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ARTICLE

Analysis of mutations in the tudor domain of the survival motor neuron protein SMN

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Autosomal recessive childhood onset spinal muscular atrophy (SMA) is a leading cause of infant mortality caused by mutations in the survival motor neuron (*SMN*) gene. The SMN protein is involved in RNA processing and is localised in structures called GEMs in the nucleus. Nothing is yet understood about why mutations in *SMN* gene result in the selective motor neuron loss observed in patients. The SMN protein domains conserved across several species may indicate functionally significant regions. Exon 3 of SMN contains homology to a tudor domain, where a Type I SMA patient has been reported to harbour a missense mutation. We have generated missense mutants in this region of SMN and have tested their ability to form GEMs when transfected into HeLa cells. Our results show such mutant SMN proteins still localise to GEMs. Furthermore, exon 7 deleted SMN protein appears to exert a dominant negative effect on localisation of endogenous SMN protein. However, exon 3 mutant protein and exon 5 deleted protein exert no such effect.

Keywords: spinal muscular atrophy; survival motor neuron; gemini of coiled bodies; tudor domains

Introduction

Childhood onset spinal muscular atrophy (SMA) is a leading cause of infant mortality, which segregates as an autosomal recessive disease with a carrier frequency of 1 in 60.[1] The disorder is characterised by the loss of lower motor neurones in the spinal cord and proximal muscle wasting. The disease is categorised into three types according to the severity and age of onset: Type I (Werdnig-Hoffmann disease) has an onset either *in utero* or shortly after birth and death usually occurs before the age of 2 years; Type II SMA has an onset after 2 years of age where individuals are never able to stand but may sit unaided; Type III (Kugelberg-Welander) SMA patients are able to stand unaided, and onset is often in adolescence.[2] All three forms of SMA are localised to chromosome 5q11.2–q13.3 in a region containing an inverted duplication and at least three genes (for review see Lefebvre[3]). Although deletions encompassing all these genes are found in SMA patients,[4–6] it is now well established that survival motor neuron (*SMN*) gene is the causative gene.[3] SMN is present in two almost identical copies in the region: the more telomeric copy of the gene is designated *SMN1* and the centromeric one *SMN2*. The *SMN1* gene is mutated or gene converted to the SMN2 sequence in

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90–98% of patients (for review see Burghes[7]). The *SMN2* gene is deleted in 3–5% of normal individuals and is therefore not associated with the disease.[4,6]

The *SMN* gene contains domains and motifs indicative of a role in RNA processing. For example, it contains a YG box motif which when mutated causes SMA.[8] This YG box is well conserved in *S. pombe*, *C. elegans* and mammalian SMN orthologues, and has been shown to be important for the oligomerisation of SMN.[9] Studies by Dreyfuss and colleagues[10–13] have demonstrated directly that SMN plays a role in RNA processing. The SMN protein localises to the cytoplasm and the nucleus in HeLa cells and is associated with RNA processing proteins by yeast two-hybrid analysis using the RGG box of the hnRNP U protein as bait.[10] Subsequently, antibodies raised against SMN1 protein showed that SMN1 in the nucleus is found in structures called GEMs (Gemini of Coiled Bodies) which are associated with coiled bodies, believed to be involved in mRNA metabolism.[10,11,12] The dominant negative mutant SMN (SMNdelN27) causes reorganisation of snRNPs in the nucleus as well as inhibiting pre-mRNA splicing *in vitro*. High levels of full length SMN stimulate splicing.[13]

The number of GEMs seen in the nucleus correlates with the level of full-length protein. These data are consistent with the lower levels of protein seen in Type I patients relative to Type II and Type III patients.[14,15] SMN has also been shown to be associated with Bcl2 although these data remain to be confirmed by other groups.[16]

Sequence analysis has led to the identification of SMN orthologues in several organisms including *C. elegans* and *S. pombe*.[8] The domains conserved across these species are likely to indicate functionally significant regions. In particular exon 3, which is proposed to encompass a tudor domain, is highly conserved.[17] Tudor domains are present in proteins, which have an RNA binding function, suggesting a role in RNA metabolism.[18]

No studies have yet been reported on the effect of mutations in the tudor domain for SMN1. We therefore decided to express SMN1 with a missense mutation in exon 3 in HeLa cells to study its ability to form GEMs. This mutation has been reported in a Type I SMA patient and results in a change of a glutamic acid residue to a lysine.[3] In addition, we also expressed SMN1 with substitution of glutamic acid 104, a conserved residue in SMN1, and in a recently described SMN-related protein/SPF30 found in spliceosomes.[18] Our data show that substitution of either of these residues does not impair GEM formation.

