Polyalanine expansion mutations in the X-linked hypopituitarism gene SOX3 result in aggresome formation and impaired transactivation

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1. ABSTRACT

Polyalanine expansion mutations have been identified in eight transcription factors that are associated with a range of congenital disorders. While some of these mutant proteins have been shown to generate cellular aggregates in heterologous cell lines, little is known about the mechanism by which these aggregates cause disease. Here we examine the aggregation and functional properties of the two known polyalanine expansion mutations associated with X-linked Hypopituitarism (XH), SOX322Ala and SOX326Ala, which contain an additional seven and eleven alanine residues, respectively. SOX322Ala and SOX326Ala proteins form cytoplasmic aggregates and nuclear inclusions in transiently transfected COS-7 and CHO K1 cells, and in transfected explant cultures of chick neural epithelium. SOX326Ala exhibits a more potent aggregation phenotype, resulting in significantly more cells with dispersed cytoplasmic and large perinuclear aggregates. SOX322Ala and SOX326Ala protein aggregates exhibit the key properties of aggresomes including vimentin redistribution, colocalisation with the Microtubule Organising Centre and sensitivity to microtubule disruption. This is the first time that aggresomes have been implicated in the aetiology of a polyalanine expansion disorder, suggesting that XH and protein conformation disorders may become manifest through similar pathological mechanisms. Further, we show that mutant SOX3 proteins have impaired transcriptional activity and reduced capacity to inhibit β-catenin/TCF-mediated transcription. These data suggest that deregulation of SOX3 target genes and inappropriate canonical Wnt signaling in central nervous system (CNS) progenitors may also contribute to dysfunction of the hypothalamic-pituitary axis in XH patients.
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2. INTRODUCTION

Many human conditions, collectively described as Protein Conformational Disorders (PCDs), are associated with the accumulation of misfolded proteins. Some of the best characterised examples of PCDs are neurodegenerative disorders such as Huntington’s disease and the Spino cerebellar ataxias which result from expansions in polyglutamine tracts (1). In these disorders, polyglutamine expansion is thought to promote the generation of misfolded intermediates which ultimately form aggregates in specific neuronal sub-types (2). Recently, it has become clear that repeat expansions in other single amino acid tracts can also cause disease. Nine human disorders have so far been shown to result from expansions in polyalanine tracts (3). All but one of these mutations occur in transcription factors and give rise to congenital malformations affecting many structures. The only polyalanine tract expansion disorder not caused by mutation of a transcription factor, oculo-phygeal dystrophy (OPMD) syndrome, results from expansions in the PABPN1 protein which is involved in mRNA polyadenylation (4). Recent expression studies of mutant polyalanine expansion proteins have demonstrated that these mutations promote the formation of cellular aggregates which localise to the cytoplasm (HOXD13) (5), the nucleus (ARX) (6) or both (FOXL2) (7). In addition, intranuclear inclusions in muscle tissue are a pathological hallmark of patients with OPMD (8). These studies suggest that aggregation is a key functional consequence of polyalanine expansion mutations. However, it is not clear whether these aggregates have a similar pathogenic role to the cytoplasmic inclusions that are associated with other PCDs.

Overexpression of several proteins associated with PCDs such as Parkin, Huntington and presenilin 1 results in the formation of large perinuclear inclusions termed aggresomes (9-11). These inclusions are characterised by their close proximity to the microtubule organising center (MTOC) and by the redistribution of the intermediate filament proteins, for example vimentin, which forms a cage-like structure around the aggregate (12). Aggresomes are believed to play a role in the processing and clearance of misfolded proteins that accumulate in the cytoplasm due to mutation, proteasomal inhibition or cellular stress. They are generated by dynein-dependent retrograde transport of microaggregates along microtubules (10, 11). Although the properties of aggresomes have principally been established using cultured cell lines, recent evidence suggests that there are similarities between aggresomes and the pathogenic lesions associated with PCDs. For example, the Lewy bodies that form in Parkinson-affected brains have the morphological appearance of aggresomes and contain centrosome components including γ-tubulin (13). Similarly, Parkinsonian Lewy bodies and the hyaline bodies in amyotrophic lateral sclerosis patients are frequently surrounded by Type-IV intermediate filaments (14). These data suggest that similar mechanisms control the formation of aggresomes in cultured cell lines and cytoplasmic lesions associated with PCDs.

