Mechanistic Insight into the Pathology of Polyalanine Expansion Disorders Revealed by a Mouse Model for X Linked Hypopituitarism

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Abstract

Polyalanine expansions in transcription factors have been associated with eight distinct congenital human diseases. It is thought that in each case the polyalanine expansion causes misfolding of the protein that abrogates protein function. Misfolded proteins form aggregates when expressed in vitro; however, it is less clear whether aggregation is of relevance to these diseases in vivo. To investigate this issue, we used targeted mutagenesis of embryonic stem (ES) cells to generate mice with a polyalanine expansion mutation in Sox3 (Sox3-26ala) that is associated with X-linked Hypopituitarism (XH) in humans. By investigating both ES cells and chimeric mice, we show that endogenous polyalanine expanded Sox3 does not form protein aggregates in vivo but rather is present at dramatically reduced levels within the nucleus of mutant cells. Importantly, the residual mutant protein of chimeric embryos is able to rescue a block in gastrulation but is not sufficient for normal development of the hypothalamus, a region that is functionally compromised in Sox3 null embryos and individuals with XH. Together, these data provide the first definitive example of a disease-relevant PA mutant protein that is both nuclear and functional, thereby manifesting as a partial loss-of-function allele.

Introduction

Trinucleotide repeat expansions are a relatively common cause of human disease. The expanded trinucleotide can occur in an untranslated region, for example in Fragile X syndrome in which a CGG repeat adjacent to the FMR1 promoter causes hypermethylation and gene silencing. Alternatively repeat expansions can occur in exonic regions and result in elongation of homopolymeric amino acid tracts. For example polyglutamine (PQ) diseases such as Huntington’s disease, are associated with long unstable PQ-encoding stretches that lead to the production of a toxic species and late onset disease characterised by the loss of a subset of neurons. In addition to PQ encoding repeats, polyalanine (PA) repeat expansion has recently emerged as a significant cause of human disease. PA expansions have been linked to nine disorders associated with PA expansions in developmentally-important transcription factors. These, and the associated disorders, are SOX3 (X linked Hypopituitarism), HOXA13 (hand–foot–genital syndrome), ARX (syndromic and non-syndromic X-linked mental retardation), HOXD13 (synpolydactyl type II), PHOX2B (congenital central hypoventilation syndrome), FOXL2 (blepharophimosis, ptosis and epicanthus inversus), ZIC2 (holoprosencephaly) and RUNX2 (cleidocranial dysplasia). The ninth is the ubiquitous RNA binding protein PABPN1 which is associated with the late onset disease oculopharyngeal muscular dystrophy (OPMD).

Despite considerable functional analyses, the mechanism by which PA expansion mutations cause disease is not completely understood. Phenotype/genotype correlations in humans and mouse models indicate that many PA alleles give rise to disease phenotypes that resemble (HOXA13, FOXL2, ZIC2) or are less severe (ARX) than null alleles, consistent with complete or partial loss-of-function (LOF). In contrast, some PA alleles cause similar but more severe disease phenotypes than null alleles consistent with toxic gain-of-function (GOF) and/or dominant negative activity (HOXD13, PHOX2B). Despite these differences in mode of inheritance, all PA proteins behave very similarly in vitro, such that over-expression in cell culture results in the generation of cytoplasmic and/or nuclear aggregates, which are likely to arise through protein misfolding. While the relevance of cellular aggregates to PA disease in general is unclear, nuclear inclusions that contain mutant PABPN1 protein are a hallmark of OPMD, suggesting that aggregates may also form in patients with PA expansion mutations in developmental transcription factors. Protein aggregation also occurs in the related polyglutamine (PQ) disorders where PQ expansion confers toxic GOF. Together, these data suggest that aggregate formation may have a pathogenic role in PA disease alleles with GOF activity. However, the critical question of whether aggre-
**Author Summary**