Experimental Methods

Mutagenesis

QuikChange™ Site-Directed Mutagenesis (Strategene, Amsterdam, Netherlands) system was used to generate E104A and E134K using SMN in pcDNA3 expression vector as template.

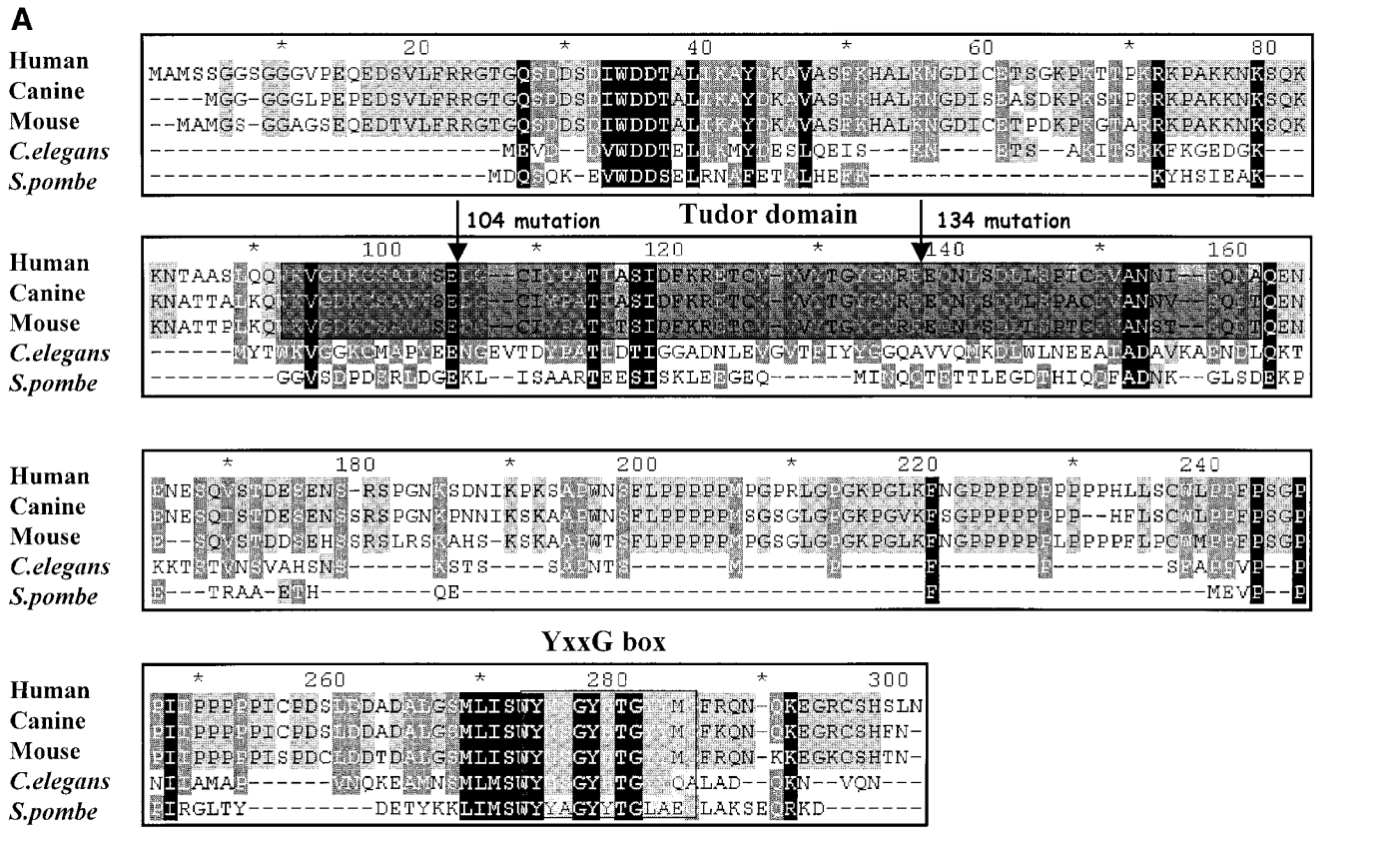
HeLa Cells in Culture and Immunofluorescence

