

Journal Pre-proof

Clinical and genetic delineation of autosomal recessive and dominant ACTL6B-related developmental brain disorders

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ACTL6B-related neurodevelopmental disorders

Journal Pre-proof

RECESSIVE

54 biallelic variants

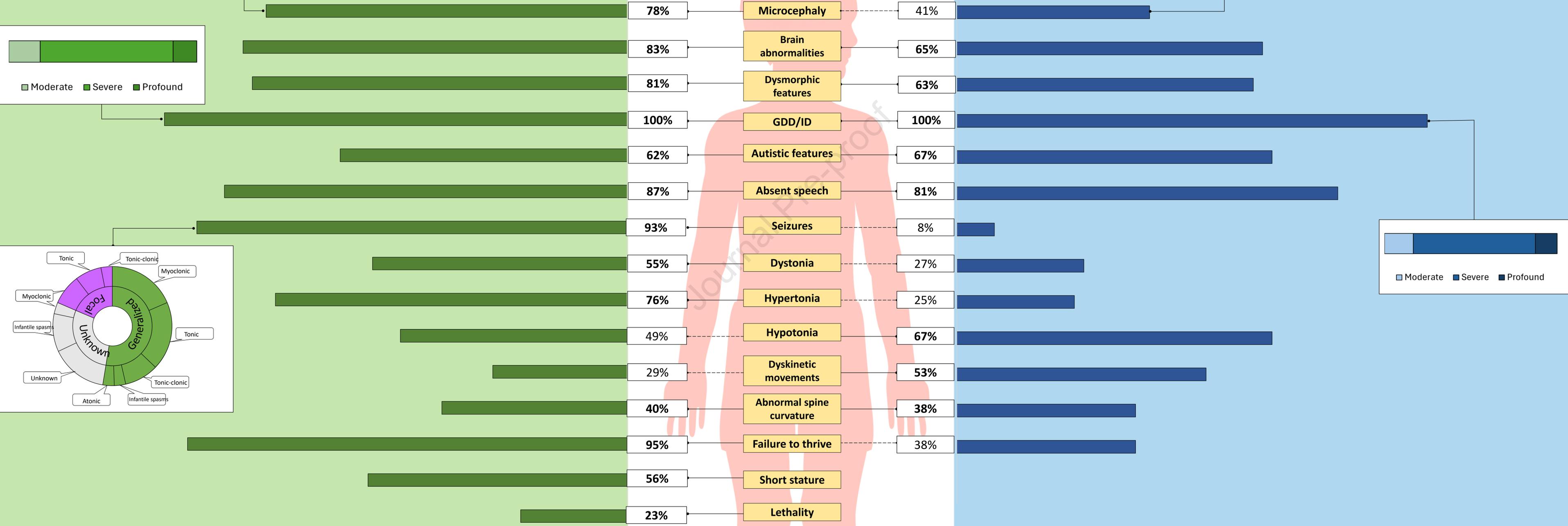
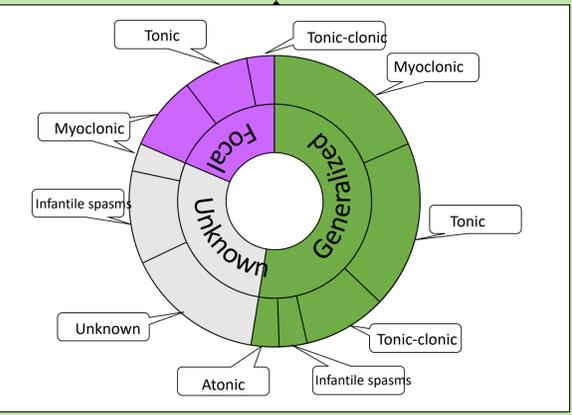
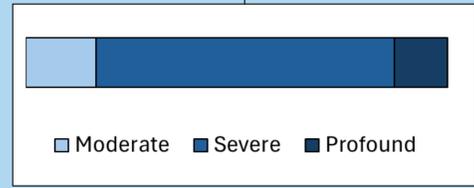
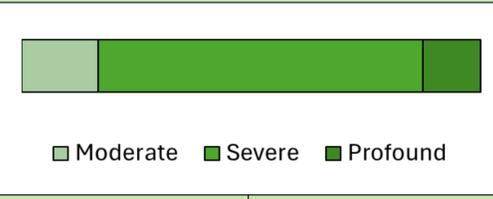
79 patients

26 patients

DOMINANT

4 monoallelic variants

27% Congenital microcephaly



***Clinical and genetic delineation of autosomal recessive and dominant
ACTL6B-related developmental brain disorders***

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Abstract (max 200 words).

Purpose: This study aims to comprehensively delineate the phenotypic spectrum of *ACTL6B*-related disorders, previously associated with both autosomal recessive and autosomal dominant neurodevelopmental disorders. Molecularly, the role of the nucleolar protein *ACTL6B* in contributing to the disease has remained unclear.

Methods: We identified 105 affected individuals, including 39 previously reported cases, and systematically analysed detailed clinical and genetic data for all individuals. Additionally, we conducted knockdown experiments in neuronal cells to investigate the role of *ACTL6B* in ribosome biogenesis.

Results: Biallelic variants in *ACTL6B* are associated with severe-to-profound global developmental delay/intellectual disability (GDD/ID), infantile intractable seizures, absent speech, autistic features, dystonia, and increased lethality. *De novo* monoallelic variants result in moderate-to-severe GDD/ID, absent speech, and autistic features, while seizures and dystonia were less frequently observed. Dysmorphic facial features and brain abnormalities, including hypoplastic corpus callosum, parenchymal volume loss/atrophy, are common findings in both groups. We reveal that in the nucleolus, *ACTL6B* plays a crucial role in ribosome biogenesis, in particular in pre-rRNA processing.

Conclusion: This study provides a comprehensive characterization of the clinical spectrum of both autosomal recessive and dominant forms of *ACTL6B*-associated disorders. It offers a comparative analysis of their respective phenotypes provides a plausible molecular explanation and suggests their inclusion within the expanding category of 'ribosomopathies'.

Keywords: *ACTL6B*, epileptic-dyskinetic encephalopathy, autism, BAFopathies, ribosomopathies

Introduction

ACTL6B (actin-like 6b, HGNC:160) encodes an actin-related protein that constitutes part of the neuronal BRG1/brm-associated factor (nBAF) complex¹. The BAF complex, also known as SWI/SNF complex, comprises at least 15 subunits that induce conformational changes in nucleosomes, regulating chromatin structure and DNA accessibility in an ATP-dependent manner^{2,3}. The nBAF complex plays a key role in epigenetic regulatory mechanisms guiding neurodevelopmental processes, including neurogenesis (cell proliferation and differentiation), cell migration, maturation, and neuronal integration⁴.

