The Conserved Glutamine-rich Region of Chick Csal1 and Csal3 Mediates Protein Interactions with Other Spalt Family Members

IMPLICATIONS FOR TOWNES-BROCKS SYNDROME*

Members of the spalt family of zinc finger-containing proteins have been implicated in development and disease. However, very little is known about the molecular function of spalt proteins. We have used biochemical approaches to characterize functional domains of two chick spalt homologs, csal1 and csal3. We show that csal1 and csal3 proteins repress transcription and that they can interact with each other. Furthermore, we found that truncated chick spalt proteins, similar to the truncated spalt protein expressed in the human congenital disorder Townes-Brocks syndrome, affect the nuclear localization of full-length spalt. Our findings have implications for the understanding of Townes-Brocks syndrome and the role of spalt genes in normal development. We propose that truncated spalt can exert a dominant negative effect and is able to interfere with the correct function of full-length protein, by causing its displacement from the nucleus. This could affect the transcriptional repressor activity of spalt and DNA binding. Spalt protein truncations could also affect the function of other spalt family members in various tissues.

This work focuses on the functional analysis of two chick homologs of the spalt family, csal1 and csal3.1 As described previously csal1 is most closely related to human Hsall1 and mouse msall1, whereas csal3 is most closely related to Hsall3 and msall3 (1–7). Spalt proteins are important in a number of developmental processes, cell fate decisions, and organogenesis. Spalt (sal) was first isolated in Drosophila and encodes a protein characterized by multiple double zinc finger motifs of the Cys2H2 type, commonly found in transcription factors (8). Expression of sal is found in various tissues in Drosophila embryos and larvae (8). Early in development sal acts as a region-specific homeotic gene and is required for the specification of head and tail regions (9). At later stages sal regulates pattern formation and cell fate decisions in the wing disc, the trachea, and in sensory organs of the peripheral nervous system (10–14). In addition it was shown recently that sal is regulated by the homeobox gene ultrabithorax (ubx), which can directly repress multiple cis-elements in the sal promoter (15).

Vertebrate sal homologs have been identified in mouse, Xenopus, and Medaka (1, 5, 16–18), where they are expressed in various tissues including central nervous system, heart, pronephros, and limb/fin buds. In human, three homologs of sal have been isolated (3, 19). Mutations in one of these genes, termed Hsall1 or SALL1, which result in a premature truncation and loss of the Cys2H2 zinc finger motifs, cause Townes-Brocks syndrome (TBS),2 an autosomal dominant disorder. TBS is characterized by anorectal, ear, and hand malformations, in particular preaxial polydactyly (2, 20). These observations show that vertebrate sal homologs play important roles in the development of a number of organ systems. In addition, mice deficient in msall1, a homolog of Hsall1, die perinatally from severe kidney failure caused by incomplete ureteric bud outgrowth. However, neither heterozygous nor homozygous mice mimic the TBS phenotype, and it was suggested that msall1 deficiency may be compensated for by msall2 and/or msall3 in mice. Alternatively, the mutant Townes-Brocks protein may exert a dominant negative effect and eliminate the function of all spalt proteins in humans (21).

The structure of spalt proteins suggested that they may act as transcriptional regulators. This was supported by the finding that a highly related Drosophila protein spalt-related (salr), can bind to DNA in vitro (22). More recently it was shown that fusion proteins of mouse sall1 and human SALL1 with the GAL4 DNA binding domain suppress transcription of a GAL4 reporter plasmid. This repression required the amino-terminal single zinc finger, which, in case of msall1, can bind to histone deacetylase (23, 24).

We have used in vitro biochemical approaches to elucidate further the molecular function of spalt proteins. We demonstrate here that chick spalt proteins, csal1 and csal3, repress transcription from a reporter plasmid. The proteins physically interact with each other in cultured cells, and the conserved glutamine-rich region located within the amino-terminal third

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§ The second chick homolog, which we previously named csal2, will now be called csal3 because it is most closely related to human Hsall3 (3, 6). In this article we have used the acronym “H” to indicate human and “m” to indicate mouse before the acronym sall for spalt-like. The nomenclature committee for the human and mouse genome consider SALL for the human and Sall for the mouse genes as sufficient.