Alanine is one of the 20 amino acid building blocks from which proteins are generated. Nearly 500 human proteins contain stretches of consecutive alanine residues ranging from 4 to 20 amino acids in length. Whilst the function of these polyalanine (PA) tracts remains unknown, they are interesting because DNA changes (mutations) that increase their length above a threshold are responsible for nine different human disorders. *In vitro* studies indicate that expanded PA proteins misfold and aggregate, suggesting that there may be a common "gain-of-function" mechanism that underpins this group of disorders. However, these data are difficult to reconcile with genetic studies, which indicate that most PA mutations cause protein loss-of-function. Therefore, to investigate the pathological mechanism of PA disorders we generated a mouse model of X-linked Hypopituitarism (XH), a disease caused by PA expansion in the Sox3 protein. Strikingly, we found that the mouse version of the disease-causing protein was almost completely cleared from cells and that aggregates do not form in *vivo*. These data explain why this type of mutation causes protein loss-of-function and reveals why nature limits the length of PA stretches.

**Results/Discussion**

In order to study the effects of disease-associated PA expansion at a cellular level we created R1 ES cells with a targeted mutation of Sox3. Homologous recombination was used to generate XY ES cells to generate a 26 alanine PA tract expansion mutation in Sox3 (Sox3-26ala). The analogous human mutation causes X-linked Hypopituitarism (XH), a disease in which hemizygous males have GH deficiency (resulting in short stature) and fully penetrant intellectual disability [13]. Importantly, we find no evidence of Sox3-26ala aggregate formation in neural derivatives of targeted ES cells *in vivo* or in neurodiffereniated ES cell cultures. Instead, the Sox3-26ala mutation leads to a massive reduction in Sox3 protein within the nucleus. We also present developmental and biochemical evidence that residual mutant protein retains some activity, indicating that Sox3-26ala functions as a partial LOF allele.

an early defect in spermatogenesis and the lack of any reported transmission of the human SOX3-26ala allele from hemizygous males [13,14]. Since we were unable to transmit the Sox3-26ala mutation through the male germline, the phenotypic consequences of the mutation were initially investigated in Sox3-26ala ↔ WT chimeric embryos. Immunostaining with a SOX3-specific antibody revealed a dramatic reduction in SOX3 protein in 13.5 dpc telencephalic ventricular zone cells expressing the Sox3-26ala allele (identified by NEO-immunoreactivity) in comparison to neighbouring WT cells (Figure 2A). The ability of the antibody to detect mutant protein was confirmed by staining COS-7 cells expressing exogenous mouse Sox3-26ala, in which large peri-nuclear and cytoplasmic aggregates were common (Figure S1). A striking reduction in mutant Sox3 protein was also observed in 7.5 dpc, 9.5 dpc, 10.5 dpc and 11.5 dpc chimeras (Figure 3 and Figure S2), indicating that this phenotype was not stage-dependent. High power microscopy failed to detect any cytoplasmic or nuclear aggregates of mutant protein but did reveal a very low level of protein in the nucleus (Figure 2A). Comparison of Sox3-26ala and Sox3-null embryonic CNS cells confirmed that the low level of Sox3-26ala protein that we observed was not due to background signal (Figure 2B). Mutant cells appeared morphologically normal and there was no apoptotic induction as assessed by staining for activated Caspase3 (Figure S3). To further characterise the cellular phenotype of Sox3-26ala mutant cells, we performed neurodiffereniation of Sox3-26ala ES cells (which are XY and therefore lack a WT Sox3 allele). Immunohistochemical analysis confirmed the overwhelming reduction of nuclear SOX3 protein in mutant cultures (Figure 2C) in which the expression of other neural progenitor markers were unaffected (data not shown). Rare cells with near normal levels of nuclear mutant protein were also detected but in no case was there evidence of protein aggregation (Figure 2C). Western blot analysis further supported the near complete loss of steady-state SOX3 protein levels in mutant cells (Figure 2D). As triplet repeat expansion mutations have been shown to affect mRNA transcription [12], we compared Sox3 message levels in WT and mutant cells *in vitro* and *in vivo* using qPCR and *in situ* hybridisation, respectively (Figure 2E-2F). No difference in Sox3 mRNA level was detected, indicating that the massive reduction in SOX3 protein levels in the mutant cells has a post-transcriptional aetiology and presumably occurs via degradation of misfolded protein [8]. This was supported by *in vitro* transcription/translation analysis which indicated that translation of the mutant transcript was unaffected by the PA mutation (Figure S4).