SOX3 is an X-linked member of the Sox (Sry-related HMG box-containing) family of transcription factors (15) which have been shown to play a key role in the development of many organs in mice and humans (16). In mice, Sox3 is expressed at high levels in the developing ventral diencephalon, a region that is critical for induction and development of the anterior pituitary primordium (Rathke’s pouch), and contains the presumptive hypothalamus (17, 18). Sox3 expression is also regionally restricted in the developing cortex, telencephalic vesicles and gonads, and in the adult is active in a subset of hypothalamic neurons (18, 19). Generation of Sox3 null mice has shown that this gene is essential for morphogenesis of the hypothalamus and pituitary, and for normal function of the hypothalamic-pituitary axis (19). Sox3/Y male mice display severe dwarfism, multiple pituitary hormone deficiencies, abnormal pituitary morphology and hypocellularity of the hypothalamus. These defects have an embryological origin and are due to abnormal development of the ventral diencephalon, which is hypoplastic and incorrectly patterned, resulting in aberrant induction of Rathke’s pouch. In addition, Sox3 null mutants display variable abnormalities of the CNS including dysgenesis of the hippocampus and corpus callosum, indicating that Sox3 is a key regulator of neuronal differentiation. This is supported by overexpression studies in chick which indicate that Sox3 acting as a transcription activator inhibits the differentiation of neural progenitors (20, 21). Currently, the target genes and downstream signaling events that are regulated by Sox3 in neural progenitors have not yet been identified. However, the Xenopus Sox3 ortholog has been shown to bind to β-catenin and inhibit TCF/β-catenin-mediated transactivation (22), implicating Sox3 in the canonical Wnt signaling pathway. The transcriptional output of this pathway is regulated by the accumulation of nuclear β-catenin which binds to LEF/TCF family proteins and activates target gene expression. Although Wnt signaling is critical for neurodevelopment in mammalian embryos (23), it is not known whether Sox3 regulates Wnt signaling output in this context.

In humans, duplications containing SOX3 have been identified in several families with X-linked Hypopituitarism (XH) (17, 24). This disorder only affects males and is characterised by variable pituitary hormone deficiency and incompletely penetrant mental retardation. Recently, a polyalanine expansion mutation in SOX3 was identified as the causative mutation in a large European pedigree with isolated Growth Hormone deficiency and mental retardation (25, 26). This mutation, a 33 bp in-frame duplication, results in the insertion of an additional 11 alanine residues into the 15 amino acid polyalanine tract (SOX326Ala). A second, shorter expansion mutation (SOX322Ala) in the same polyalanine tract has recently been identified in an XH family with panhypopituitarism and normal cognitive function (17). While epitope-tagged SOX322Ala and SOX326Ala have been shown to mislocalise to the cytoplasm (5,17), the type of aggregates that these mutant proteins generate is not known. Further, the relative aggregation potential of SOX322Ala and SOX326Ala proteins...
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and their propensity to form in developing neuroectoderm (their normal cellular context) has not been investigated. To address these questions, and to determine the functional consequence of polyalanine expansion mutations in SOX3, we expressed untagged and epitope-tagged mutant proteins in heterologous cell lines and developing neuroectoderm. We find that polyalanine expansion in SOX3 leads to the formation of aggresomes, which, to our knowledge, is the first time that these cellular aggregates have been directly implicated in polyalanine expansion disorders. Further, we show that mutant proteins exhibit significantly reduced transcriptional activity and have reduced capacity to inhibit TCF/β-catenin-mediated transactivation. These data provide novel mechanistic insights into the molecular pathology of XH and implicate canonical Wnt signaling and transcriptional dysregulation in the aetiology of this disorder.