2 The abbreviations used are: TBS, Townes-Brocks syndrome; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; GALADBD, GAL4 DNA binding domain; HA, hemagglutinin; HEk, human embryonic kidney; HH, Hamburger and Hamilton; HSV, herpes simplex virus; TK, thymidine kinase, TRITC, tetramethylrhodamine isothiocyanate.

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of spalt proteins is necessary for this interaction. We also demonstrate that "TBS-like" truncated chick proteins can still interact with wild-type protein and with each other. We show that truncated csal1 protein is localized to the cytoplasm of cells, whereas the full-length protein is exclusively nuclear. In addition, in the presence of truncated spalt proteins, nuclear localization of csal1 is abrogated. Together these findings provide new evidence for the molecular mechanism underlying TBS.

EXPERIMENTAL PROCEDURES

Probes and in Situ Hybridization—Digoxygenin-UTP-csal1 and FITC-UTP-csal3 antisense RNA probes were generated, and in situ hybridization was carried out as described previously (4, 6). Probes were detected separately, and anti-digoxygenin antibody coupled to alkaline phosphatase (Roche Molecular Biochemicals) was developed with 5-bromo-4-chloro-3-indolyl-phosphate (Roche) at 350 μg/ml. Embryos were then washed extensively, fixed in 4% paraformaldehyde, heat treated at 65 °C, and anti-FITC antibody coupled to alkaline phosphatase (Roche) was detected with Magenta Phos (Sigma) at 175 μg/ml.

Plasmid Constructs—To generate an in-frame fusion with the GAL4 DNA binding domain the open reading frames of csal1 were subcloned into p1012GAL4 using HindIII and XhoI, and csal3 was subcloned using EcoRI and XhoI. The cloning vector, p1012GAL4 and the reporter plasmids, pG5TKCAT and pG0TKCAT, were a gift from Dr. Neil Perkins, University of Dundee (25).

To construct HA- and FLAG-tagged spalt proteins we performed PCR mutagenesis and replaced the internal stop codon with an XhoI site for cloning into pCMV1-HA or pCMV1-FLAG (26). Truncations and deletions were generated using site-directed mutagenesis to introduce internal XhoI sites by PCR into the tagged spalts. PCR products were digested with DpnI (Roche) and transformed into competent DH5α E. coli using standard protocols. Plasmids were digested with XhoI and the vector religated to generate tagged truncations.

Cell Transfections and CAT Assays—HEK293 cells were transfected with 12.5 μg of the reporter plasmid and 2.5 μg of the GAL4 fusion construct by calcium phosphate and CAT assays carried out as described (27, 28). Briefly, protein extract of transfected cells was prepared in lysis buffer (0.25 M Tris, pH 7.8) by repeated freeze-thaw cycles, protein concentration was determined using the Bradford method (Bio-Rad), and equal amounts of protein (10 μg) were incubated with [3H]chloramphenicol and acetyl-CoA for 20 min at 37 °C. Reaction products were separated on a silica TLC plate with 90% chloroform and 10% methanol; after drying the plates conversion of [3H]chloramphenicol to [acetyl-3H]chloramphenicol was quantitated using a Molecular Dynamics PhosphorImager.