Taken together, these results indicate that Sox3-26ala is a LOF allele and argue strongly against a role for aggregation in the pathogenesis of XH. To further investigate LOF as the mechanism of disease, we compared the development of the hypothalamus, infundibulum and anterior pituitary in Sox3-26ala chimeric embryos to Sox3 heterozygous embryos that carry a complete LOF (null) allele. Sox3+/− embryos exhibit a defect in infundibular development that results in aberrant induction and bifurcation of the anterior pituitary primordium, Rathke’s Pouch (RP) [15] accompanied by expansion of the floor of the ventral diencephalon (Figure 3). Similar defects in infundibular morphology and pituitary localisation have also been identified in XH patients [16]. Importantly, 13.5 dpc and 11.5 dpc high percentage Sox3-26ala ↔ WT chimeras exhibited dysmorphology of the infundibulum, Rathke’s Pouch and ventral diencephalon that was indistinguishable from Sox3+/− embryos (Figure 3). These abnormalities were also present in low percentage chimeras, although were less severe consistent with the loss of SOX3

Mouse Model of SOX3 Polyalanine Expansion Mutation
Table 1. Failure of Sox3-26ala targeted ES clones to transmit through the germline.

| Clone     | Number of chimeras | Percentage chimerism (range) | Number of pups born | Percentage of ES-derived pups (agouti) |
|-----------|--------------------|------------------------------|---------------------|---------------------------------------|
| WT        | 15                 | 5–100                        | 50                  | 18                                    |
| Neo       | 4                  | 50–90                        | 35                  | 49                                    |
| Sox3-26ala-1 | 6                  | 5–50                         | 127                 | 0                                     |
| Sox3-26ala-2 | 4                  | 60–90                        | 81                  | 0                                     |
| Sox3-26ala-3 | 3                  | 20–60                        | 53                  | 0                                     |
| Sox3-26ala-4 | 4                  | 75–95                        | 156                 | 0                                     |

Figure 1. Generation of Sox3-26ala ES cells. Scale representation of the Sox3 locus, targeting vector and recombinant alleles (A). Probing of BglII digested DNA from ES cell clones with the 5’ probe yielded an 8.8 kb fragment from the WT locus and a 5.9 kb fragment when the Neo cassette was recombined into the Sox3 locus (Sox3-26ala or Neo). B) Representative Southern blot of 3 clones including a targeted clone (Sox3-26ala-3) is shown. C) PCR using primers spanning the alanine expansion (red arrows in A) was used to distinguish whether targeted clones carried the expansion and gave a 219 bp product instead of 186 bp as seen in WT.

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function being responsible for this phenotype. Notably, there was an obvious reduction in SOX3 protein in mutant (NEO+) cells thereby confirming that PA expansion has a functional impact in neural cells that are directly implicated in XH pathology.

Having established that Sox3-26ala behaves as a LOF allele, we next considered whether the low level of remnant SOX3 protein in mutant cells was functional. To investigate this, we initially performed transactivation assays in COS-7 cells using wild type and mutant human and mouse SOX3 expression constructs and a luciferase reporter containing four SOX consensus motif (SOCM) binding sites. Both mouse and human SOX3-26ala proteins showed activity in this assay that was significantly higher than background (Figure 4A). However, consistent with previous reports [7,16], this activity was much lower than WT SOX3 protein. To investigate whether this reduction was caused by lower nuclear protein levels or an inherent defect in mutant protein
transactivation activity, we measured that relative amount of WT and mutant SOX3 protein in the nucleus of transfected cells by Western Blot (Figure 4B). We observed a reduction of similar magnitude to the reduced luciferase output, suggesting that the mutant protein that is present in the nucleus has similar activity to WT. To investigate whether the residual nuclear SOX3-26ala protein is functional *in vivo* we compared the phenotypes of 7.5 dpc chimeras generated from either *Sox3* or *Sox3*-26ala ES cells, as it has been reported that *Sox3*-null ES cell, WT embryo chimeras exhibit severe gastrulation defects [15]. As previous analysis of *Sox3*-null ES cell chimeras was not performed with R1 ES cells (the parent line used to generate the 26ala mutant ES cells), we generated R1 *Sox3*-null ES cells for this experiment. Consistent with previous reports, a high proportion (52%) of *Sox3*-null ES cell chimeras generated abnormal gastrulae (Figure 5). In contrast, only 19% of *Sox3*-26ala ES cell chimeras were morphologically abnormal which was not significantly different to the proportion of abnormal chimeric embryos generated using *Sox3*-flox control ES cells (Figure 5). Immunostaining of *Sox3*-26ala chimeras revealed a reduction in SOX3 protein levels that was similar to later embryonic stages (Figure S1). These data indicate that nuclear SOX3-26ala protein is functional *in vivo* and is sufficiently abundant to prevent overt defects in gastrulation. We therefore conclude that *Sox3*-26ala is a partial LOF allele.