3. METHODS

3.1. Plasmid construction

The plasmid pcDNA3.1 SOX3 contains the SOX3 open reading frame (ORF, NM 005634) cloned into pcDNA3.1/myc-His (Invitrogen). The plasmid pcDNA3.1 SOX322Ala carries the SOX322Ala ORF amplified from an affected male (25). The plasmid pcDNA3.1 SOX326Ala was constructed via site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Stratagene) of pcDNA3.1 SOX326Ala using the following primers: 5’ CAG CGG CCT GCC TCC CGG TCG CGG CGC GGC ACC GGG AGA CGG GC 3’ and 5’ GC GGC CGC GGC AGC GGC GGC C 3’. To produce SOX326Ala, 0.5 µg of pcDNA3.1/myc-His SOX3, pcDNA3.1/myc-His SOX322Ala and pcDNA3.1/myc-His SOX326Ala PCR products from pcDNA3.1 SOX3, pcDNA3.1 SOX322Ala, or pcDNA3.1 SOX326Ala were amplified using the primers N2 Fwd 5’ CCG GAA TTC TCA GAT GTG GGT CAG CG 3’ and C1 Rev 5’ CGG GAA TAC CTA GTG GTG CCT CGG A 3’ and ligated to pcDNA3.1/myc-His. The pcDNA3.1/V5-His SOX3, pcDNA3.1/V5-His SOX322Ala and pcDNA3.1/V5-His SOX326Ala plasmids were TA cloned using PCR products from pcDNA3.1 SOX3, pcDNA3.1 SOX322Ala or pcDNA3.1 SOX326Ala using the primers Sox3 Fwd 5’ ATG CGG CCT GCC TCC CGG TCG CGG CGC GGC ACC GGG AGG CAG GAG GCC GCT G 3’ and Sox3 Rev-stop 5’ GAT GCC TCC CGG CAC CGG TGC CGC CGC TGC CGC GGC C 3’. The plasmids pMES SOX3, pMES SOX322Ala, and pMES SOX326Ala were made by ligating the EcoRI digested ORFs from pcDNA3.1 SOX3, pcDNA3.1 SOX322Ala, or pcDNA3.1 SOX326Ala to EcoRI digested pMES (27). All plasmids were sequenced to verify identities.

3.2. Aggregation Assays

COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). CHO-K1 cells were grown in DMEM, 10% FCS, supplemented with proline. COS-7 or CHO-K1 cells were grown in chamber slides (Nunc) and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature, 48 h post-transfection with Lipofectamine (Invitrogen). For the microtubule destabilization experiment, cells were cultured for 32 h post-transfection and subjected to 1 µM nocodazole or DMSO (Sigma) for 16 h before fixing. Cells were permeabilized and blocked with 0.1% Triton X-100 in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA, Sigma) at room temperature for 3 h. The primary antibodies, mouse anti-Myo V (Invitrogen) and mouse anti-V5 (Invitrogen) were diluted 1:250, mouse anti-Vimentin (Sigma) was diluted 1:1500, mouse anti-γ-tubulin (Sigma) was diluted 1:50, and rabbit anti-Sox3 (T. Edlund, Umea University, Sweden) was diluted 1:2000 in 1% BSA, PBS. Cells were incubated with primary antibodies overnight at 4°C. The secondary antibodies, goat anti-mouse IgG Alexa Fluor 488 (1:500 Molecular Probes), goat anti-rabbit IgG Alexa Fluor 488 (1:500 Molecular Probes), or goat anti-mouse IgG Alexa Fluor 594 (1:1000 Molecular Probes) were diluted in 1% BSA, PBS. Cells were incubated with secondary antibodies at room temperature for 3 h. The cells were washed and mounted with Vectashield containing 4',6-Diamidino-2-phenylindole (DAPI, Vector Laboratories, CA). The samples were examined with a fluorescence microscope IXL 70 (Olympus). Each experiment was repeated three times and 300 transfected cells from random fields were analyzed each time.

3.3. Chick Electroporation

Hamburger and Hamilton Stage (HH) 7-8 (3-5 somite, E1.25) chick embryos (Research Poultry, Research, Australia) were electroporated with pMES SOX3, pMES SOX322Ala, and pMES SOX326Ala as described previously (28). Neural explants were cultured on fibronectin coated plates in Ham’s F12 containing 10% FCS for 48 hours at 37°C before fixation with 4% PFA in preparation for immunohistochemistry. Images were captured using a Leica TCS SP2 confocal microscope.