ACTL6B (also known as BAF53B) is specifically found in nBAF complexes of post-mitotic neurons where it appears to play a critical role in activity-dependent dendritic outgrowth and neuronal maturation⁵. Deficits in distinct subunits of this complex have been linked to a range of syndromic or non-syndromic neurodevelopmental disorders (NDD). Pathogenic loss-of-function (LoF) or dominant negative (DN)/gain-of-function (GoF) variants in numerous subunits, such as *ARID1A*(HGNC:11110), *ARID1B*(HGNC:18040), *ARID2*(HGNC:18037), *SMARCC2*(HGNC:11105), *SMARCD1*(HGNC:11106) (LoF) and *SMARCA4*(HGNC:11100), *SMARCE1*(HGNC:11109), *SMARCB1*(HGNC:11103)(DN/GoF), cause Coffin-Siris syndrome which is an autosomal dominant disorder characterized by intellectual disability/global developmental delay (ID/GDD), speech impairment, feeding difficulties, coarse facial features, hypertrichosis, hypoplastic fifth finger and toenails, and corpus callosum agenesis⁶⁻⁸ (MIM 135900, 614607, 614608, 614609, 616938, 617808, 618027, 618362 and 618779). Furthermore, pathogenic heterozygous variants in *SMARCA2*(HGNC:11098) are associated with two different conditions: Nicolaides-Baraitser syndrome, characterized by severe GDD/ID, early-onset seizures, short stature, dysmorphic facial features, and sparse hair (MIM 601358), and a congenital disorder that comprises distinct facial features, blepharophimosis

and GDD (Blepharophimosis-Intellectual impairment Syndrome or BIS, MIM 619293)^{9,10}. Pathogenic variants in *BCL11A* are associated with an autosomal dominant intellectual developmental disorder with dysmorphic features and asymptomatic persistence of foetal haemoglobin, referred to as Dias Logan syndrome (MIM 617101)¹¹, while monoallelic variants in *BCL11B* are linked to either intellectual developmental disorder with dysmorphic facies, speech delay, and T-cell abnormalities (MIM 618092) or severe combined immunodeficiency with intellectual disability, spasticity and craniofacial abnormalities (MIM 617237)¹². Biallelic pathogenic variants in *ACTL6B* have been associated with a subtype of developmental and epileptic encephalopathy (DEE) characterized by severe GDD, epilepsy, cerebral atrophy, and abnormal central system myelination (DEE76, MIM 618468)¹³⁻¹⁷. Additionally, two recurrent *de novo* missense *ACTL6B* variants have been linked with a dominant NDD characterized by intellectual disability, and severe speech and gait disturbances (Intellectual developmental disorder with severe speech and ambulation defects, MIM 618470)¹². Thirty-nine patients from 31 unrelated families carrying either biallelic (29 patients) or *de novo* (10 patients) variants have been reported in six separate publications since 2015. Nevertheless, a comprehensive study describing the full range of genotypic and phenotypic manifestations associated with *ACTL6B* variants is lacking.

In a high-throughput study, *ACTL6B* was tentatively assigned to the nucleolus¹⁸, a multi-layered biomolecular condensate where the initial steps of ribosome biogenesis occur. Ribosome biogenesis disorders, collectively referred to as ‘ribosomopathies’, encompass an emerging class of syndromes, including neurological disorders, caused by pathogenic variants in ribosomal proteins or in ribosomal assembly factors¹⁹. Despite partial elucidation of the nBAF complex and *ACTL6B* protein's role in neuronal development, especially in association with DEE or NDD, no study has suggested *ACTL6B*'s involvement in ribosome biogenesis so far. In this study, we define the spectra of *ACTL6B*-related disorders in 105 patients from 90

unrelated families, including 65 newly described patients harbouring either biallelic (n=50) or *de novo* (n=16) variants in *ACTL6B*. In addition, we report a significant role of *ACTL6B* in ribosome biogenesis, providing a likely molecular explanation for the associated disorders. Thus, we propose that *ACTL6B*-related disorders are new forms of “ribosomopathies”.

Methods

Patient ascertainment: clinical and genetic evaluation

The affected individuals were identified through extensive data sharing with collaborators and database screening of several diagnostic and research genetic laboratories worldwide, as well as using GeneMatcher²⁰. Informed consents were obtained from all study participants and/or their parents or legal guardians. Genome/exome sequencing was performed on genomic DNA extracted from blood in different diagnostic or research laboratories worldwide. When necessary, candidate variants were confirmed via Sanger sequencing in additional family members, especially in cases of presumed *de novo* variants or informative families with multiple affected or unaffected siblings. Comprehensive clinical data, including epileptic phenotype details (seizure duration/frequency) and family histories, were systematically collected from new and reported cases by recruiting clinicians and documented in structured data forms. . Where available, electroencephalogram (EEG) recordings, clinical photos, videos, and brain MRI scans were reviewed by a multidisciplinary team comprising child neurologists, epileptologists, dysmorphologists, and neuroradiologists.

In silico analysis

Information on the ACTL6B protein was obtained from the AlphaFold Protein Structure database²¹. Protein tertiary structures were visualized using PyMOL (Schrödinger, LLC) and the variants were modelled in Coot²². All variants were standardized to NM_016188.5 /GRCh38/hg38.

Cellular studies

Cells were cultured under standard conditions. Knockdown experiments were carried out by reverse transfecting SH-Sy5y cells with DsiRNA silencers (IDT), followed by total RNA extraction for subsequent denaturing RNA electrophoresis and Northern blot analysis. Western blotting was performed on cell lysates collected 72 hours post-DsiRNA treatment, with proteins separated by SDS-PAGE and transferred to a nitrocellulose membrane for detection using the BAF53B polyclonal antibody. Immunofluorescence assays were performed on SH-Sy5y and U2OS cells using antibodies targeting ACTL6B and nucleolar components. The acquired images were analysed using FIJI (ImageJ). Detailed explanation of the methods is available in the 'Supplementary Methods' section of the Supplementary Materials.

Results

Clinical Characterisation of Disease Spectrum

The comprehensive cohort, encompassing both autosomal recessive and dominant cases, comprises 105 patients from 90 unrelated families, with 62 females and 42 males. Of these, 65 individuals are newly described, with ages at last evaluation ranging from 3 months to 29 years (median 4, IQR 6.5). Table 1 provides an overview of the key clinical findings.