Immunoprecipitation—HEK293 cells were transfected with 10 μg of each plasmid (27). After 48 h protein was extracted with Nonidet P-40 lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Igepal) in the presence of complete protease inhibitor (Roche) by incubating on ice for 20 min and centrifugation at 13,000 rpm for 5 min (29). Protein extracts were preclariyed with 15 μl of protein A-agarose (Sigma) for 20 min at 4 °C and then immunoprecipitated with 10 μl of either anti-HA.11 (Babco) or anti-FLAG M2 (Sigma) and shaken for 1 h at 4 °C. After this, 30 μl of protein A-agarose was added and shaken for 1 h at 4 °C. The protein A-agarose pellet was washed four times in 1 ml of Nonidet P-40 lysis buffer and boiled in 20 μl of Laemmli buffer before SDS-PAGE separation and Western blotting. Western blots were performed with the same antibodies and detected with either a secondary anti-mouse IgG-horse-radish peroxidase (Jackson) or protein A-horseradish peroxidase (Sigma) using the enhanced chemiluminescence detection reagent (Amer sham Biosciences).

Immunofluorescence and Microscopy—COS-7 cells were grown on coverslips and transfected with 5 μg of each plasmid by calcium phosphate (27). After 48 h cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and proteins were detected with primary antibodies, anti-HA.11 (Babco) or anti-FLAG polyclonal (Sigma), and anti-mouse-FITC and anti-rabbit–TRITC secondary antibodies (Jackson). Immunostaining was analyzed using a Zeiss Axiosplan2 and a Leica TCS SP2 confocal microscope.

RESULTS

Coexpression of csal1 and csal3 in Limb Development—We have shown previously that csal1 and csal3 are expressed during limb development (4, 6). In the present study we have used double in situ hybridization to determine the extent to which csal1 and csal3 transcripts might be coexpressed. Both chick spalt genes were detected in developing wing and leg buds from Hamburger-Hamilton (HH) (30) stages 23–25 (Fig. 1, A–D). At HH23, csal1 and csal3 transcripts were both expressed in the distal most region of limb buds with a slightly broader expression of csal1 (Fig. 1A). Subsequently, csal3 expression became more highly restricted, and both transcripts overlapped in posterior limb bud mesenchyme (Fig. 1, B and C). At HH25, csal3 expression began to decrease, and faint purple staining was identified in a patch of mesenchyme cells (Fig. 1D, white line tracing). Transcripts of both genes were also detected in other areas of the embryo, in particular the developing neural tube and the tail bud (Fig. 1C). This raises the possibility that csal1 and csal3 could interact functionally during development.

GAL4 Fusions of Csal1 and Csal3 Mediate Transcriptional Repression—It was demonstrated recently that fusion proteins of both human and mouse sall1 with the GAL4 DNA binding domain (GAL4DBD) can repress transcription from a GAL4 responsive reporter plasmid (23, 24). To assess whether chick spalt proteins can also function as transcriptional repressors, we performed similar experiments and fused the GAL4DBD to the amino-terminal end of chick csal1 and csal3. These plasmid constructs were cotransfected into HEK293 cells along with a reporter construct, G5TKCAT, which contains five GAL4 binding sites in front of the HSVTK promoter driving CAT gene expression (25). CAT activity was quantitated and compared with the CAT activity obtained with the plasmid vector containing only the GAL4DBD, which was set to be 100%. This showed that, similar to human and mouse sall1, csal1-GAL4DBD and csal3-GAL4DBD fusion proteins can repress transcription, 4-fold and 6.5-fold, respectively (Fig. 2, left two columns). To test whether transcriptional repression depends on DNA binding we cotransfected GAL4 spalt fusion proteins with a reporter plasmid lacking GAL4 binding sites (G0TKCAT; Fig. 2, right two columns). In this situation we observed an ~2-fold reduction in transcription activity.