This study represents the first investigation of the disease-associated *Sox3*-26ala mutation under the control of the endogenous locus in a whole animal setting. The complete lack of aggregates in SOX3 positive CNS zones (including the hypothalamic-pituitary axis) provides strong evidence that aggregation is
not a feature of the human disease. In contrast, our data demonstrate that PA expansion results in a dramatic reduction in SOX3 protein due to a post-translational defect. Given that PA expansion proteins co-localise with chaperones in vitro and that aggregation is promoted by pharmacological inhibition of the proteasome [8], it seems likely that mutant SOX3 protein misfolds and is cleared from the cell. Of note, the small amount of protein that remains translocates to the nucleus and is functionally active. The level of protein that remains is sufficient for some developmental contexts but not others (i.e. gastrulation but not pituitary induction). This context-specific threshold of SOX3 protein may explain why XH patients with SOX3 PA mutations, but not null mutations, have been identified.

In direct contrast to the Hoxa13 (+7ala) spdh spontaneous mouse model in which mutant protein is mislocalised to the cytoplasm [8], our data provide the first definitive example of a disease-relevant PA mutant protein that is both nuclear and functional, thereby manifesting as a partial LOF allele. Interestingly, mutant protein levels in the Hoxa13 and Arx PA mouse models, which also exhibit LOF inheritance, are diminished but not abolished by western blot and whole mount immunodetection [1,17]. This raises the possibility that nuclear localisation of suboptimal levels of functional protein may be a feature of several PA diseases. In the case of ARX, the PA mutant mouse model has been reported to have normal nuclear localisation of mutant protein in some tissues, but to have a reduction in the total number of positive cells [17,18]. Given our data, an alternative interpretation is that cells expressing ARX are not lost but are unable to be detected due to misfolding and clearance of the mutant protein. In situ hybridisation of Arx transcripts should provide a means of discriminating between these possibilities.

In addition to SOX3-26ala, XH is also associated with a 7 alanine expansion mutation (SOX3-22ala) in the same polyalanine tract [16,19,20]. Although SOX3-22ala is occasionally associated with mild learning difficulties [20], it is interesting to note that the infantile behaviour/severe MR that is fully penetrant in SOX3-26ala-carrying males is not found in affected males with the SOX3-22ala expansion mutation, suggesting that the shorter expansion is less severe. Given our data showing a massive depletion of SOX3 protein in Sox3-26ala mutant cells in vivo, we would predict that SOX3-22ala protein would also be reduced (compared to WT) but to a lesser extent than the SOX3-26ala protein. This higher level (and therefore activity) of SOX3-22ala compared to SOX3-26ala would be sufficient for "cognitive" CNS development. Consistent with this idea, the SOX3-22ala protein is less prone to aggregate formation in cell culture [7,16], although one must be cautious in

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Figure 4. SOX3-26ala from mouse and human retains transactivation activity. A) COS-7 cells were transfected with pcDNA3.1 expression vector containing either mouse Sox3, human SOX3, mouse Sox3-26ala, human SOX3-26ala or an empty vector control. Values represent mean normalised luciferase values plus standard deviation of four independent experiments measured 48 hours after transfection. Student’s T-tests (two tailed, unequal variance) of SOX3-26ala from human or mouse compared to empty vector show a statistically significant increase in luciferase activity. B) Nuclear protein lysates prepared from duplicate plates 48 hours after transfection show that less SOX3 is detected in the nucleus of cells expressing both mouse and human SOX3-26ala, pcDNA3.1-EGFP transfected cells were used as a control and prepared for both nuclear protein and whole cell extracts (WCE). Blotting for Histone H3, indicates equal loading and blotting for α-Tubulin shows an absence of cytoplasmic contamination in nuclear preparations. Transfection efficiency was determined by co-transfecting EGFP and counting positive cells prior to harvesting and found to be equal for all plasmids. doi:10.1371/journal.pgen.1003290.g004