Autosomal recessive ACTL6B-related developmental and epileptic-dyskinetic encephalopathy

The autosomal recessive cohort comprises 46 females and 32 males from 65 families, with age at last evaluation ranging between 3 months and 17 years (median 4, IQR 7). Consanguinity was reported in 40 families. Compound heterozygous biallelic variants occurred in 6 of the 16 families without a historical report of consanguinity. Pregnancy and delivery were unremarkable for 26 of the 34 patients with available information. Prenatal manifestations such as foetal abnormal or slow movements, oligohydramnios, and breech presentation, occurred in two foetuses requiring caesarean section. Preterm birth was reported in eight infants, while forty-one infants were born at term and one post-term (median 39 weeks, IQR 1.75). Birth parameters, including length and weight, when available, were within normal ranges for 85% and 89% of the newborns, respectively, while head circumference was reduced only in one case. Infantile hypotonia manifested in 34 infants (34/45, 76%). Failure to thrive (FTT) was documented in 95% of the children (53/56) and was likely due to feeding difficulties, often detected at birth or in infancy (42/46, 91 %) and requiring the use of nasogastric tube or gastrostomy (n=17).

Seizures manifested in 93% of the children (70/75) with the onset ranging from birth to 11 years (median age 2 months, IQR 6). A majority (81%) experienced their first seizure within the first year of life and 34% within the first month. In four individuals (6%) seizures occurred at birth and probably antenatally as well. Patients presented with both focal (n=18) and generalized (n=40) onset of seizures. Two exhibited focal onset seizures with subsequent generalization, while five experienced only focal-onset seizures, and twenty-six exclusively had generalized seizures. Seizure types were variable and included myoclonic (n=30, 8 focal, 17 generalized, 5 not specified), tonic (n=27, 8 focal, 17 generalized, 2 not specified), tonic-

clonic (n=11, 2 focal with secondary generalization, 10 generalized), atonic (n=3) and epileptic spasms (n=12, 2 generalized, 10 not specified). Seizure type and onset were not specified for sixteen and thirteen individuals, respectively. When data on frequency were available, 92% of the patients presented seizures daily (up to 20-25/day) and clustering was reported in 64% of the cases (20/31). Seizure duration ranged from a few seconds to 5 minutes, and status epilepticus was reported in ten children (12/32, 38%). When information on treatment was present, seizures were considered intractable in 88% of the patients. Treatment often included a combination of two to five antiseizure medications (ASMs), as seizures were rarely controlled by a single ASM (4/34, 12%). Although a distinct treatment pattern could not be determined, effective approaches often involved the combination of sodium valproate and other ASMs. Detailed information on the epileptic phenotype and individual treatment is available in Supplementary Table S3.

EEG abnormalities were recorded in 96% of the patients (53/55), including focal, multifocal, and diffuse epileptiform discharges, slowing of background activity, and subcortical changes. Notably, one patient exhibited EEG abnormalities despite the absence of clinical seizures. Although there were no distinctive or shared patterns among the abnormalities, certain age-related characteristics were found. A comprehensive summary of the results can be found in Supplementary Materials.

GDD and ID were reported in all the affected individuals (79/79, 100%). Motor delay was observed in 95% of individuals (62/65), with 74% failing to achieve unsupported sitting and 82% unable to accomplish walking (Figure 1D). A significant proportion (61/70, 87%) showed a complete lack of speech and language development, while other eight individuals (8/70, 11%) exhibited delayed speech, limited just to a few words. Ten patients had regression of motor and cognitive abilities (10/46, 22%). When formal cognitive assessments were performed, the degree of intellectual disability varied from moderate (n=3), to severe (n=29) and profound

(n=22). Behavioural abnormalities included attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), aggressivity, and sleep disturbance. Autistic-like features, such as sound hypersensitivity and stereotypic hand movements, were noted in 26 patients (26/42, 62%).

At the last evaluation, 78% of the patients (45/58) exhibited microcephaly (Z score range -7.5 - $+1.5$ SD). Notably, head circumference within normal ranges at birth suggested postnatal deceleration of head growth, indicative of progressive microcephaly. Tragically, 23% of patients succumbed to the disorder in infancy or early childhood (median age 2.83 years). The neurological examination showed limb hypertonia and spasticity in a substantial percentage of cases (53/70, 76%). Dystonia was observed in 55% of individuals (32/58), accompanied by increased deep tendon reflexes (35/52, 67%), muscle weakness (33/43, 77%), and axial hypotonia (34/70, 49%). The ophthalmological evaluation revealed a variable presence of refractive defects (n=9), strabismus (n=3), and nystagmus (n=4) (16/31, 52%).

Neuroimaging studies were conducted on 63 individuals. Among these, 28 underwent independent review, while radiology reports were available for 35. Of those with imaging available for independent review, most cases had low-resolution snapshots of brain MRIs and three had DICOM files. 52 individuals (83%) exhibited abnormal findings, while 11 reportedly had a normal study (not independently reviewed). Of the reviewed cases (n=28), the most common abnormalities included hypoplastic corpus callosum (96%), widened subarachnoid spaces (89%), global brain atrophy (86%), ventricular enlargement/ventriculomegaly (57%), white matter volume loss (57%), mega cisterna magna/enlarged cisterna magna (50%), hypomyelination (43%), and patchy white matter signal abnormalities/focal white matter lesions (21%).

Facial photographs and/or videos were reviewed for 29 children from 23 families (Figure 2A). The predominant facial dysmorphic features observed in autosomal recessive *ACTL6B*-related NDD included narrow forehead/bifrontal/bitemporal narrowing (79%), full or broad nasal tip (76%), thick/full lower lip vermilion (65.5%), and broad, tall, or pointed chin (79%). Detailed facial dysmorphic features for all patients are shown in Supplementary Table S5 and tabulated using a unique Human Phenotype Ontology identification number to indicate the frequency of each feature. Supplementary Table S5 also includes patients for whom photographs were not available for assessment.