Physical Interactions of Csal1 and Csal3—We next wanted to investigate whether csal1 and csal3 can interact to form higher order structures. We generated HA- and FLAG-tagged versions of both chick spalt proteins (Fig. 3). HEK293 cells were cotransfected with these constructs, and immunoprecipitations were performed using protein extracts followed by Western blot analysis. As a control, protein extracts from cells transfected with either csal1-HA or csal3-FLAG were immunoprecipitated with both anti-HA and anti-FLAG antibodies. In neither case did we observe nonspecific cross-reactivity, and protein was detected on a Western blot only after immunoprecipitation with the appropriate antibody (Fig. 4A, lanes 3 and 6).
found that immunoprecipitation of csal1-FLAG with an anti-FLAG antibody resulted in coprecipitation of csal1-HA-tagged protein, demonstrating a physical interaction between multiple csal1 proteins (Fig. 4B, lane 6). Similarly, immunoprecipitation of csal3-FLAG with an anti-FLAG antibody resulted in coprecipitation of csal3-HA-tagged protein, suggesting that csal3 forms homo-oligomers (Fig. 4C, lane 6). Because csal1 and csal3 are coexpressed in limb development we next examined whether they could interact with each other. Using protein extracts of cells transfected with csal1-HA and csal3-FLAG expression constructs we could detect csal1-HA protein after immunoprecipitation of csal3-FLAG, demonstrating that csal1 and csal3 can interact in HEK293 cells (Fig. 4D, lane 6).

The Glutamine-rich Domain of Spalt Mediates Protein Interactions—To identify domains in csal1 and csal3 which can mediate spalt protein interactions, we generated truncated versions of the proteins and tested their ability to interact in communoprecipitation assays (Fig. 3, A and B). Initially we produced HA-tagged versions of csal1 containing amino acids 1–806 and 1–290. Both of these truncations retain the amino-terminal single zinc finger and the glutamine-rich region but lack some or all of the C\_\text{2H}\_\text{2} double zinc finger motifs. Mutations in Hsall1 which cause TBS result in the loss of all C\_\text{2H}\_\text{2} double zinc finger motifs (2). Immunoprecipitation of csal1-1–806-HA with an anti-HA antibody resulted in coprecipitation of full-length csal1-FLAG (Fig. 5A, lane 6), and immunoprecipitation of csal1-1–290-HA with an anti-HA antibody resulted in coprecipitation of full-length csal1-FLAG, demonstrating that the first 290 amino acids are sufficient to mediate interactions with full-length csal1 (Fig. 5A, lane 5). Similarly, immunoprecipitation of csal3-3HA with an anti-HA antibody resulted in coprecipitation of the truncated csal3-1–282-FLAG protein, indicating that amino acids 1–282 of csal3 are sufficient to interact with full-length csal3 (Fig. 5B, lane 4). Next we investigated whether csal1-1–290 and csal3-1–282 can interact with each other. We found that immunoprecipitation of csal1-1–290-HA resulted in coprecipitation of csal1-1–290-FLAG and csal3-1–282-FLAG, respectively (Fig. 5C, lanes 5 and 6). Together these experiments demonstrate that TBS-like amino-terminal truncations of csal1 and csal3 are sufficient to mediate interactions with full-length proteins and with each other.

