector pCS (Turner and Weintraub, 1994).

We used XTRPS1 3′ sequences to identify a mouse EST clone and the radiolabeled 400 bp insert to probe a mouse embryonic gut cDNA library, as described previously (Malik and Shivdasani, 2000). The single partial cDNA clone thus isolated was extended by RACE, and full-length mTRPS1 cDNA was assembled by cloning in pCS. The mTRPS1 sequence indicated that the maternally expressed form of XTRPS1 lacks amino acids 362–617, which is present only in the zygotic product in *Xenopus*. To clone this fragment of XTRPS1 we performed RT–PCR on stage 32 embryos using primers GGAAACACCAAGTACTTTCGC and TGTGACTTTATTGTCCAGGTGGAA. Although the amplified fragment, which is highly conserved between *Xenopus* and mammals (Figure 1), allowed us to deduce the full protein sequence of zygotic XTRPS1, we did not assemble a separate expression construct for this isoform; experiments described in this report use either full-length mTRPS1 or the maternal form of XTRPS1.

Plasmid constructs

Xenopus Mixer/Mix.3, β-catenin, GATA4 and human p45 Tcf-1 expression plasmids were gifts from Paul Mead and Xi He (Children's Hospital, Boston, MA), Todd Evans (Albert Einstein College of Medicine, New York) and Hans Clevers (University Hospital, Utrecht, The Netherlands), respectively. XTRPS1ΔN encodes amino acids 806–1272 of XTRPS1, XTRPS1ΔNΔC320 encodes amino acids 806–952, and XTRPS1ΔNΔC119 encodes amino acids 806–1153. These constructs were subcloned in pCS with an in-frame ATG codon using standard techniques. XGATA4+C119 and XGATA4tr+C119 are fusion constructs of XGATA4 (amino acids 1–352 and 1–274, respectively) with the terminal 119 amino acids (1153–1272) of XTRPS1, also in pCS. XTRPS1mut and XTRPS1ΔNmut were generated by PCR with the alteration of two Cys residues within the GATA-type zinc finger to yield the sequence FGANAL in place of FCANCL. Similarly, in mTRPS1mut the sequence VGNAGG is altered to VCNACG. Constructs P1–P4 (Figure 7) are truncation mutants of mTRPS1 that mimic disease isoforms reported by Momeni *et al*. (2000).

Antibodies and immunofluorescence

Antisera against TRPS1 were prepared by serially immunizing rabbits with purified His_6-tagged proteins containing amino acids 427–669 of XTRPS1 and amino acids 301–567 of mTRPS1. Specific reactivity against the immunogens was confirmed by immunoblotting (data not shown).

Proteins were overexpressed in COS cells by transfection of 1 μg of plasmid DNA using 2.5 μl of FuGENE6 (Roche Biochemicals, Indianapolis, IN) on chamber slides. After 24 h the cells were washed with phosphate-buffered saline (PBS), fixed for 20 min in 3% paraformaldehyde in PBS, and incubated sequentially for 5–10 min in 50 mM NH_4Cl in PBS and 0.1% Triton X-100 in PBS with intermittent washes. After blocking for 1 h in 10% goat serum, samples were incubated sequentially for 1 h with the appropriate TRPS1 antiserum or pre-immune serum (1:100) and fluorescein-conjugated goat anti-rabbit IgG (1:1000), each diluted in PBS/5% fetal calf serum. The slides were

then washed, stained for 3 min in DAPI (Sigma), mounted, and photographed under UV microscopy.

Electrophoretic mobility shift assay (EMSA)

Proteins were translated *in vitro* using the above plasmid constructs and the TNT-coupled wheat germ extract system (Promega, Madison, WI); protein identity and sizes were confirmed by immunoblotting using a rabbit antiserum against XTRPS1. EMSAs were performed as described previously (Shoichet *et al*., 2000) in a final volume of 20 µl at 4°C using 1/10 volume *in vitro* translated protein and [γ-^{32}P]ATP end-labeled double-stranded oligonucleotide with the sequence 5′-CTGGGGACAGATAAGCTACAGC-3′. A 100-fold excess of competitor oligonucleotides (GATC, 5′-CTGGGGACAGATCAGCTACAGC-3′) or 1 µl of anti-XTRPS1 or pre-immune serum was incubated with the extracts for 20 min prior to addition of the probe. Reaction products were resolved on 5% polyacrylamide gels at 200 V for 2 h at 4°C.

Transcriptional activation assays

Transient transfection assays were performed in COS cells as described before (Shoichet *et al*., 2000). The αD3 and αD4 (mutant) promoter constructs, provided by Todd Evans (Albert Einstein College of Medicine), contain multiple repeats of the sequence AGATAA or ACTGAA, respectively, upstream of firefly luciferase cDNA. The Tcf-responsive reporter OT (da Costa *et al*., 1999) was provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Transfections were performed in duplicate using FuGENE6 (Roche), 0.025 µg of β-galactosidase plasmid, 0.05 µg of reporter plasmid and 2–4 µg of XGATA4 or TRPS1 expression plasmid or empty vector. Relative luciferase activity was determined by comparing averaged luciferase:β-galactosidase ratios, and was expressed relative to results from control transfections with the empty vector. Results are representative of at least four independent experiments.