HeLa cells were grown and transfected as previously.[18] Haemagglutinin (HA) antibody (Boehringer Mannheim, Mannheim, Germany) was used at 1:1500 dilution. We generated and purified a rabbit polyclonal antibody raised against a peptide corresponding to amino acid residues 170–187 in exon 4 of SMN, (SMN-ex4). SMN-ex4 was used at 1:500 dilution. SMN-C3 antibody[14] was used at 1:1000 dilution. Antibodies were incubated as previously[18] with HeLa cells and visualised using fluorescently labelled secondary antibody. Indocarbocyanide (Cy3) and fluorescein isothiocyanate (FITC) were at 1:1000 dilution. 4,6-diamidino–2-phenylindole (DAPI) counter staining was carried out by applying DAPI-Vectashield mounting medium and placing in humidity chamber overnight at 4°C.

Results and Discussion

In order to study the effect of the patient mutation at position 134 on the SMN1 protein (see Figure 1), a SMN1 mammalian expression construct was generated. The mutation was introduced using a PCR-based site-directed mutagenesis approach (see Experimental Methods). A second SMN1 expression construct was generated with a missense mutation at SMN1 amino acid position 104. This residue is absolutely conserved in human, mouse, zebrafish, *S. pombe* SMN (Figure 1a), and in human and *Drosophila melanogaster* SMNrp (Figure 1b). This construct contained a substitution of a glutamic acid residue 104 to an alanine (E104A). These two constructs were used for transfecting HeLa cells to study their localisation in GEMs. The detection of expressed protein in cells was performed using anti-HA antibodies. The controls used were SMN full-length (exon 1–7) construct, SMN exon 7 deleted construct (leading to loss of GEM formation), an SMN exon 5 deleted construct, where GEM formation is not lost, (exon deletion control).

Figure 2 shows the results of the transfection of SMN constructs into HeLa cells. The missense mutations in exon 3 of SMN clearly do not lead to loss of GEM formation (Figure 2a, 2c). The over-expression of SMN