3.4. Transcription and TOPFLASH Assays

Transcriptional activities of SOX3, SOX322Ala and SOX326Ala were determined using the Dual-Luciferase Reporter Assay System (Promega). A total of 1.25 µg of plasmid DNA was transfected into COS-7 cells cultured in 12-well plates using Lipofectamine according to manufacturer’s protocol. All transfections were preformed in triplicate and contained 0.2 µg luciferase reporter SOC M, 0.05 µg of Renilla luciferase plasmid pRL-CMV and 0.1-1.0µg of pcDNA3.1, pcDNA3.1 SOX3, pcDNA3.1 SOX322Ala or pcDNA3.1 SOX326Ala expression constructs (Promega). The firefly luciferase and Renilla luciferase activities were determined after 48 h according to manufacturer’s protocol on a FluorStar Optima (BMG technologies). Relative luciferase activity is the ratio of firefly activities to Renilla activities normalised to pcDNA3.1. Transfections were carried out four times and values are mean ± standard error of the mean (S.E.). For the TOPFLASH assay, HEK 293 cells grown in DMEM, 10% FCS, in a 12-well plate were transfected in triplicate using FuGene6 (Roche) following the manufacturer’s instructions. 0.5 µg pcDNA3.1 SOX3, pcDNA3.1 SOX322Ala or pcDNA3.1 SOX326Ala was transfected along with 0.1 µg β-catenin plasmid, 0.1 µg TOPFLASH, and 0.05 µg pRL-CMV plasmid. Cultures were lysed 48 h post-transfection and firefly luciferase and Renilla luciferase activities were determined as for the transcription
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Figure 1. SOX3\textsuperscript{22Ala} and SOX3\textsuperscript{26Ala} form aggregates in COS-7 cells. COS-7 cells transfected with pcDNA3.1 SOX3, SOX3\textsuperscript{22Ala} or SOX3\textsuperscript{26Ala} expression constructs were immunostained with \(\alpha\)-SOX3 antibody (green) and counterstained with DAPI (blue). Left column: SOX3/Alexa Fluor 488. Middle column: DAPI staining of nuclei. Right column: Merge. (A-C) Nuclear fluorescence obtained with SOX3. (D-F) Cytoplasmic aggregates generated by SOX3\textsuperscript{22Ala}. (G-I) Punctate nuclear aggregates formed by SOX3\textsuperscript{22Ala}. (J-L) Perinuclear aggregate generated by SOX3\textsuperscript{26Ala}. (M) SOX3\textsuperscript{26Ala} has a greater aggregation activity than SOX3\textsuperscript{22Ala}. COS-7 cells were transiently transfected with pcDNA3.1 SOX3, SOX3\textsuperscript{22Ala} or SOX3\textsuperscript{26Ala}. 48 hrs post-transfection, cells were immunostained using \(\alpha\)-SOX3 antibody and the nuclei visualised using DAPI. Nuclear expression is represented by white bars. Cytoplasmic, perinuclear, and nuclear aggregates are denoted by black, grey and striped bars, respectively. Values represent means \(\pm\) S.E. of three independent experiments. SOX3\textsuperscript{22Ala} cytoplasmic aggregate and perinuclear aggregate numbers are significantly different from that of SOX3\textsuperscript{26Ala} by Student’s paired \(t\)-test (*\(P<0.05\)).

4. RESULTS

4.1. SOX3\textsuperscript{22Ala} and SOX3\textsuperscript{26Ala} generate cytoplasmic and nuclear aggregates \textit{in vitro} and \textit{in vivo}

To compare the molecular properties of wildtype and polyalanine mutant SOX3 proteins, we transiently transfected untagged SOX3, SOX3\textsuperscript{22Ala} and SOX3\textsuperscript{26Ala} expression constructs into COS-7 cells. The subcellular localisation of exogenous protein was determined by immunofluorescence using an \(\alpha\)-Sox3 polyclonal antibody (19). Wildtype SOX3 protein was localised exclusively to the nucleus (Figure 1A-C,M). In contrast, SOX3\textsuperscript{22Ala} and SOX3\textsuperscript{26Ala} proteins frequently formed aggregates which fell into three groups: small cytoplasmic aggregates distributed throughout the cytoplasm which were often concentrated near the nucleus (Figure 1D-F), small nuclear inclusions...
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Figure 2. SOX326Ala forms aggregates in chick neural epithelium. Cultured in ovo electroporated chick embryo neural explants were immunostained for SOX3 (green, left column) and counterstained with DAPI (blue, middle column). Merged images are shown in the right column. (A-C) nuclear SOX3 expression, (D-F) cytoplasmic aggregates of SOX326Ala, (G-I) nuclear inclusions of SOX326Ala and (J-L) large perinuclear aggregates of SOX326Ala.

