Differentiation

Supplementary data for:

Myhre syndrome is caused by dominant-negative dysregulation of SMAD4 and other co-factors

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Supplementary methods

Cellular fractionation

HEK293T cells were rinsed twice in cold PBS. Cells were then scraped and centrifuged at 450xg for 5 minutes. Cells were then lysed in 5x PCV (packed cell volume) of Lysis buffer (10 mM HEPEs pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, DTT and protease inhibitors) for 15 minutes on ice. The cells were centrifuged for 5 minutes and 420xg, and resuspended in 2xPCV. Cells were lysed using narrow-gauge hypodermic needle. The lysed cells were centrifuged for 20 minutes at 10000xg. The supernatant (cytoplasmic fraction) was removed to a fresh tube, and the nuclear pellet was resuspended in Extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, DTT, protease inhibitors). Nuclear pellet was incubated for 30 minutes and centrifuged for 5 minutes at 20000xg.

Case reports

Patient 1

Patient 1 is an eight-year-old boy born to healthy non-consanguineous parents. He presented with short stature, fusion of the second and third cervical vertebrae (Fig. 1a, Fig. S1a,b), left cryptorchidism, unilateral inguinal hernia, and attention deficit hyperactivity disorder. He was initially reported with mild developmental delay, but this was not confirmed formally, and subsequent clinical review showed normal development. Ophthalmic assessment revealed esotropia with anisometropic amblyopia and significant tortuosity to retinal vasculature, but there were otherwise no concerns with his vision. The patient was initially reported to have reduced hearing; however, formal hearing testing was normal. He has no history of seizures, cardiopulmonary symptoms, or gastrointestinal problems. There is no known history of genetic disorders in the family.

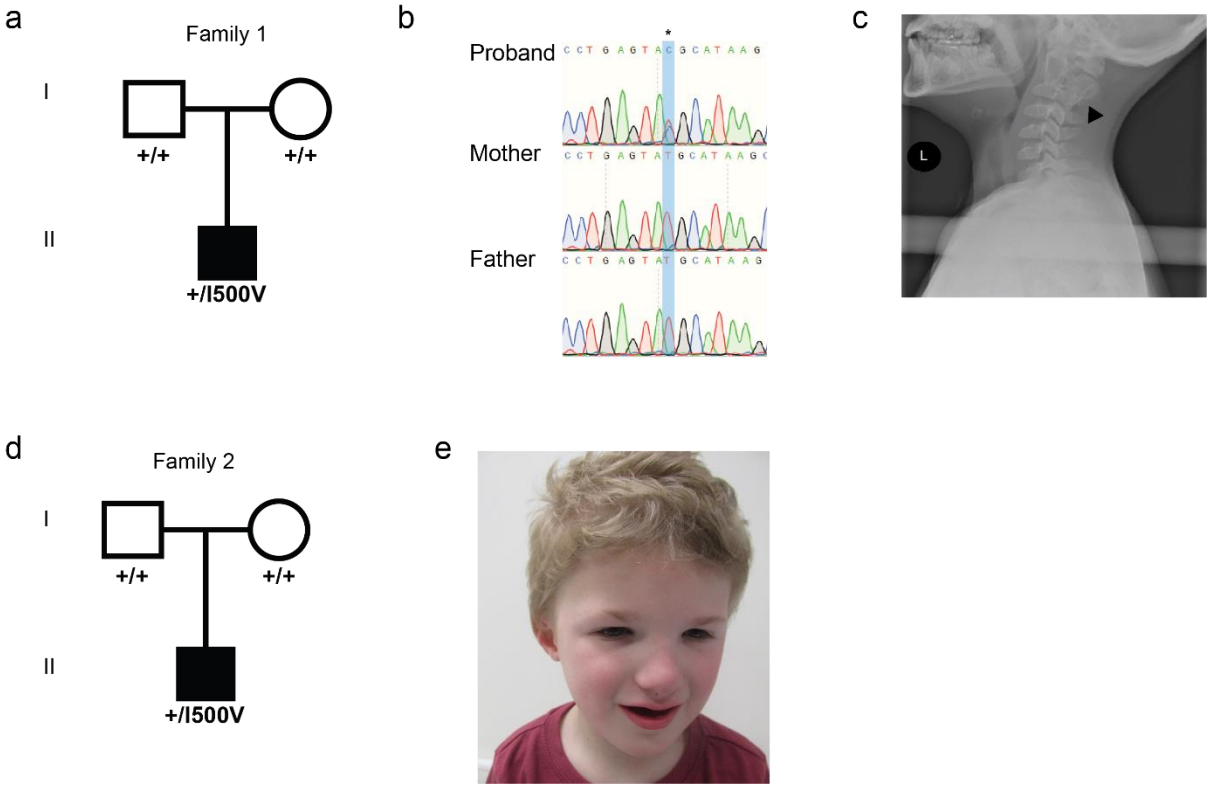
At 8 years of age, his height was 113.4 cm (<1st centile) and weight 20.3kg (3rd centile). He was reportedly normocephalic. He had no cleft lip or palate. He was not dysmorphic. His nuchal skin was normal and he had no cutaneous lesions. He had brachydactyly. Examination of his neurological, cardiac, respiratory and gastrointestinal systems was normal.

Skeletal survey revealed the segmentation defects of the cervical vertebrae, but no other bony abnormalities were reported. His bone age was 8 years at the chronological age of 8 years and 10 months. Research exome sequencing and analysis of the child-parents trio identified a *de novo* missense variant in NM_005359.6(SMAD4): c.1498A>G p.(Ile500Val). A novel *de novo* stop-gain variant in the gene *EXOC8* (NM_175876(EXOC8): c.2026C>T: p.(Gln676*)) was also found in this patient. Heterozygous truncating variants in *EXOC8* are not known to cause disease (Coulter et al. 2020).