We generated further deletions of both csal1 and csal3 to identify the domain within the amino terminus which mediates physical interactions between chick spalt proteins. HA-tagged csal1 truncations were generated, encompassing amino acids 1–65, 1–218, 1–240, and FLAG-tagged csal3 truncations were generated encompassing amino acids 1–85, 1–230, and 1–252. As depicted in the schematic representation csal1-1–65 and csal3-1–85 contain only the amino-terminal single zinc finger motif, csal1-1–218 and csal3-1–230 contain in addition the intervening region up to the glutamine-rich domain, and csal1-1–240 and csal3-1–252 terminate immediately after the glutamine-rich domain. We also generated a FLAG-tagged csal3 construct with an internal deletion of the glutamine-rich domain, termed csal3-ΔQ-FLAG (Fig. 3B). Immunoprecipitations followed by Western blot analysis demonstrated that only csal1-1–240 and csal3-1–252 proteins were able to interact with csal1 and csal3 proteins. Immunoprecipitation of csal1-1–240-HA resulted in coprecipitation of csal1-1–290-FLAG (Fig. 6A, lane 6). In contrast, immunoprecipitation of either csal1-1–218-HA or csal1-1–65-HA did not result in coprecipitation of csal1-1–290-FLAG (Fig. 6A, lanes 7 and 8, respectively). Furthermore, immunoprecipitation of csal1-1–240-HA resulted in coprecipitation of csal3-1–282-FLAG (Fig. 6B, lane 6). As expected, immunoprecipitation of csal1-1–218-HA and csal1-1–65-HA did not result in coprecipitation of csal3-1–282-FLAG (Fig. 6B, lanes 7 and 8). In similar experiments we found that
only csal3-1–252 was able to interact with other spalt proteins (Fig. 6, C and D). Immunoprecipitation of csal3-HA resulted in coprecipitation of csal3-1–252-FLAG (Fig. 6C, lane 6). In contrast, immunoprecipitation of csal3-HA did not result in coprecipitation of either csal3-1–230-FLAG or csal3-1–85-FLAG (Fig. 6C, lanes 7 and 8, respectively). The bands migrating at a slightly higher molecular mass to csal3-1–252 observed in Fig. 6, C and D, lanes 5–12, are caused by detection of IgG light chains with protein A and are also seen in mock transfected controls (Fig. 6, C and D, lanes 5 and 9). Immunoprecipitation of csal1-1–290-HA resulted in coprecipitation of csal3-1–252-FLAG (Fig. 6D, lane 6) but not csal3-1–230-FLAG or csal3-1–85-FLAG (Fig. 6D, lanes 7 and 8, respectively). Finally, immunoprecipitation of csal1-HA does not result in coprecipitation of csal3 protein, which lacks the 22 amino acids encompassing the glutamine-rich region (csal3-3Q-FLAG, Fig. 6E, lane 6). These experiments show that the conserved glutamine-rich domain in csal1 and csal3 is necessary for interaction with other spalt proteins.

Subcellular Localization of Csal1 and Csal3—We examined the subcellular localization of the two spalt proteins in COS-7 cells, transfected with tagged versions of both chick spalt proteins. Using anti-HA or anti-FLAG antibodies and fluorescence microscopy, we detected csal1-HA exclusively in the nucleus (Fig. 7, A, C, and D), whereas csal3-FLAG was found exclusively in the cytoplasm (Fig. 7, F–H). Confocal microscopy confirmed that csal3-FLAG was excluded from the nucleus (data not shown). Interestingly, when csal1-HA and csal3-FLAG were cotransfected we observed a clear change in the subcellular localization of csal1-HA. In the presence of csal3, csal1 was detected in the cytoplasm colocalizing with csal3-
Physical Interactions between Spalt Proteins

Expression of Truncated Spalt Proteins Affects the Localization of Csal1—We coexpressed full-length csal1-HA with csal1-1–290-FLAG to determine what effect this might have on protein distribution. In contrast to full-length csal1, truncated csal1 protein (csal1-1–290) was no longer restricted to the nucleus but instead could be detected throughout the cell (Fig. 7, M–P). When this truncated form of csal1 was cotransfected with full-length csal1 protein we detected full-length csal1 in the cytoplasm with apparently increased levels immediately around the nucleus (Fig. 7, Q–T; compare Q and A). In contrast, the csal1 truncation that lacks the glutamine-rich region (csal1-1–218) was no longer able to relocalize full-length csal1 protein from the nucleus to the cytoplasm (Fig. 7, U–X, compare U, Q, and A). These experiments indicate that TBS-like amino-terminal truncations of spalt proteins alter the normal localization of full-length csal1, and this effect is mediated by the glutamine-rich region. To confirm that the glutamine-rich region is required for relocalization, we cotransfected csal1-HA and csal3-ΔQ-FLAG. Expression of csal3-ΔQ-FLAG was observed throughout the cell, including expression in the nucleus. However, expression of csal3-ΔQ-FLAG did not result in a change of csal1-HA localization, which remained exclusively nuclear (Fig. 7, Y–B′, compare B′ and L).