(SOX3 polyalanine expansions) expression construct (data not shown). The aggregation activity of SOX322Ala and SOX326Ala protein is therefore not restricted to heterologous cell lines and readily occurs in vivo in an appropriate cellular context.

4.2. Cytoplasmic aggregation activity of SOX3 mutants is proportional to the length of the polyalanine tract

It has previously been reported that the cytoplasmic aggregation activity of HOXD13 polyalanine mutant proteins is proportional to the length of the polyalanine tract (5). To determine whether SOX3 polyalanine expansion mutants also exhibit this property, we scored SOX3, SOX322Ala, and SOX326Ala expressing COS-7 cells for aggregation phenotype (n=3 experiments). Cells expressing wildtype SOX3 invariably exhibited diffuse nuclear-restricted fluorescence (Figure 1M). Approximately 42% of cells expressing SOX322Ala contained aggregates 48 hrs after transfection which included multiple small cytoplasmic aggregates (28%), large perinuclear aggregates (4%) or condensed aggregates in the nucleus (10%). Aggregate formation was significantly more frequent in cells expressing SOX326Ala protein, occurring in approximately 88% of transfected cells (Figure 1M). Perinuclear aggregate formation was significantly higher (8-fold) in SOX326Ala expressing cells, with approximately 32% of transfected cells manifesting a single large, round, aggresome-like inclusion (Figure 1J-M). Multiple small cytoplasmic aggregates were also significantly more frequent in SOX326Ala expressing cells (49% versus 28%, p< 0.05). Nuclear inclusions occurred with similar frequency in SOX322Ala and SOX326Ala expressing cells. These data indicate that increased length of polyalanine tracts in SOX3 promotes the formation of aggresome-like bodies and dispersed cytoplasmic aggregates.

4.3. SOX322Ala and SOX326Ala form aggresomes

The large perinuclear inclusions generated by SOX322Ala and SOX326Ala mutant proteins have marked morphological similarity to aggresomes formed by other proteins (9-11). Aggresomes exhibit three key features: redistribution of the intermediate filament vimentin, localization to the MTOC and reduced formation in cells exposed to microtubule disruption agents such as nocodazole (12). We therefore assayed SOX322Ala and SOX326Ala cytoplasmic aggregates for these properties. As a positive control, we used GFP-250 (GFP fused to a 250 amino acid fragment of p115), which has been previously shown to form aggresomes (11). As shown in Figure 3A-C, vimentin forms a cage around the GFP-250 aggresome in COS-7 cells. Analysis of SOX322Ala and SOX326Ala expressing COS-7 cells containing large perinuclear aggregates revealed that vimentin was concentrated at the site of the inclusion body and in some cases had formed a cage-like structure that is typical of aggresomes (Figure 3G-I). Similar results were obtained using CHO K1 cells (data not shown). In contrast, cells containing diffuse nuclear localisation, loose cytoplasmic aggregates, or nuclear inclusions did not exhibit altered vimentin distribution (data not shown). To determine the proximity of SOX322Ala and SOX326Ala aggregates to the MTOC, we performed additional co-localisation studies using the...
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Figure 3. SOX326Ala perinuclear aggregates are associated with vimentin redistribution. COS-7 cells transiently transfected with GFP-250, pcDNA3.1 SOX3 or SOX326Ala were immunostained 48 hr post-transfection for SOX3 (green), vimentin (red), and counterstained with DAPI (blue). Column 1: GFP-250 or SOX3 staining. Column 2: Vimentin staining. Column 3: Merged images. (A-C) Vimentin forms a cage around the GFP-250 aggresome. (D-F) Perinuclear Vimentin localisation in a SOX3-expressing cell (G-I) Vimentin collapsed around the SOX326Ala perinuclear aggregate.