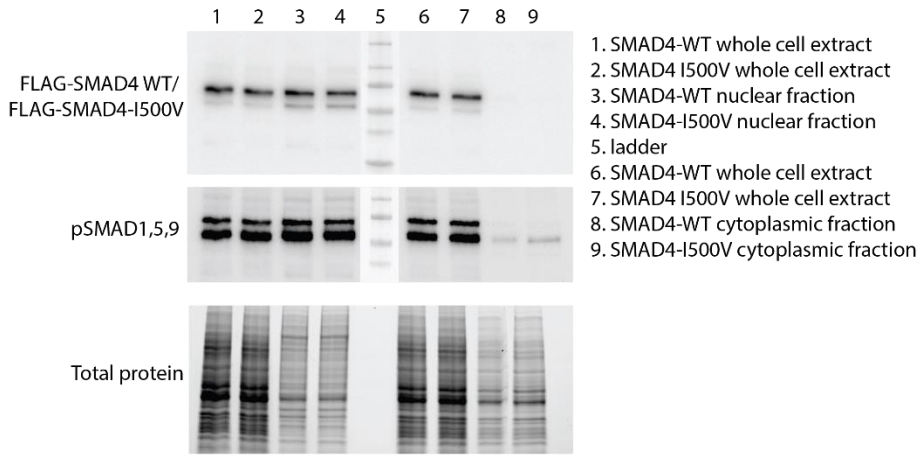
Patient 2

Patient 2 is a six-year-old boy referred to the genetics clinic with global developmental delay, autism spectrum disorder, attention deficit hyperactivity disorder, intellectual disability, hypotonia and short stature (Fig. 1b, Fig. S1c). He walked at 2 years of age, had fine motor difficulties, and was non-verbal. Examination revealed height of 110.9 cm (10^{th} centile), weight 18.3 kg (25^{th} centile) and head circumference 51 cm (50^{th} centile). He had deeply set eyes, short philtrum, thin vermilion of the upper lip, low columella, prominent ears, and prognathism. He had thickened skin over palms of hands and soles of feet and limited range of motion of the joints. MRI of the brain and chest X-ray were normal. No cardiac phenotypes were observed in the patient. The NM_005359.6(*SMAD4*): c.1498A>G p.Ile500Val variant was identified in the patient by commercial gene panel sequencing. The parents of the patient were not available for genetic testing.

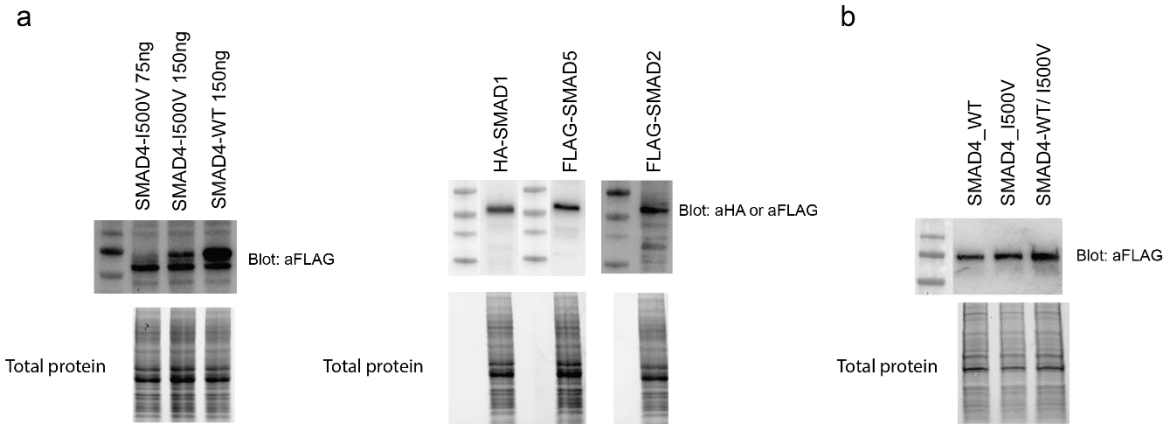
Supplementary fig 1 (a,b,c) Pedigree, Sanger sequencing, X-ray of the cervical vertebra showing the C2-C3 segmentation defects (arrowhead) for patient 1. (d,e) Pedigree of patient 2 and facial dysmorphism of patient 2 with deeply set eyes with down-slanted palpebral fissures, prominent ears, low columella, short philtrum, thin upper lip vermilion, and prognathism.



Supplementary fig 2 Expression of SMAD4-WT and SMAD4-I500V in nuclear and cytoplasmic cell compartments. Blots were probed with anti-FLAG or anti-pSMAD1,5,9.



Supplementary fig 3 Transfection controls for (a) figure 2 and (b) figure 4. Lysates from transfected cells for either the luciferase assays or qPCR were run on TGX-stain-free gels and probed with anti-FLAG or anti-HA to detect protein products.



Supplementary fig 4 Gene expression of (a) *SMAD4*, (b) *ID3*, (c) *COL1A1*, (d) *CTGF*, (e) *SMAD6* and (f) *SMAD7* were assessed in untransfected and transfected cells in basal, TGFβ1- or BMP4- treated conditions at 2 hours and 48 hours. Differences in expression between *WT-SMAD4* and *I500V* transfected cells in different treatment groups were statistically analyzed by two-way ANOVA and presented as mean ± SD, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, n=3.