Autosomal dominant ACTL6B-related neurodevelopmental disorder

The cohort of cases with autosomal dominant (AD) inheritance comprises 16 females and 10 males, with their age at last evaluation ranging from 1 to 29 years (median 6 years, IQR 9). Consanguinity was reported in only 2 families. Limited information was available on the prenatal/perinatal period. When gestational age at birth was provided, delivery occurred after a full-term pregnancy in 93% of the cases, with one baby having a pre-term delivery. For those cases where birth parameters of length, weight, and occipitofrontal head circumference were provided, three newborns presented with normal ranges, while the other three were small for gestational age or had low birthweight, and three presented with microcephaly at birth (3/10, 30%). Most babies (87%) exhibited infantile hypotonia (13/15). Feeding difficulties and FTT were present in 53% and 38% of the cases, respectively. Epilepsy occurred only in two children (2/25, 8%). One child presented with infantile spasms at 3 months, which subsided at 18 months, unresponsive to vigabatrin, and subsequently developed focal tonic-clonic seizures with secondary generalization at 18 years. In the other affected individual, generalized seizures began at 1.6 years of age, with each episode lasting 30 seconds and occurring daily (up to

5/day). In both cases, seizures were responsive to ASMs. EEG recordings were not available in either of the cases. EEG abnormalities were reported in only one patient with no clinical seizures (1/8; 12.5%), Particularly, irregular, slow-wave/sharp-wave complexes and diffusely disorganized and slow background activity were documented. All affected children manifested GDD and ID (26/26). Motor delay was documented in 96% of the patients. Half of the cohort had not achieved independent sitting or walking at the last evaluation, with median ages of achievement of unsupported sitting and walking being 2 and 4 years, respectively (IQR 0.8 and 3.5). Absent speech was reported in 81% of the children (21/26), while in the other five children (19%) speech development was delayed (median age at first words 1.5). When formal cognitive assessment was performed, the degree of intellectual disability ranged from moderate (n=4), severe (n=17) to profound (n=3). Autistic features were present in 14 patients (67%). At the last evaluation, less than half of the patients (9/22, 41%) had microcephaly. Neurological examination showed axial hypotonia in 67% of the patients (14/21), and variable presence of hypertonia (5/20, 25%) and dystonia (4/15, 27%). Fourteen patients with AD disorder underwent neuroimaging studies, with twelve having only radiology reports available and two whose imaging were independently reviewed. Brain abnormalities were detected in 86% of the cases who had neuroimaging performed and common findings included: prominence of the subarachnoid spaces (29%), hypoplastic corpus callosum (21%), brain atrophy (21%), ventricular enlargement (21%), hypomyelination (21%), and mega cisterna magna (14%) (Supplementary Table S4). Facial photographs and/or videos from 10 children across 10 families were subjected to review (Figure 2B). Based on this assessment the most prevalent facial dysmorphic features include narrow forehead/bifrontal/bitemporal narrowing (60%), full or broad nasal tip (60%), thick/full lower lip vermilion (50%), and broad, tall, or pointed chin (70%). Detailed facial dysmorphic features for all patients are presented in Supplementary

Table S5, tabulated using unique HPO IDs to demonstrate the frequency of each feature. Supplementary Table S5 also includes patients without available photographs for assessment.

Identification of biallelic and monoallelic *ACTL6B* variants delineate an allelic series.

A total of 58 *ACTL6B* variants (n=54 biallelic; n=4 monoallelic) have been identified. Of those, 30 were novel and not previously reported in the literature. An overview of the molecular findings is available in Figure 3 and Supplementary Table S6-S7. Notable, all identified variants were consistently predicted as detrimental across a suite of in silico tools, impacting highly evolutionarily conserved residues. According to the American College of Medical Genetics (ACMG) classification, 35 were classified as pathogenic, 20 as likely pathogenic, and 3 as variants of uncertain significance (VUS). The biallelic variants identified in this study were found to be inherited from unaffected heterozygous parents. Specifically, among the patients, 60 inherited the variant in a homozygous state, while 14 patients inherited it in a compound heterozygous state. The molecular spectrum hereby described includes 24 nonsense variants (comprising 8 frameshift and 16 stop-gain), 18 missense variants, 7 splicing variants and 5 deletions.

Remarkably, all biallelic variants were either absent or found at very low allele frequencies in multiple variant frequency databases, ranging from 0.0-0.00002, (refer to Supplementary Table S6 for details). Notably, six variants were observed in more than one family. Particularly, variants p.(Phe147del) and p.(Ter427AspextTer33) were found in eight and four independent families from different countries, respectively. This underscores the global relevance of these specific variants in the context of *ACTL6B*-disorders. These variants were divided into distinct groups based on their consequence, including missense, in-frame deletion, truncating,

frameshift, and pre-mRNA splicing. Comprehensive comparisons were conducted across these genotypic groups and within individual variants in terms of clinical features. No evident genotype-to-phenotype correlation was identified in the recessive cohort (Figure 3). Four monoallelic variants were detected in the cohort, and all were *de novo* missense variants that were absent in several genetic variant frequency databases (listed in Supplementary Table S7). Notably, three of the variants occurred at the residue p.Gly343 in 24 independent families, indicating this location as a recurring hotspot for mutational events within a CpG dinucleotide²³. Interestingly, two of the variants lead to the same amino acid change p.(Gly343Arg).

ACTL6B is a nucleolar protein required for efficient ribosome biogenesis.

The subcellular distribution of endogenous ACTL6B was established by performing immunostaining in two distinct cell lines, namely SH-Sy5y (neuroblastoma) and U2OS (osteosarcoma). In neuronal cells (SH-Sy5y), ACTL6B exhibited a distinctive localisation pattern around the protein Pescadillo Ribosomal Biogenesis Factor 1 (PES1), forming a ring. PES1 is known to label the granular sub-compartment (GC) of the nucleolus, which is one of its most cortical parts. The ACTL6B ring colocalized perfectly with Nucleophosmin (NPM1), another component of the GC (Figure 4A). Therefore, ACTL6B is positioned at the periphery of the GC of the nucleolus, as further supported by similar observations in U2OS cells where ACTL6B also displayed a ring-like distribution (Figure 4B). To determine whether ACTL6B plays a role in ribosome biogenesis, particularly in pre-rRNA processing, SH-Sy5y cells were transfected with two independent silencers (#1 and #2) targeting the mRNAs encoding ACTL6B. After a 3-day incubation, total RNA was extracted and analysed by northern blotting using a probe detecting major pre-rRNA processing intermediates (Figure 4C). Ethidium

bromide staining of the agarose gel was also conducted to visualize the large mature rRNAs (18S and 28S) (Figure 4D). Efficient protein depletion was confirmed through Western blot analysis with a specific antibody (Figure 4E).