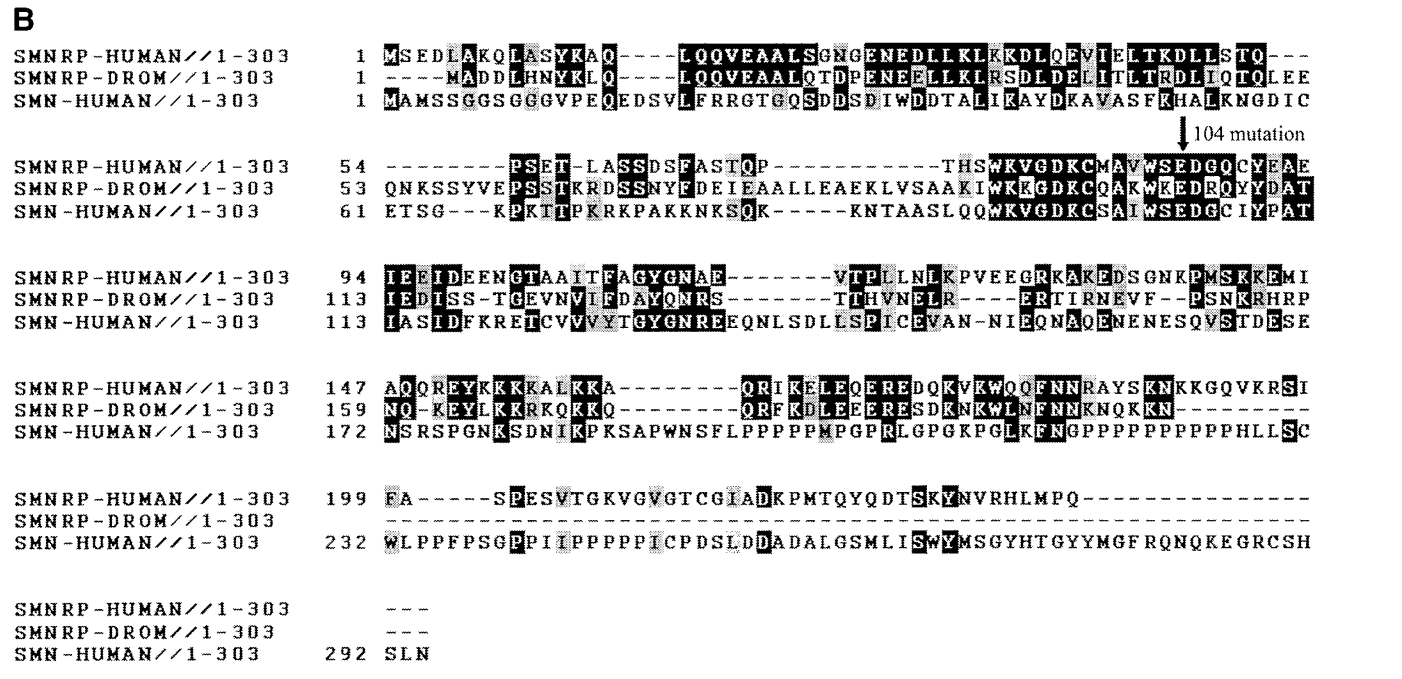


Figure 1 a: *Multiple alignment of SMN orthologous.* **b:** *Multiple alignment of human SMN-related protein (SMNRP),* Drosophila melanogaster *SMNRP, and human SMN; constructed using Clustal-X; showing the location of missense mutation in the SMN tudor domain, used in over-expression studies.*