MTOC marker γ-tubulin. Cells expressing GFP-250 generated aggresomes which always formed in close proximity to the MTOC (Figure 4A-C) as did SOX322Ala and SOX326Ala perinuclear aggregates (Figure 4G-I). Dispersed cytoplasmic aggregates were most abundant in the cytoplasmic region adjacent to the MTOC (Figure 4D-F). To determine the impact of microtubule disruption on SOX326Ala aggregate formation, we treated transfected COS-7 cells with nocodazole and scored the cells for aggregation phenotype. Nocodazole treatment led to a significant reduction in the proportion of cells containing SOX326Ala (31% to 5%) and GFP-250 (80% to 12%) perinuclear aggregates (Figure 4J). These data demonstrate that SOX322Ala and SOX326Ala perinuclear aggregates exhibit the key properties of aggresomes.

4.4. Polyalanine mutations in SOX3 reduce transactivation

To determine the impact of polyalanine expansion mutations on SOX3 function, we compared the transcriptional activity of wildtype and mutant SOX3. Expression constructs for SOX3, SOX322Ala and SOX326Ala proteins were transfected into COS-7 or CHO K1 cells together with the luciferase reporter plasmid SOCM that has the Sox Consensus Motif upstream of luciferase. Increasing quantities of SOX3 activated the SOCM reporter up to approximately five-fold in a dosage-dependent manner (Figure 5A). In contrast, SOX322Ala and SOX326Ala exhibited significantly less transcriptional activity. We therefore conclude that polyalanine expansion mutations in SOX3 result in impaired transactivation activity.

4.5 Polyalanine mutations in SOX3 alleviate repression of β-catenin-mediated transcription

Previous studies have indicated that Xenopus Sox3 can inhibit β-catenin/TCF-mediated gene activation by competing with TCF factors for binding to β-catenin (22). We therefore tested whether SOX3 and the polyalanine expansion mutants SOX322Ala and SOX326Ala also modulate β-catenin-TCF-mediated transcription using the TOPFLASH reporter assay (29). HEK 293 cells cotransfected with the TOPFLASH reporter construct and β-catenin led to constitutive activation of the β-catenin-TCF target gene luciferase (Figure 5B). This activation was suppressed more than eight-fold by the co-transfection of wildtype SOX3 (Figure 5B). Increasing amounts of SOX3 led to a dose-dependent reduction in activation of TOPFLASH by exogenous β-catenin (data not shown). In contrast, SOX322Ala and SOX326Ala exhibited less than two-fold repression of β-catenin/TCF transcription. Western blot analysis revealed similar levels of exogenous SOX3 protein accumulation (data not shown). These data suggest that human SOX3 modulates β-catenin-mediated signaling and that polyalanine expansion mutations have significantly reduced repression activity.

5. DISCUSSION

Polyalanine expansion mutations in the transcription factor SOX3 have recently been associated with the congenital neuroendocrinological disorder XH. However, little is known about the pathological mechanism of these mutations. Here we show that mutant SOX3 proteins containing expanded polyalanine tracts aggregate in the cytoplasm and nucleus and generate aggresomes. This is the first time that a disease-causing protein containing a polyalanine expansion mutation has been shown to generate aggresomes and suggests that polyalanine and protein conformation disorders such as Alzheimers and Parkinsons may become manifest through similar pathological mechanisms. Further, we show that mutant SOX3 proteins have impaired transcriptional activity and reduced capacity to inhibit β-catenin/TCF-mediated transcription. These data suggest that deregulation of SOX3 target genes and inappropriate canonical Wnt signaling in CNS progenitors may also contribute to dysfunction of the hypothalamic-pituitary axis in XH patients.

Two types of cytoplasmic aggregates were present in cell lines and neuroectodermal tissue expressing the SOX322Ala and SOX326Ala mutant proteins: small punctate “microaggregates”, which were typically distributed throughout the cytoplasm, and larger perinuclear aggresomes. Recent expression studies in cell lines have shown that other mutant transcription factors associated with polyalanine expansion disorders including HOXD13, HOXA13, RUNX2 and FOXL2 also form dispersed cytoplasmic aggregates (5). This phenotype was also reported in COS cells expressing epitope-tagged
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Figure 4. SOX3 (SOX326Ala) perinuclear aggregates colocalize with γ-tubulin and are sensitive to microtubule disruption. COS-7 cells transiently transfected with pcDNA3.1 SOX3, SOX322Ala or SOX326Ala were immunostained 48 hr post-transfection for SOX3 (green), γ-tubulin (red), and counterstained with DAPI (blue). Column 1: GFP-250 or SOX3 staining. Column 2: γ-tubulin staining. Column 3: GFP-250/SOX3+ DAPI + γ-tubulin merged images. (A-C) γ-tubulin, a marker for the MTOC, colocalizes with the GFP-250 aggresome. (D-F) γ-tubulin does not colocalise with SOX322Ala cytoplasmic aggregates. (G-I) γ-tubulin colocalizes with the SOX326Ala perinuclear aggregate. (J) Microtubule destabilization significantly decreases GFP-250 and SOX326Ala aggresome formation and decreases cytoplasmic aggregate formation. Black and white bars denote DMSO and nocodazole treatment, respectively. Error bars are S.E. and statistical significance was determined by Student’s paired t-test at P<0.05.