While the levels of large mature rRNAs were not significantly affected by *ACTL6B* depletion, notable inhibitions in pre-rRNA processing were observed. Specifically, there was an accumulation of the 30S pre-rRNA (indicating inhibition of cleavage at site A0 in pathway 2, see Figure 4F) and an accumulation of the 21S pre-rRNA (indicating inhibition at site C). These observed phenotypes were consistent with both silencers. Additionally, other phenotypes were observed only with one of the two silencers (#2), including a reduction in the primary transcript, the 47S (possibly reflecting an increased turnover), and a mild reduction of 12S (suggesting inhibition at site 3' and/or increased turnover). In conclusion,

In conclusion, *ACTL6B* is a protein that localizes primarily at the periphery of the nucleolus where it is important for efficient pre-rRNA processing and thus for optimal ribosomal subunit biogenesis.

Discussion

This study provides a comprehensive overview of *ACTL6B*-related disorders, including those associated with rare NDD inherited as autosomal recessive (biallelic) and dominant (monoallelic) traits. The core phenotype observed in both AR and AD disorders manifests as moderate-to-profound GDD/ID, minimal or absent speech, and autistic features. Overall, our findings are consistent with previously reported cases and emphasize the cardinal features and clinical synopsis of the disorder. A critical distinguishing factor between AR and AD disorders lies in the presence or absence of an epileptic phenotype. Strikingly, seizures occurred in 93% of the recessive cases, 88% of which were classified as intractable seizures. In contrast, only

two patients with monoallelic variants presented an epileptic disorder, effectively managed by ASMs. Such a marked contrast suggests a potential link to distinct pathomechanisms underlying these disorders. Unravelling the influence of these mechanisms on epilepsy's onset, progression, and response to treatment holds promise for tailoring more effective therapeutic interventions for affected individuals.

However, other clinical features exhibited a higher prevalence in the AR disorder when compared to the AD cases. Patients with AR disorder frequently presented FTT (95%) and short stature (56%), whereas FTT was less commonly observed in the AD cohort (38%), and instances of short stature were never reported.

Motor delay was documented in nearly all cases of both AR (95%) and AD (96%) disorders. Nevertheless, the severity of the delay differed between the two cohorts. The percentage of achievement of motor milestones (such as walking and sitting) was notably lower in the AR group (18-27%) compared to the AD group (39-70%). Intellectual disability (ID) was universally observed in all patients (100%). Absent or limited speech was documented in the majority of cases with AR and AD disorders. There was a notable difference in the degree of ID, with a higher proportion of patients with biallelic variants exhibiting profound ID (41%) compared to the AD cohort (13%). However, both groups presented with a spectrum of severity ranging from moderate to profound.

During the neurological assessment, a substantial number of patients with biallelic variants exhibited increased tendon reflexes (69%) and hypertonia/spasticity (77%), while axial hypotonia was present in nearly half of the cases (48%). Conversely, individuals with de novo variations predominantly showed axial hypotonia (67%), with fewer cases presenting hypertonia or spasticity (25%) and hyperreflexia (44%). Dystonia was more frequent in AR

disease (54% vs 27%), while dyskinetic movements were relatively more common in AD disorder (53% vs 30%). Autistic features were consistently present in both cohorts (62-67%).

Neuroradiological abnormalities were observed with in both groups, though the patients with AR disorder presented with a higher prevalence of abnormalities and more consistent findings of hypoplastic corpus callosum, parenchymal volume loss/atrophy, and prominence of the subarachnoid spaces. It is crucial to note a limitation in this comparison due to the restricted availability of imaging for AD cases. Only two MRI snapshots were accessible for independent review, both of which unveiled the same abnormalities found in the AR cohort.

Upon examination of the available photographs, the most common facial features observed in both groups consist of a narrow forehead/bifrontal/bitemporal narrowing, full or broad nasal tip, thick/full lower lip vermilion, and broad, tall, or pointed chin. Our evaluation indicates the absence of a recognisable facial gestalt for either AR or AD *ACTL6B*-related NDD.

This study emphasizes the cardinal and distinct features of the two *ACTL6B*-related disorders (DEE76 and IDDSSAD), providing a differential diagnosis for BAFopathies, which includes various NDDs resulting from mutations in different subunits of the BAF complex (Supplementary Table S8, Supplementary Figure 1). Monoallelic variants in genes encoding BAF complex subunits are linked to different forms of syndromic intellectual development disorders, including Coffin-Siris's syndrome and Nicolais-Baraitser syndrome. While these disorders share commonalities with *ACTL6B*-related disorders, including GDD/ID, microcephaly, short stature, and autistic features, they are distinguishable by characteristic facial and hand/digital features. Unlike *ACTL6B*-related disorders, epilepsy is usually not a prominent feature in Coffin-Siris syndrome and Nicolais-Baraitser syndrome and movement disorders or dystonia are not reported. Epilepsy is also extremely rare in the *ACTL6B* dominant cohort, suggesting its rarity in dominant BAFopathies.

Another autosomal recessive BAFopathy, involving biallelic variants in *SMARCD2*, has been reported. However, its distinct manifestations, centered around recurrent infections and hematologic abnormalities, set it apart from *ACTL6B*-related disorders. Although GDD/ID is observed, limited knowledge about its association with neurodevelopment complicates establishing a precise differential diagnosis. The nBAF complex plays a crucial role in various neurodevelopmental processes, influencing neurogenesis in terms of both cell proliferation and differentiation, cell migration, maturation, and integration of neurons. Recent studies have shed light on the involvement of the neuronal BAF complex in development of ASD. Pathogenic variants in genes encoding BAF complex subunits, such as *ARID1B*, *SMARCA2*, *SMARCC1*(HGNC:11104), and *SMARCA4*, have been linked to an elevated risk of ASD. Autistic features were frequently observed in both AR and AD cases of *ACTL6B*-related disorders, providing additional evidence of the connection between nBAF complex dysfunction and ASD. However, the intricate relationship between the two remains not fully understood, warranting further research to elucidate the underlying mechanisms.

Ribosome biogenesis dysfunction has been linked to tissue-specific disorders impacting primarily the brain, the blood, and the bones²⁴. Most of these diseases are deeply rooted in the embryonic development, and the brain disorders are no exception. It was notably shown using different animal models (frog, mouse, etc.) that optimal ribosome biogenesis is instrumental for proper maturation of neural crest cells, which are important for the development of the bone structure of the head and of the peripheral nervous system, among others^{25,26}. The importance of ribosome biogenesis in neurological disorders was also illustrated by mutations in a gene encoding the methyltransferase *METTL5-TRM112* acting on the small ribosomal subunit rRNA, the 18S. The absence of this methyltransferase leads to production of ribosomes lacking a single methyl group²⁷. This could be considered a rather insignificant difference considering the ribosome is a multi-mega-kDa nanomachine, yet it is enough to cause GDD, microcephaly

and associated manifestations²⁸, including ID and unbalanced walking, which could be recapitulated in a mutant mouse and fly, respectively^{29,30}.