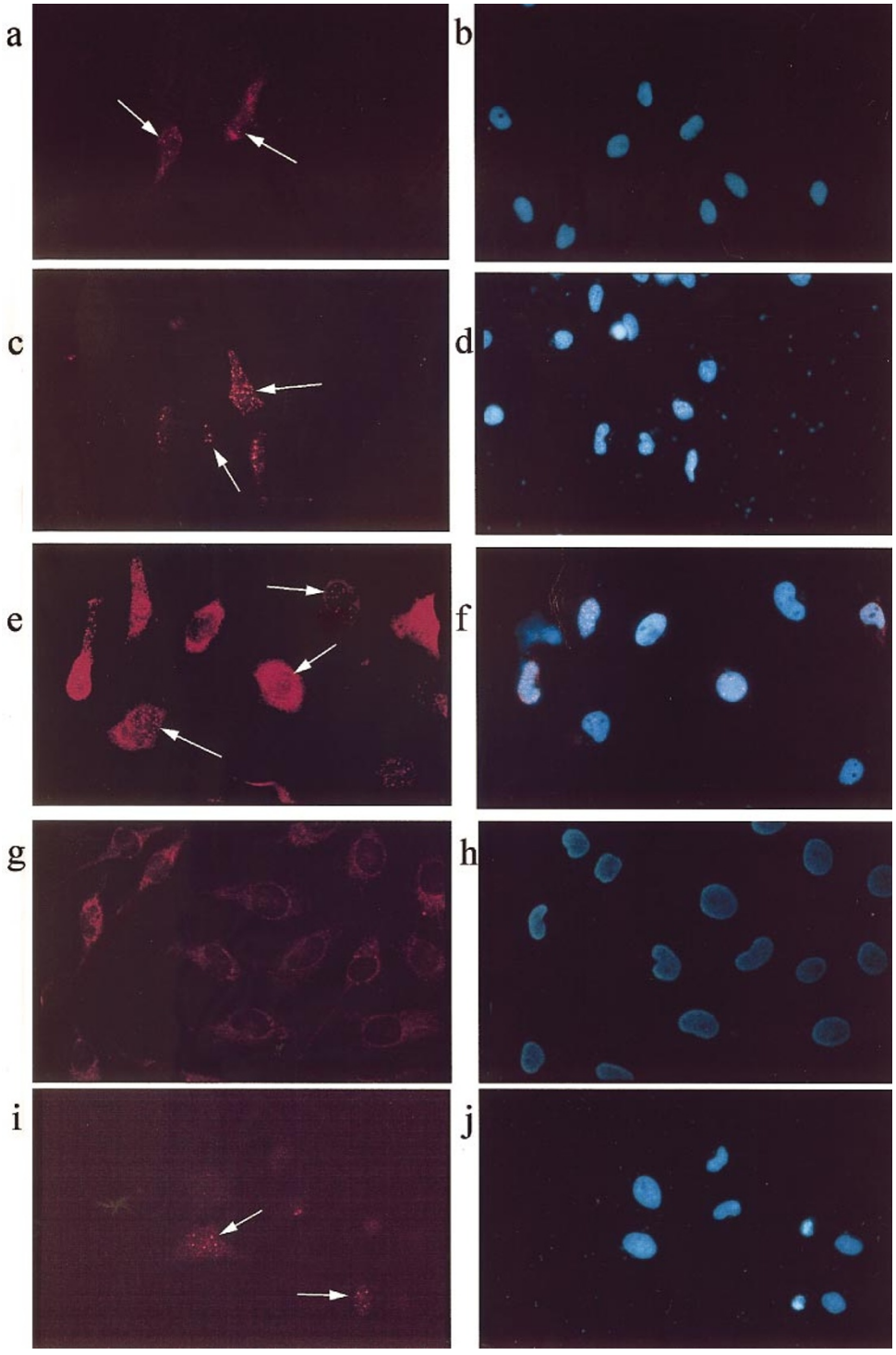


Figure 2 *Transfection of HA-tagged-SMN constructs into HeLa cells (arrows indicate the GEMs). Mag. 400 ×.* **a** *missense mutation E134K;* **c** *missense mutation E104A;* **e** *full length SMN;* **g** *SMN exon 7 deleted;* **i** *SMN exon 5 deleted;* **b, d, f, h, j** *Triple band filter: DAPI(blue) stain showing the nucleus of both transfected and non-transfected cells. The transfected cells over-expressing HA-tagged-SMN protein are detected by HA antibody and visualised with secondary Cy3 (red) antibody.*