**DISCUSSION**

The experiments presented here demonstrate for the first time that spalt proteins can interact with each other. The conserved glutamine-rich domain is necessary to mediate protein interactions of csal1 and csal3, two chick spalt family members. Furthermore, we determined the subcellular distribution of tagged csal1 and csal3 proteins and found that the localization of full-length csal1 is altered in the presence of csal3 or truncated forms of csal1. Relocalization required the conserved glutamine-rich domain. We discuss our findings and their implications for spalt function during normal embryogenesis and in TBS.

Structural Motifs of Spalt Proteins—The structure of spalt proteins, in particular the presence of multiple zinc finger motifs of the C2H2 type, suggests a role as a transcription factor. This is supported by the finding that Drosophila spalt-related protein can bind DNA and by the recent evidence demonstrating the ability of human and mouse sall1 to repress transcription from a heterologous reporter (23, 24). Transcriptional repressor activity of sall1 is mediated by the amino-terminal zinc finger, which was shown to recruit histone deacetylase (24). This is in contrast to human sall1, where transcriptional repression was observed with GAL4DBD fusions, but this was independent of histone deacetylase (23). The amino-terminal zinc finger domain is highly conserved in all vertebrate members of the spalt family described so far, suggesting that transcriptional repression may be a common property of different spalts from different species. Consistent with this, we found that both csal1 and csal3 repress transcription. However, our results suggest that only part of the sal-mediated transcriptional repression activity depends on direct binding to the active promoter. In addition, there may be a contribution that is independent of DNA binding. The mechanisms underlying this phenomenon need further investigation, ideally in the context of a native target promoter of sal proteins, which has yet to be identified.

We found that csal3 is expressed in the cytoplasm rather than in the nucleus of transfected COS-7 cells. This seems to
Fig. 7. Subcellular localization of csal1 is affected by csal3 and by truncated csal1 proteins. Immunofluorescence of COS-7 cells transfected with tagged proteins, csal1-HA (A–D), csal3-FLAG (E–H), csal1-HA and csal3-FLAG (I–L), csal1-1–290-FLAG (M–P), csal-HA and csal1-1–290-FLAG (Q–T), csal1-HA and csal1-1–218-FLAG (U–X), csal1-HA and csal3-FLAG (Y–B’). Cells were stained with an FITC-coupled anti-HA antibody (A, E, I, M, Q, U, and Y), or a TRITC-coupled anti-FLAG antibody (B, F, J, N, R, V, and Z), or with 4,6-diamidino-2-phenylindole (C, G, K, O, S, W, and A’). Merged images are shown in D, H, L, P, T, X, and B’. The areas in which HA-tagged and FLAG-tagged proteins overlap appear in yellow. In all transfections equal amounts of each plasmid were used (5 µg).

rule out a role as a transcriptional repressor in vivo. However, it is conceivable that stimulation of cells with an appropriate growth factor could cause translocation of csal3 into the nucleus where it could act on DNA. Alternatively, the role of csal3 could be to act as a negative regulator of csal1 function. In cells coexpressing both csal1 and csal3 we observed that csal1 is cytoplasmic and the removal of csal1 from the nucleus could provide a mechanism for abrogating its functions. These explanations are not exclusive, and csal3 could regulate csal1 by its effect on subcellular localization and also have a role as a transcriptional repressor in its own right.

The glutamine-rich region is highly conserved in all invertebrate and vertebrate spalt family members isolated so far, but its function has never previously been characterized. Our experiments demonstrate that this domain is required for interaction of csal1 and csal3 with themselves and with other spalt proteins. The glutamine-rich region is sufficient to mediate spalt interactions, when present within the amino terminus. Furthermore, in the absence of this domain, we do not observe any interactions between the spalts, demonstrating that this domain is necessary. It is unclear at present whether glutamine-rich motifs of two spalt molecules bind directly to each other or to another motif within the amino terminus or whether they mediate binding to an unrelated protein, which will then in turn interact with another spalt molecule.