Our study expands the genotypic spectrum of both *ACTL6B*-associated disorders, introducing 26 biallelic and 1 monoallelic variant that were not previously reported. All biallelic variants are anticipated to result in Loss of Function (LoF), according to Bell et al⁵, and are distributed across the protein in intra-domain structured regions³¹. Conversely, monoallelic variants impacting the amino acid residue p.Gly343 are expected to exert Dominant Negative (DN) effects, influencing the affinity to nucleosomal DNA by introducing a side chain upon mutation variation to arginine or tryptophan^{5,31}. *ACTL6B* is among the first genes of the multisubunit protein BAF complex for which both recessive and dominant variants are pathogenic, with distinct consequences on protein function (LoF vs DN). Pathogenic monoallelic variants in other BAF complex genes are predicted to result in either LoF (*ARID1A*, *ARID1B*, *ARID2*, *SMARCC2*, *SMARCD1*) or DN/GoF (*SMARCA4*, *SMARCE1*, *SMARCB1*). Furthermore, disruptions in various structural hubs of the BAF complex (e.g., histone binding, catalytic ATPase, ARP module) might contribute to the variability in symptoms and severity observed in different BAFopathies³¹.

This study also investigates the role of *ACTL6B* in ribosome biogenesis and provides a likely molecular explanation for *ACTL6B*-related disorders. *ACTL6B* had been assigned tentatively to the nucleolus¹¹, a multilayer biomolecular condensate where ribosome biogenesis is initiated. Our study has confirmed the localization of *ACTL6B* in the nucleolus and has revealed the protein is enriched in a ring at the periphery part of the organelle. *In vitro*, experiments on model cells demonstrated that mature rRNAs levels were not grossly affected upon *ACTL6B* depletion. However, the protein is required for efficient pre-rRNA processing at specific cleavage steps, therefore its dysfunction does compromise important steps of ribosome biogenesis. Ribosome biogenesis disorders, collectively referred to as

ribosomopathies, designate an emerging class of syndromes, including neurological disorders, caused by a pathogenic variant in ribosomal proteins or in ribosomal assembly factors. The association between ribosome biogenesis dysfunction and NDDs has only started to unveil. Future research is warranted to establish and much remains unknown about these disorders' key features. As our understanding evolves, *ACTL6B*-associated disorders are proposed to be included among the expanding spectrum of ribosomopathies (Supplementary Table S9).

While our study provides important insights into *ACTL6B*-related disorders, it is important to acknowledge the potential limitations arising from our relatively small and homogenous sample size. This constraint may not fully encompass the genetic and phenotypic diversity present within the broader population affected by these conditions. Nevertheless, our work offers a comprehensive overview of *ACTL6B*-related disorders, including the disease spectrum, genotype-phenotype relationships, and differential diagnosis based on mutational status. These insights contribute valuable information for improving clinical diagnosis and guiding therapeutic approaches. Additionally, our study proposes a probable molecular explanation for the pathology: inefficient ribosome biogenesis. This process has been previously linked to defects in the brain (neurodevelopmental and neurological issues) and bones (skeletal development, short stature, etc.). Therefore, our findings improve the understanding of the *ACTL6B*-related syndrome and contribute to the expanding knowledge of diseases associated with ribosome biogenesis dysfunction.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. For families R9, R16 and D5, data is available

in the National Genomics Research Library v5.1, Genomics England (doi:10.6084/m9.figshare.4530893/7. 2020).

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Author contributions

Conceptualization: E.C., T.Q., C.R., S.E., D.L., H.H., R.M.; Data curation: E.C., D.M., M.S., A.R., P.S., A.M.D.L., R.M.; Formal analysis: E.C., T.Q., D.L., R.M.; Funding acquisition: H.H., J.R.L., J.E.P., D.M., D.P., R.D., P.S., T.M.; Methodology: E.C., T.Q., C.R., M.S., R.D., A.M.D.L., S.E., D.L., R.M.; Supervision: D.L., H.H., R.M.; Writing-original draft: E.C., T.Q., M.S., R.D., A.M., D.L., R.M.; Writing-review & editing: all authors.

Ethics declaration

Individuals (and/or their legal guardians) recruited in a research setting gave informed consent for their research participation. Those individual research studies received approval from the Review Boards and Bioethics Committees at University College London Hospital (project 06/N076) and the other institutions involved in this study. Permission for inclusion of their anonymized medical data in this cohort, including photographs, was obtained using standard forms at each local site by the responsible referring physicians.

Conflict of interest

S.V.M. and A.B. are employees of GeneDx. L.H. is employee at 3billion. The remaining authors declare no competing interests.

Additional Information

The online version of this article contains supplemental material, which is available to authorized users.

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Figure Legends

Figure 1: Overview of dominant and recessive *ACTL6B*-related disorder

- a. Bar plot showing the frequency (%) of the main clinical features associated with *ACTL6B*-related disorder (on the left: recessive disorder; on the right: dominant disorder).
- b. Radar plot displaying the overlapping or distinguishable features of recessive and dominant disorder.
- c. Degree of global developmental delay (GDD)/intellectual disability (ID) in the disorders.
- d. Age span of achievement of developmental milestones in the recessive and dominant cohort compared to normal range. On the right, triangular radar plot showing the percentage of patients who achieved milestones at last evaluation.

Figure 2: Dysmorphology, neuroimaging and epileptic findings in *ACTL6B*-related disorder

- a. Facial photographs of 18 newly reported patients with recessive disorder.
- b. Facial photographs of 4 newly reported patients with dominant disorder.
- c. Box plot displaying head circumference measurements in standard deviations (SD) to the norm.
- d. Bar plot showing the frequency (%) of the main dysmorphic features.
- e. Bar plot showing the frequency (%) of the main neuroimaging findings.
- f. Epileptic phenotype of patients with recessive disorder. On the left, bar plot documenting age at onset; in the middle, sunburst chart displaying seizure types, on the right, box plots documenting seizure duration (seconds) and frequency (seizures/day).