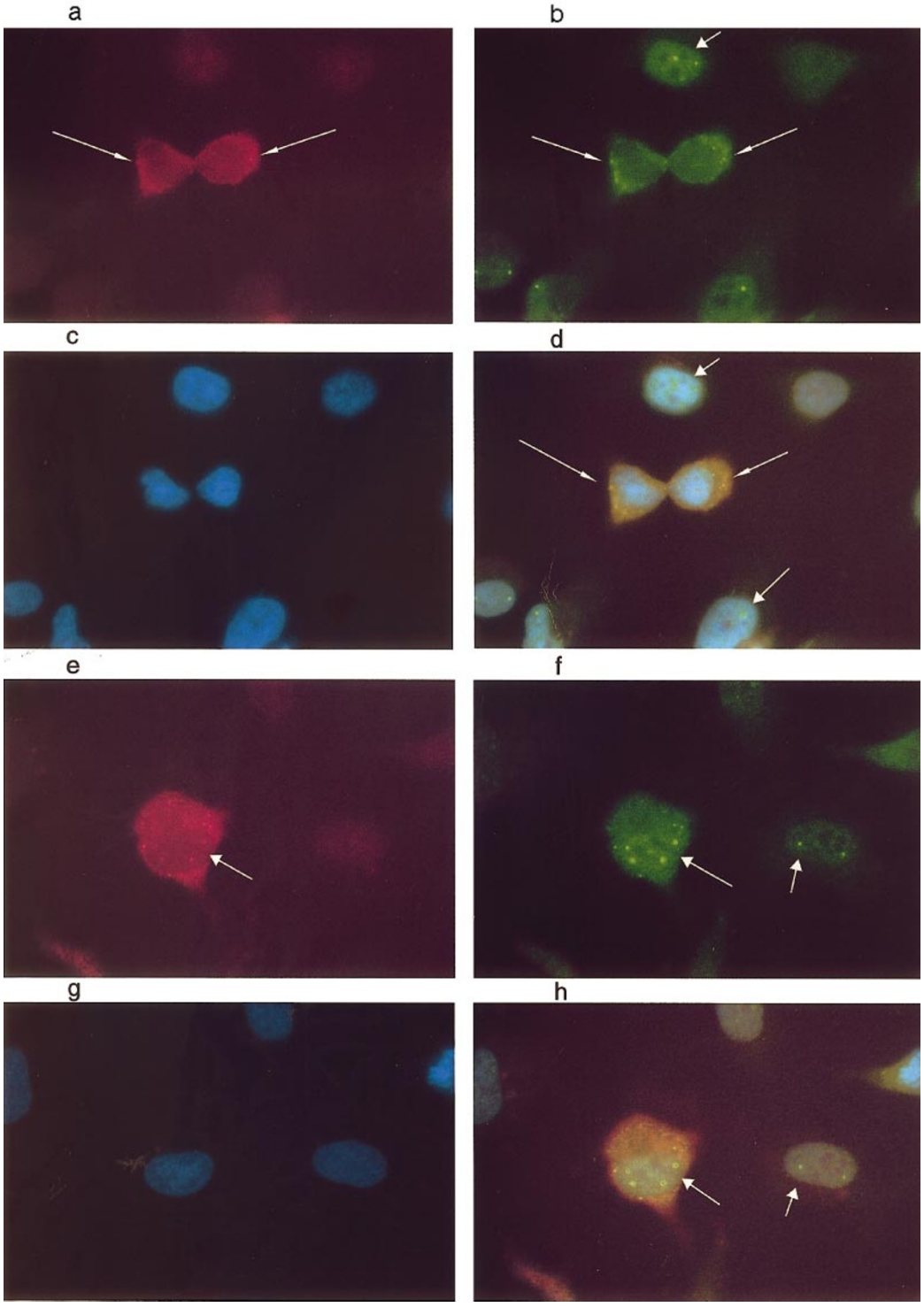


Figure 3 *Transfection of HA-tagged-SMN constructs into HeLa cells. Examining the effect of over-expression of SMN protein on the endogenous SMN protein. Mag. 1000 ×.* **a–d** *SMN exon 7 deleted construct. The two central cells are over-expressing the SMN7del leading to loss of GEM formation. The non-transfected cells: top centre and on bottom of picture show endogenous SMN and GEMs, detected by SMN-ex4 antibody.* **e–h** *SMN exon 5 deleted construct. Double antibody labelling:* **a, e** *showing the over-expressed HA-tagged SMN protein detected by HA antibody and visualised with secondary Cy3 (red) antibody.* **b**, **f** *SMN-ex4 antibody detecting both the endogenous and over-expressed protein, (FITC, green).* **c, g** *DAPI stain showing the nucleus of both transfected and non-transfected cells.* **d, h** *Triple band filter. Thick arrows pointing at GEMs, thin arrow pointing at cytoplasmic aggregates.*

proteins also leads to formation of cytoplasmic aggregates. However, deletion of exon 7 abolishes GEM formation consistent with the importance of the YG box[8] and residues in this region for SMN oligomerisation.[9] Furthermore on the same time course, the relative intensity of signal from exon 7 deleted over-expressed SMN protein in the cytoplasm (Figure 2g), to other over-expressed SMN proteins here (Figure 2a, b, c, l) appears weaker. This might be a reflection on the instability of exon 7 truncated protein or perhaps due to the inability of the protein to be transported across the nuclear member efficiently to form GEMs.

It has been proposed that SMN mutant proteins might exert a dominant negative effect on SMN function.[13,16] The deletion of the first 27 amino acids of SMN has such an effect.[13] In SMA patients the ratio between levels of truncated mRNA and protein from SMN2 to full length SMN mRNA[4] and protein[15] is increased. The truncated or mutant SMN protein may be able to interact with normal SMN protein and interfere with its function. We studied the over-expression of exon 3 mutant and the exon 5 deleted and exon 7 deleted SMN proteins to determine if they exerted such an effect *in vivo*. Double antibody labelling of transfected HeLa cells was carried out.

Figure 3 shows loss of endogenous SMN's ability to form GEMs in the nucleus after over-expression of exon 7 deleted SMN protein. The over-expressed exon 7-deleted SMN has a dominant negative effect on the endogenous SMN, resulting in loss/decrease in number of GEMs. The over-expression of full length SMN1, E134K mutant and SMN exon 5 deleted has no such effect and can lead to an increased number of GEMs. The over-expressed SMN tagged proteins were detected with anti-HA antibody (Figure 3 panels (a) and (e)). The SMN-ex4 antibody detected both the over-expressed and the endogenous protein (Figure 3 panels (b), (f)). This allowed tracking and localisation of the over-expressed mutant proteins in relation to normal endogenous protein. There was no detectable difference between the localisation of the over-expressed SMN proteins detected by HA-tagged antibody to endogenous SMN detected by SMN-ex4 antibody. However, over-expression of exon 7 deleted protein seems to interfere with endgenous SMN, as the SMN-ex4 antibody (Figure 3b) detected no GEMs. This appears to be dose dependent as transfected cells weakly staining with HA-tagged antibody still show some GEM formation. However the cells with highest expression of SMN7del are unable to form any GEMs. This provides direct evidence for deleted exon 7 protein exerting a dominant negative effect. The over-expression of E134K, exon 5 deleted, and full length protein have no such effect, they in fact result in an increased number of GEMs. In addition to SMN-ex4 antibody we also repeated these experiments using SMN-C3 antibody, for detection of endogenous SMN protein, and obtained a similar staining pattern.

In conclusion, an exon 3 missense mutation does not inhibit GEM formation, and is unable to exert a dominant negative effect on the formation of these structures. It is likely that the SMN tudor domain performs roles other than in GEM formation. Impairment of one of these functions still results in SMA as seen in one Type I patient. One possibility is that the ability of SMN to bind with one of its interacting proteins is lost. Further studies are under way to determine if mutations in the tudor domain would lead to loss of interaction with any of the SMN interacting proteins.

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