Microinjection and embryo manipulations

Capped RNA was transcribed from linearized plasmids *in vitro* using the mMESSAGE mMachine kit (Ambion, Austin, TX) and confirmed by gel electrophoresis. *Xenopus* oocytes were fertilized *in vitro*, dejellied in 3% cysteine, rinsed in 0.1× Modified Marc's Ringer's solution (MMR; 1× MMR is 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES and 0.1 mM EDTA) and transferred to 3% Ficoll in 0.5× MMR. RNA (4.6 nl; ⩽1 ng) was injected into the animal pole at the one-cell stage. Animal caps were dissected between Nieuwkoop–Faber stages 8.5 and 9.5 (Nieuwkoop and Faber, 1967), and expression of proteins corresponding to injected RNAs was confirmed by immunoblot analysis (data not shown). Animal explants were cultured overnight in 0.5× MMR supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin, then transferred to 0.1× MMR, cultured at 21°C until untreated tadpoles reached stages 38–40, and then processed for RT–PCR analysis.

RT–PCR

Total RNA was extracted from *Xenopus* animal explants or whole embryos using RNAzolB (Tel-Test, Friendswood, TX) and reverse transcribed. RT–PCR (annealing temperature 61°C) was carried out in the presence of 0.1 µCi of [α-^{32}P]dCTP to improve sensitivity and the number of PCR cycles was varied to ensure that amplification was in the linear range. PCR products were resolved on 4% polyacrylamide gels. Primers (5′ to 3′) and sizes of the amplified products are: XGATA5 (332 bp), GCCAGACGAATACACCTACAG and AGAAACCCA-AGATACCACCAT; mTRPS1 (350 bp), AGTCTGCATCTCTTACCC-AGC and AGGAGCAGGTCAATGGAAGC; or as reported previously for EF-1α, IFABP and LFABP (Shoichet *et al*., 2000). Normalization was confirmed by RT–PCR amplification of EF-1α or ODC mRNA.

mRNA in situ hybridization

Paraffin-embedded tissue sections (10 µm) were prepared from mouse embryos after overnight fixation in 4% paraformaldehyde at 4°C. Two separate, partially overlapping 1.2 kb digoxygenin (DIG)-labeled antisense probes corresponding to the 5′ UTR and coding sequence of mTRPS1 were prepared with a DIG-labeling kit (Boehringer). Hybridization was carried out at 55°C for 12–16 h, and sections were washed three times in 0.2× SSC at 60°C, then twice in PBS containing 2 mg/ml bovine serum albumin and 0.1% Triton X-100 (PBT) at ambient temperature. Samples were blocked with 10% goat serum (Gibco) in PBT for 1 h, incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer) diluted 1:2000 in PBT and 10% goat serum, and washed three times in PBT. Slides were incubated in NBT/BCIP solution (Boehringer) for 12–48 h, washed, and counterstained with nuclear fast red for 15–45 s.

Northern analysis

Total RNA was isolated from selected fetal mouse tissues, resolved on a 1% formaldehyde–agarose gel, transferred to Duralon-UV membranes (Stratagene, La Jolla, CA), and hybridized overnight at 68°C with a radiolabeled probe corresponding to the mTRPS1 5′ UTR + coding nucleotides 1–1128. Membranes were washed in 0.1× SSC/0.1% SDS, and exposed for autoradiography.

Chromosomal localization

We performed Southern blot analysis using 94 progeny from the Jackson Laboratory BSS interspecific backcross [(C57BL/6JEi × SPRET/Ei)F_1 × SPRET/Ei] (Rowe *et al*., 1994). Segregation of *Trps1* alleles was followed using an *Msp*I restriction fragment length polymorphism (C57BL/6JEi, 6.7 kb; SPRET/Ei, 5.0 kb) and a 300 bp PCR product probe (primers GAGTGAGGTCCTGACAAGCGATAAC and AGACC-ATGAACCGTCTTCTGTTGT). Typing data are deposited in the Mouse Genome Database (accession No. J:66178) and can be accessed at http://www.jax.org. FISH was carried out as described (Kroll *et al*., 2000). A bacterial artificial chromosome (BAC) probe (217G18) containing the gene was isolated by screening the CalTech BAC library (Shizuya *et al*., 1992) by PCR with the primers CCAAGGAGAAAACGAAGGC-ACCACCAAATG and GGGGCCTGCATAGGAACAATGCACAAG, and confirmed by PCR to contain *hTRPS1*. BAC and alpha-satellite centromere probes were labeled with DIG- or biotin-conjugated nucleotides, respectively, using the Bioprime system (Life Technologies, Bethesda, MD) and co-hybridized against normal human lymphocyte metaphase spreads for 16 h at 37°C in the presence of excess human COT-1 DNA (Life Technologies). Slides were washed in 0.5× SSC at 73°C for 5 min and hybridization was detected using rhodamine-conjugated anti-DIG antibody and FITC-conjugated avidin (ONCOR, Gaithersburg, MD).

Accession numbers

Sequence data have been submitted to the DDBJ/EMBL/GenBank database under the accession Nos AF346836, AF346837 and AF346838.

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