Figure 3: Genetic findings and genotype-phenotype correlations

- a. Schematic drawing illustrating the gene structure of *ACTL6B* and the location of the biallelic and monoallelic variants identified. Green pentagon: missense variant. Red square: stop gain. Purple triangle: splice variant. Blue diamond: in frame deletion. Grey octagon: frameshift.
- b. Ribbon representation of a three-dimensional model of *ACTL6B* obtained with AlphaFold. The four missense variants identified in *ACTL6B* were modelled and are displayed in red. By analogy with actin, ATP is shown bound in the catalytic cleft of *ACTL6B*, and the four subdomains that characterize the reference actin fold are labelled (mauve-background circles).
- c. Partially transparent surface and ribbon representations of *ACTL6B*, showing the four missense variants in red. *ACTL6B* is a subunit of the BAF chromatin remodeler related to the yeast SWI/SNF remodeler, whose three-dimensional structure has been determined using cryo-electron microscopy. *ACTL6B* is most closely related to actin-related protein7 (ARP7) of yeast SWI/SNF. In the structures of yeast SWI/SNF, ARP7 makes contacts with other remodeler subunits. By analogy with ARP7, we show the surface of *ACTL6B* predicted to be involved in inter-subunit contacts within the BAF remodeler (yellow).
- d. Schematic table representation of the missense variants identified in *ACTL6B* and their localization/interactions.
- e. Sequence of *ACTL6B* highlighting residues undergoing missense variants (red) and residues likely to be involved in inter-subunit contacts within the BAF remodeler (yellow). The diagrams in parts d and e were generated with the program PyMOL (Schrödinger, LLC).
- f. Phenotype genotype correlations in the recessive cohort. Variants are grouped in the following categories: 1) missense, 2) in frame deletion, 3) truncating, 4) frameshift, 5) pre-

mRNA splice site, and 6) indel. Green: feature present; yellow: feature not present; blank: information not available. Blue rectangle: homozygous; blue triangle: compound heterozygous.

Figure 4: ACTL6B is a nucleolar protein required for efficient pre-rRNA processing.

a. Subcellular distribution analysis of ACTL6B in SH-SY5Y cells. Cells were labelled with an antibody specific to ACTL6B (in green) and co-stained with an antibody specific to the nucleolar protein Nucleophosmin (NPM1) or pescadillo (PES1) (in red). In the merge panels, the blue signal corresponds to the nucleoplasm (labelled with DAPI). Scalebar, 1 μ m.

b. ACTL6B was immunostained (in green) with a specific antibody in U2OS cells. Blue, DAPI. Scalebar, 1 μ m.

c. Pre-rRNA processing analysis upon ACTL6B depletion in SH-SY5Y cells. Cells were depleted with 25 nM siRNA for 3 d. Total RNA was extracted and separated on a denaturing gel, transferred to a nylon membrane, and processed for northern blotting with probes recognizing 5' ETS, ITS1 or ITS2 sequences. Two silencers (#1 and #2) targeting a distinct region of ACTL6B transcripts were used independently.

d. Mature rRNA analysis of samples described in panel c. Ethidium bromide staining revealed the steady-state levels of mature 18S and 28S rRNAs.

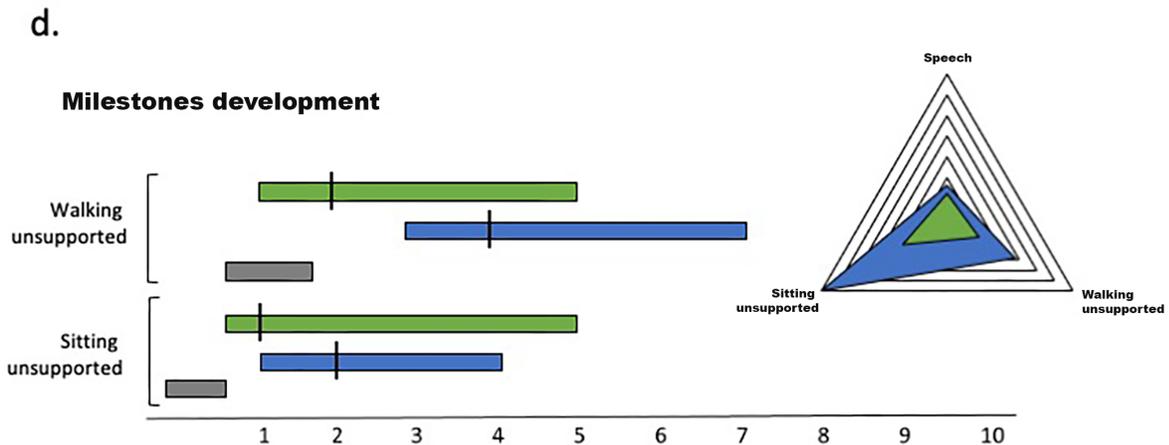
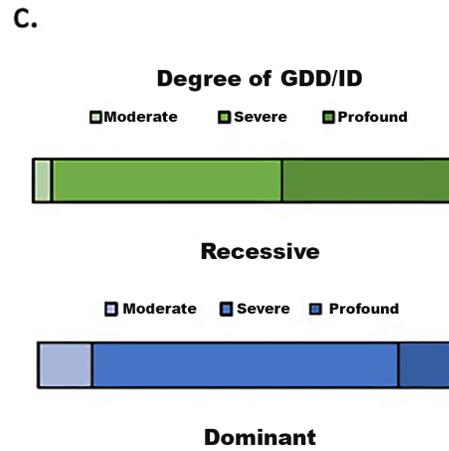
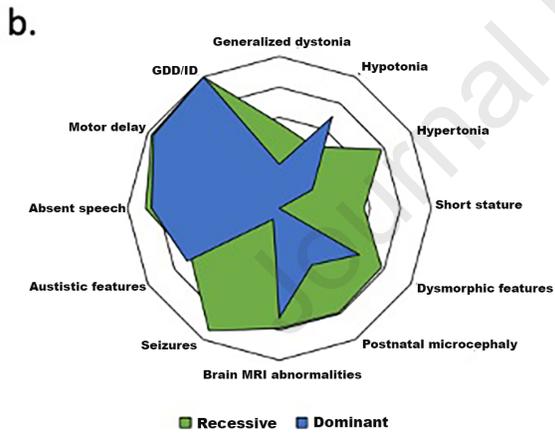
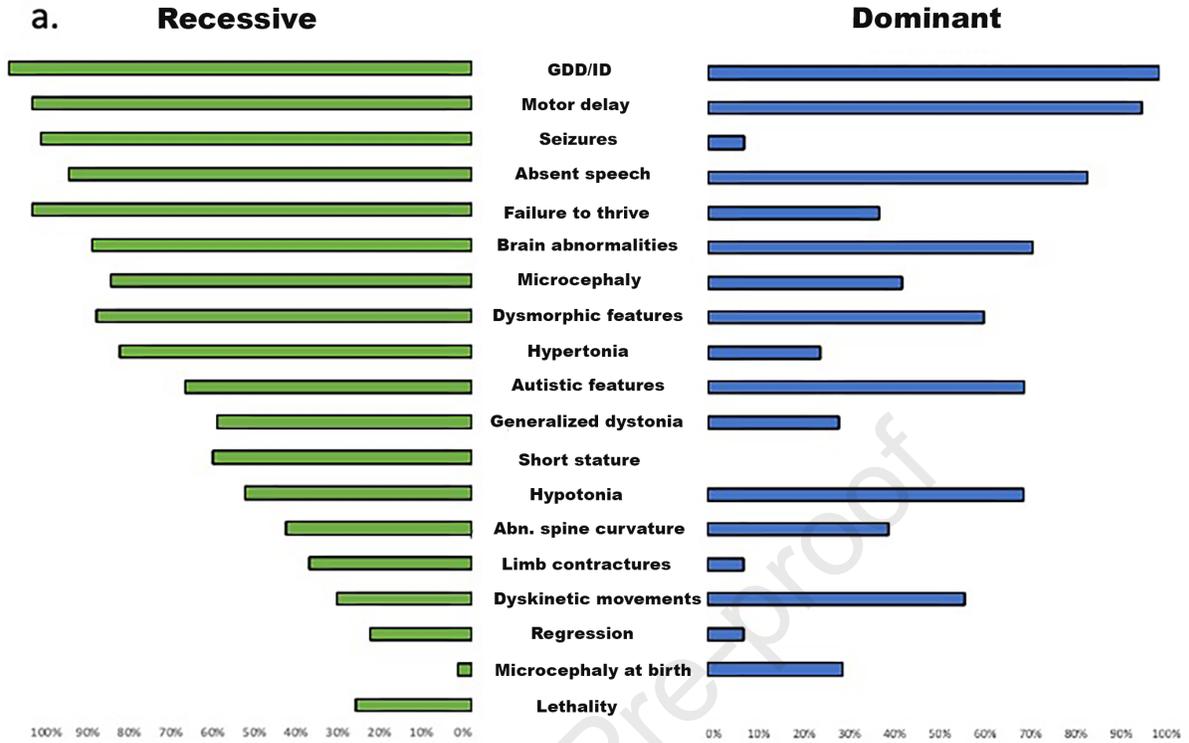
e. Western blot analysis confirming efficient protein depletion. The antibody used are described in the Materials and Methods. As control for loading, the blot was probed for β -actin.