SOX326Ala (5) and SOX322Ala (17). While these reports indicate that aggregation is a common consequence of polyalanine expansion, the mutant proteins were not shown to generate aggresomes. Aggresomes form in response to the accumulation of misfolded proteins in the cytoplasm and are generated by many mutant proteins associated with neurodegenerative diseases including Parkin, α-synuclein, synphilin-1, presenilin 1 (10), peripheral myelin protein PMP22 (30), superoxide dismutase (31). Aggresomes are generated by dynein-dependent retrograde transport of cytoplasmic microaggregates resulting in their perinuclear localisation and close proximity to the MTOC. The large perinuclear aggregates generated by SOX322/26Ala proteins exhibit the three key properties of aggresomes: altered distribution of the intermediate filament protein vimentin, colocalization with the MTOC and a significantly reduced formation in conditions that promote microtubule disruption. These data indicate that polyalanine expansion proteins are capable of generating aggresomes and suggest that polyalanine and polyglutamine expansions disorders may become manifest through similar pathological mechanisms. Our findings also raise the possibility that cellular aggregates may be a pathological feature of XH patients. The ability of mutant SOX3 to generate cytoplasmic and nuclear aggregates in developing neuroectodermal cells provides support for this possibility. However, this issue may be difficult to resolve as XH is a rare condition and there are relatively few SOX3-expressing cells in the CNS after birth (19). The generation of an XH mouse model may therefore provide the best approach to explore the pathogenic significance of aggregate formation.

Given the propensity of transcription factors harbouring polyalanine expansions to generate cytoplasmic aggregates, why is it that only SOX3 appears to generate aggresomes? One possibility is that SOX3 has an inherently high aggregation activity, perhaps due to the presence of three additional polyalanine tracts in the N-terminus, which results in the rapid formation of aggresomal bodies. Indeed, perinuclear aggregates were readily detected in SOX322Ala and SOX326Ala expressing cells 24 hours after transfection (data not shown). As aggresomes form when the capacity of the cell to degrade protein aggregates via the proteasome is exceeded (32), it is also possible that extended culture periods of some polyalanine expanded transcription factors may be required to generate aggresomes. On this note, it has been shown that mutant HOXD13 cytoplasmic aggregates at 48-hours post transfection did accumulate in close proximity to the MTOC (as was also observed for SOX322Ala and SOX326Ala aggregates) suggesting their active recruitment to this cellular compartment to form a “pre-aggresomal” inclusion (5). Comparative expression studies of SOX3 and other mutant proteins containing polyalanine expansions in a range of cellular contexts will be required to resolve this issue.