Figure 5. Residual nuclear SOX3-26ala protein rescues a gastrulation defect of Sox3-null <-> WT chimeric embryos. Sox3-26ala <-> WT chimeras are normal at 7.5 dpc (gastrulation) unlike Sox3-null <-> WT chimeras. A total of 15 Sox3-flox<->WT ES chimeras, 31 Sox3-null<->WT chimeras and 21 Sox3-26ala<->WT chimeras were blind scored by two independent operators as morphologically normal or abnormal. The average score for each embryo was used to plot the percentage of abnormal embryos for each condition and chi squared analysis was performed with Sox3-flox<->WT embryos used to set expected outcomes. Significantly more Sox3-null<-> WT chimeras were abnormal (p = 0.0001) while Sox3-26ala<->WT chimeras did not deviate from expected (p = 0.95). An example of normal morphology is shown for Sox3-flox<->WT and Sox3-26ala<->WT chimeras and an abnormal Sox3-null<->WT chimaera is also shown that exhibits distortion of the ectodermal layer and apparent expansion of cells at the primitive streak and the adjacent extra-embryonic region. doi:10.1371/journal.pgen.1003290.g005
interpreting pathological mechanism from over expression studies in heterologous cell lines. While it would be useful to directly compare SOX3-26ala and SOX3-22ala levels in vivo, a mouse model of the SOX3-22ala mutation has not been generated. However, given that it is now possible to generate neural precursors directly from patients via iPSC cells, it would be interesting to compare the SOX3 protein levels in neuroprogenitors derived from SOX3-22ala and SOX3-26ala carrying males. This approach might also be informative for PA disorders in general, particularly given that increased disease severity is generally associated with longer PA expansions [21].

Based on these and other published data, we propose that both GOF and LOF PA disease alleles are associated with a primary defect in protein folding but that a critical difference in the capacity of cells to clear mutant protein results in either the accumulation of mislocalized cytoplasmic protein (GOF) or a diminished level of functional nuclear protein (LOF). As indicated by earlier reports [21], the former mechanism provides scope for dominant-negative and toxic GOF through cytoplasmic sequestration of endogenous binding partners. In the latter, we propose that misfolded protein is efficiently cleared by the cell without significant perturbation of other cellular processes, manifesting as LOF or partial LOF, depending on the amount of residual functional protein. Factors that determine whether or not a cell is able to efficiently clear the misfolded protein could include the level of expression, local concentration differences within the cell, the efficiency of the degradation pathway within different affected cell types and the length of the PA expansion. We propose that the aggregates seen when SOX3-26ala and all other PA expansion proteins are expressed in vitro reflect the overloading of the cell with mutant protein such that normal degradation pathways are overwhelmed. Resolution of the factors that determine whether a cell is able to clear or tolerate mutant protein will have broader implications for proteinopathies such as Alzheimers Disease and Huntington Disease in which only subsets of cells display aggregation. For the future, it will be interesting to determine the behaviour of mutant protein in other PA mouse models with GOF and LOF inheritance and, where possible, in patient-derived induced pluripotent stem (iPS) cell derivatives.