Role of Spalt Proteins in Development—We showed previously that the onset of csal1 expression at HH17 in wing and leg bud precedes that of csal3. During subsequent stages of limb bud outgrowth up to HH25, csal1 expression is restricted to the distal third of wing and leg bud and the apical ectodermal ridge. After HH26, csal1 expression was no longer detected in limb buds. In contrast, csal3 is only transiently expressed in HH18 wing buds, expression can be detected again from HH23–HH26 in a region of posterior mesenchyme (4, 6). Here we showed csal1 and csal3 transcripts overlap in a distinct region of the developing limb buds (Fig. 1), and full-length csal1 and csal3 proteins interact in coimmunoprecipitation experiments (Fig. 4D). These observations raise the distinct possibility that csal proteins may also form homo- and heterodimers in vivo and interact functionally during embryogenesis to pattern the developing limb.

We showed here that csal1 and csal3 show a distinct subcellular distribution with csal1 being mainly nuclear and csal3 mostly found in the cytoplasm. Although this localization to separate compartments of the cell seems to preclude a physical interaction in vivo we found that the distribution of csal1 changes in presence of csal3 (Fig. 7). Thus it is interesting to speculate that in embryonic development csal3 protein may act as a modifier of csal1 by altering csal1 localization and consequently affecting its function in the nucleus. Both chick spalt genes are coexpressed in a subset of limb mesenchyme cells, and limb development is affected in patients with TBS.

Implications for Townes-Brocks Syndrome—Expression of a truncated Hsall1 protein in the human congenital syndrome TBS has indicated an important role for spalt in normal development. To date it is unclear whether the complex phenotype in
TBS is caused by haploinsufficiency or a dominant negative effect of the truncated protein. Interestingly, mouse embryos with homozygous deletions of csal1 have apparently normal limbs and show no sign of the polydactyly and other phenotypes that are typically seen in TBS patients. Instead they display a severe kidney phenotype. The heterozygous mice appear normal (21), and this finding argues against haploinsufficiency as the underlying mechanism. Instead, the TBS gene product may act in a dominant negative manner. In this scenario, it would not be surprising that complete loss of msall1 function does not mimic the TBS phenotype because other members of the family may be able to compensate.

There is growing evidence that the Townes-Brocks protein retains important functional domains (23, 24, and this report), and this is more consistent with the possibility that the TBS phenotype is caused by dominant negative interference with the normal function of other proteins. The truncated Hsall1 proteins expressed in TBS patients are equivalent to csal1-1–290 and csal3-1–298. All proteins include the most amino-terminal single zinc finger as well as the glutamine-rich region, whereas they have lost all other zinc finger motifs (2, Fig. 3). Our experiments show that csal1-1–290 and csal3-1–298 can still interact with full-length and truncated forms of csal1 and csal3 in coprecipitation assays (Figs. 5 and 6). In addition, both truncations localize to the cytoplasm and affect the nuclear localization of full-length csal1 in COS-7 cells (Fig. 7). Thus our experiments strongly suggest that the mutant Hsall1 proteins expressed in TBS patients may retain the ability to interact with other members of the spalt family. Therefore, the phenotype may in part be the result of a functional interference of truncated Hsall1 protein with different spalt proteins in different tissues.

There are other possible mechanisms by which a dominant negative effect might be created. As described in this paper, truncated csal1 has the ability to relocate full-length csal1. Second, truncated spalt may sequester corepressors, such as histone deacetylase, or it may form inactive complexes unable to bind DNA. All of these could have a role in TBS, and further work is required to establish which of these mechanisms are operating.

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The Conserved Glutamine-rich Region of Chick Csal1 and Csal3 Mediates Protein Interactions with Other Spalt Family Members: IMPLICATIONS FOR TOWNES-BROCKS SYNDROME

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