While the aggregation products of SOX322Ala and SOX326Ala expressing cells were identical, significant differences in the frequency of aggregate formation and aggregation type were observed. SOX326Ala exhibited a more potent aggregation phenotype, resulting in significantly more cells with dispersed cytoplasmic and large perinuclear aggregates. In this respect, SOX3 appears to be similar to HOXD13 in which a correlation between alanine tract length and aggregation activity was also demonstrated (5). Together these data suggest that polyalanine tract length is the major factor in determining aggregation activity. Generally, these repeat length differences in HOXD13 are reflected in the clinical...
Figure 5. (A) SOX3^{22Ala} and SOX3^{26Ala} exhibit a significant reduction in transactivation activity in comparison with SOX3. COS-7 cells were transfected with 0.1, 0.5 or 1.0 µg of pcDNA3.1 SOX3, pcDNA3.1 SOX3^{22Ala} or pcDNA3.1 SOX3^{26Ala} expression vectors (increasing quantities are indicated by the gradient bar on the horizontal axis). Statistically significant differences (p<0.05) between mutant and wildtype SOX3 transactivation levels are indicated by an asterisk (*). Values represent means ± S.E. of four independent experiments. (B) SOX3 represses β-catenin-mediated signaling. HEK 293 cells were transfected with expression (β-catenin, SOX3, SOX3^{22Ala}, SOX3^{26Ala}) and luciferase reporter (TOPFLASH, FOPFLASH) constructs as indicated. SOX3^{22Ala} and SOX3^{26Ala} have significantly less repression activity (*).
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phenotypes of patients with SPD, with longer repeat expansion mutations giving rise to more severe symptoms (33). A similar phenotype-genotype correlation holds for XH patients with respect to cognitive function; affected males expressing SOX3<sup>26Ala</sup> are mentally retarded as opposed to SOX3<sup>22Ala</sup> males who are not cognitively impaired (17, 26). In contrast, endocrine dysfunction is much more severe in affected males that carry the SOX3<sup>22Ala</sup> mutation (panhypopituitarism) compared with SOX3<sup>26Ala</sup> males (isolated Growth Hormone Deficiency). Phenotypic differences in these kindreds may be influenced to some degree by their genetic background, as is suggested by Sox3 loss-of-function studies in mice (19).

Relatively little is known about the impact of polyalanine expansion mutations on transcriptional activity. Given that mutations in transcription factors are responsible for all known congenital disorders associated with polyalanine expansion, resolving this issue may provide insight into the molecular pathology underlying many polyalanine expansion disorders. In this study we showed that SOX3<sup>22Ala</sup> and SOX3<sup>26Ala</sup> have significantly reduced transcriptional activity compared with wildtype SOX3. Similar reductions in transactivation have recently been shown for SOX3<sup>22Ala</sup> (17) and a five residue polyalanine tract expansion mutation in PHOX2B (34), suggesting that reduced transcriptional activity may be a common functional consequence of polyalanine expansion. How might this occur? Clearly, aggregation of mutant protein in the cytoplasm would be expected to reduce the amount of protein available for transcriptional activation. In addition, a dominant negative mechanism may operate, in which mutant protein aggregates sequester wildtype protein. This appears to be the case for HOXD13 which, when co-expressed with mutant (+14 Ala) protein in COS-1 cells, localises to cytoplasmic mutant protein aggregates (5). We found, however, that SOX3<sup>22Ala</sup> and SOX3<sup>26Ala</sup> aggregates did not sequester wildtype SOX3 or the other SOXB1 family members, SOX1 and SOX2 (data not shown). In addition, it is also possible that polyalanine tract expansion may alter the conformation of SOX3 protein and impair its ability to interact with transcription cofactors. In vitro studies indicate that polyalanine tracts form β-sheets (35). A 7 or 11 alanine expansion in SOX3 could therefore alter protein conformation through abnormal β-sheet formation, potentially hindering interactions with cofactors.

Binding partners for several members of the SOX family of transcriptional factors have been identified (16), underlining the importance of cofactors in nuclear localisation and transcriptional regulation of SOX proteins. Xsox3 has been shown to directly interact with β-Catenin and to inhibit β-catenin/TCF-mediated transcription in a TOPFLASH assay (21). Our studies indicate that human SOX3 also inhibits β-catenin/TCF-mediated transcription, suggesting that SOX3 may bind to β-catenin <em>in vivo</em> to inhibit canonical Wnt signalling. Interestingly, transgenic reporter mice indicate that Wnt/β-catenin signalling is active in the CNS in many areas that overlap with Sox3 expression and require Sox3 function, including the ventral diencephalon/infundibulum (36). Furthermore, β-catenin overexpression in the developing CNS results in abnormal morphogenesis of the cerebral cortex (37), indicating that correct levels of β-catenin are essential for normal neurogenesis. Given the markedly reduced repression of β-catenin/TCF-mediated transcription activity exhibited by the SOX3<sup>22Ala</sup> and SOX3<sup>26Ala</sup> proteins, we hypothesize that targets of canonical Wnt signaling may be inappropriately regulated in XH patients, leading to the neuroendocrinological defects associated with this congenital disorder.

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7. REFERENCES


SOX3 polyalanine expansions


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