**Methods**

ES cell targeting

**Sox3-26ala allele.** The targeting vector was based on a Sox3-Neo floxed vector published previously in which GFP and the loxP sites were removed and a 36 bp insertion was introduced to the existing 42 bp alanine encoding tract [15]. This was electroporated into 129 strain-derived (R1) mouse ES cells and 1000 G418 resistant clones were screened by Southern blotting. Integration at the Sox3 locus was determined by a shift in a BglII fragment from 8.8 kb to 5.9 kb when probed with a 5’ Sox3 probe [15]. Clones were subsequently screened for the alanine expansion using PCR primers flanking the alanine tract; 5’-GAACGCATGCTACAGGAGG-3’ and 5’-GTCCGAGTGTTGCTCAGG-3’, β-Actin (89 bp) 5’-CTGGCTTGACGGCCAGG-3’ and 5’-GTTCCTCATACCCCGGAAAGG-3’. Sox3 expression was normalised to β-Actin and expressed as relative quantity (RQ) using ABI software. Embryonic chimerism was determined against a standard curve of Sox3 dosage generated from adult gDNA of WT and Sox3-null mixed 0:100, 25:75, 50:50, 100:0. Loading was corrected using primers against Sox1 (171 bp) 5’-GACGTGGATTGGCTATGAGAAGACAATC-3’ and 5’-CCTCTCGAGGCGGAGGTTATATT-3’ and Ngn3 (120 bp) 5’-CCCCAGAGCACACACTACCT-3’ and 5’-AGTCATGCCACATTCTCTGTCG-3’. Chimerism in Figure 2C was estimated by NEO IF and ISH.

**ISH and IF**

Embryos were fixed in 4% PFA overnight and CDM bodies were fixed for 15 minutes. Both were then equilibrated in 30% sucrose overnight, set in OCT compound and cryo-sectioned on a Leica CM1900 at 10 μm. ISH on fixed frozen sections was performed using a Sox3 probe from a fragment generated with the following primers; 5’-AGGGCTTGGACACGTACAGC-3’ and 5’-AGGGCCCTGGACACGTCACAG-3’ and a Neo probe from a fragment amplified with the following primers; 5’-GATCCAGTGCCTCAAGGAAGGAGTACGAGGTCG-3’ and 5’-GGCTATCGGTTCGGTATTATATT-3’. Images were captured on a Zeiss Axioscope upright microscope with AnalySIS software, using a 2.5× (Zeiss Neofluor; NA0.5) objective lens. Primary antibodies and dilutions were goat anti-sOX3 (R&D #AF2509;1:150) and rabbit anti-NPTII (Millipore #06-747, 1:130). Imaging of IF was performed on a Zeiss Axioimplan2 upright microscope with Axiosview software, using a 20× (Zeiss Achromat; NA0.75) or a 100× (Zeiss Achromat; NA1.3) objective lens.

**Western blotting**

Day 4 N2B27 neurodifferentiated ES cells were lysed in RIPA buffer supplemented with protease inhibitors. SDS loading buffer was added and the samples were resolved by 10% SDS-PAGE. Duplicate gels were subjected to immunoblot analysis using anti-sOX3 (1:2500) and anti-α-Tubulin (Sigma #T8203, 1:2500) antibodies since both detect proteins at ~40 kD. Signals were developed using ECL substrate (West Pico, Pierce). For nuclear protein lysates, cells were subjected to hypotonic lysis (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.4% NP-40, 10% Ficoll-400, 1 mM DTT, 1 mM PMSF, 1× protease inhibitors), centrifugation to pellet nuclei that were then washed in wash
buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 150 mM KCl, 10% Ficoll-400, 1 mM DTT, 1 mM PMSF, 1× protease inhibitors) and lysed in nuclear extract buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.5 mM EDTA, 20% glycerol, 0.42 M KCl, 1 mM DTT, 1 mM PMSF, 1× protease inhibitors).

Luciferase transcription assay

Transcriptional activities of mouse and human SOX3 and SOX3-26ala were determined using the Dual-Luciferase Reporter Assay System (Promega). 1.0 µg of plasmid DNA was transfected into COS-7 cells using Lipofectamine according to manufacturer’s instructions. All transfections were performed in triplicate and contained luciferase reporter Sox Consensus Motif (SOCM; 4×AACAAG) [7], Renilla luciferase plasmid pRL-CMV and one of pcDNA3.1, pcDNA3.1 hSOX3, pcDNA3.1 hSOX3-26ala, pcDNA3.1 mSOX3 or pcDNA3.1 mSOX3-26ala expression constructs (Promega). The firefly luciferase and Renilla luciferase activities were determined after 48 h on a FluorStar Optima (BMG technologies). Relative luciferase activity is the ratio of firefly to Renilla normalised to pcDNA3.1. The assay was repeated four times. Statistical analysis was performed using Student’s T-test (two tailed, unequal variance).

Ethics statement

Animal experiments were approved by the University of Adelaide Animal Ethics Committee. All studies were conducted in accordance with the principles of animal replacement and reduction and experimental refinement. Animals were monitored daily for evidence of illness and, if distressed, were culled immediately by cervical dislocation by an experienced investigator/animal technician.

Supplementary methods

Cell free transcription/translation. Cell free transcription/translation was performed using the TNT Coupled Reticulocyte Lysate System (Promega, #L4610) as per manufacturer’s instructions. Plasmids used were 250 ng of either pcDNA3.1 hSOX3, pcDNA3.1 hSOX3-26ala, pcDNA3.1 mSOX3 or pcDNA3.1 mSOX3-26ala. Reactions were resolved on a Bio-Rad 10% precast gel (#456-1033), transferred to PVDF membrane, exposed to phosphor-screen and scanned.

COS-7 aggregation assay. COS-7 cells were grown on glass coverslips, transfected with one of pcDNA3.1, pcDNA3.1 hSOX3, pcDNA3.1 hSOX3-26ala, pcDNA3.1 mSOX3 or pcDNA3.1 mSOX3-26ala using Fugene6 (Roche) and stained with SOX3 antibody and DAPI two days later. At least 100 cells were scored randomly from each condition. Results are shown as the mean of three independent experiments and error bars represent one standard deviation.

Supporting Information

Figure S1 Mutant SOX3-26ala protein aggregates in vitro. COS cells were transfected with mouse or human pcDNA3.1 SOX3 or pcDNA3.1 SOX3-26ala expression plasmids and two days later cells were fixed and stained with a SOX3 antibody (Green) and DAPI (Blue). Cells were scored according to the localisation of the SOX3 staining as nuclear, cytoplasmic or peri-nuclear for which representative examples are shown. At least 100 cells were scored from each condition and the experiment was repeated three times. Error bars show one standard deviation from the mean. Student’s T-tests between WT and mutant for each category for both mouse and human revealed statistically significant differences with a P value of less than 0.05 in each case.

Figure S2 SOX3-26ala protein is cleared from neuroprogenitor cells in vitro. Sox3-26ala cells (arrow) WT chimeras were cut at 7.5 dpce (transverse section through neural groove), 9.5 dpce (transverse section through neural tube) or 10.5 dpce (sagittal section through ventral diencephalon) and stained with antibodies against SOX3 and NEO. Mutant Sox3-26ala cells (NEO positive cells; arrow heads) have low SOX3 staining. Confocal microscopy was performed for 7.5 dpce and 9.5 dpce embryos on a Leica SP5 spectral scanning confocal microscope with AnalySIS software and a 100× (Leica HCL PL-APO; NA1.4) objective. 10.5 dpce embryos were imaged using a Zeiss Axioplan 2 epifluorescence microscope as described in the methods.

Figure S3 SOX3-26ala expressing cells show no signs of apoptosis. Coronal section of a 13.5 dpce Sox3-26ala cell WT chimera stained with antibodies against SOX3 and Activated Caspase3 (BD Pharmingen, #59565, 1:1000). Boxed area in overlay shows a rare Activated Caspase3 positive cell, while the arrowhead indicates a patch of mutant cells (low SOX3) in which no Activated Caspase3 positive cells are seen.

Figure S4 A) Phosphor image of an in vitro transcription/translation reaction of mouse and human SOX3 WT and 26ala expression constructs. SOX3 protein of the expected size is indicated by the arrow. Two strong bands were generated by the mouse plasmids, possibly reflecting the inclusion of an upstream ATG in the plasmid, while human plasmids generated only one strong band at the expected size. SOX3-26ala for both mouse and human runs slightly higher than WT protein reflecting the expanded polyalanine tract. In addition, both mouse and human SOX3-26ala have a high molecular weight species that appears to be an aggregated form of SOX3 (⋆, -) indicates no template control. B) Immunoblotting using anti-SOX3 antibody (R&D #AF2569) generates a near identical pattern to the phosphor image in (A), thus confirming that the antibody is able to detect mutant and wild type protein with comparable efficiency.

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Author Contributions

Conceived and designed the experiments: PT JH SP NR DM LR. Performed the experiments: JH SP NR DM LR. Analyzed the data: JH PT SP NR DM LR. Wrote the paper: PT JH.

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