f. Pre-rRNA processing pathway depicting major intermediates and probes used. See doi: 10.1016/j.biochi.2012.02.001³² for details.

Table 1. Overview of ACTL6B-related clinical features.

	HPO codes	Recessive cases			Dominant cases		
		Newly reported	Previously reported	Total	Newly reported	Previously reported	Total
Gender	-	29 F, 20M	17F, 12M	46F, 32M	8F, 8M	8F, 2M	16F, 10M
Median age at last follow-up (IQR)	-	4.875 (7.25)	2.25 (3.75)	4 (7)	6 (2.87)	5.7 (4)	6 (9)
Zygoty	-	42 H, 8 CH	23H, 6 CH	65 H, 14 CH	15DN	10DN	25DN
Growth							
Congenital Microcephaly	HP:0011451	1/27(4%)	-(0/7)	1/34 (3%)	2/9 (22%)	1/2 (50%)	3/11 (27%)
Progressive microcephaly	HP:0000253	24/34 (71%)	21/24 (88%)	45/58 (78%)	4/12 (33%)	5/10 (50%)	9/22 (41%)
Feeding difficulties	HP:0011968	41/45 (91%)	+(1/1)	42/46 (91%)	8/15 (53%)	n/a	8/15 (53%)
Failure-to-thrive	HP:0001508	40/42 (95%)	13/14 (93%)	53/56 (95%)	5/13 (38%)	n/a	5/13 (38%)
Short stature	HP:0004322	16/29 (55%)	7/12 (58%)	23/41 (56%)	- (0/14)	n/a	-(0/16)
Hypotonia in infancy	HP:0008947	33/44 (75%)	+(1/1)	34/45 (76%)	14/16 (88%)	n/a	13/15 (87%)
Motor development							
Motor delay	HP:0001270	45/48 (94%)	17/17 (100%)	62/65 (95%)	16/16 (100%)	8/9 (89%)	24/25 (96%)
Unsupported sitting not achieved	-	32/45 (71%)	5/5 (100%)	37/50 (74%)	3/10 (30%)	1/3 (33%)	4/13 (30%)
Unsupported walking not achieved	-	36/45 (80%)	5/5 (100%)	41/50 (82%)	7/12 (58%)	4/7 (57%)	11/19 (61%)
Intellectual development							
Absent speech	HP:0001344	39/48 (81%)	22/22 (100%)	61/70 (87%)	12/16 (75%)	9/10 (90%)	21/26 (81%)
Delayed speech	HP:0000750	8/48 (17%)	-	8/70 (11%)	4/16 (25%)	-	4/26 (15%)
GDD/ID	HP:0001263 HP:0001249	50/50 (100%)	29/29 (100%)	79/79 (100%)	16/16 (100%)	10/10 (100%)	26/26 (100%)
Mild-to-moderate	-	2	1	3	4	-	4
Severe	-	19	10	29	7	10	17
Profound	-	21	1	22	3	-	3
Autistic features	HP:0000729	17/32 (53%)	9/10 (90%)	26/42 (62%)	7/13 (54%)	7/8 (88%)	14/21 (67%)
Neurological examination							
Seizures	HP:0001250	44/49 (90%)	26/26 (100%)	70/75 (93%)	1/16 (6%)	1/10 (10%)	2/26(8%)
Increased tendon reflexes	HP:0001347	27/40 (68%)	8/12 (67%)	35/52 (67%)	4/9 (44%)	n/a	4/9 (44%)
Hypotonia	HP:0001252	24/45 (53%)	10/25 (40%)	34/70 (49%)	9/15 (60%)	5/6 (83%)	14/21 (67%)
Hypertonia/spasticity	HP:0001276	31/45 (69%)	22/25 (88%)	53/70 (76%)	5/15 (33%)	-(0/5)	5/20 (25%)
Muscle weakness	HP:0001324	31/41 (76%)	+(2/2)	33/43 (77%)	11/16 (69%)	n/a	11/16 (69%)
Dystonia	HP:0001332	19/42 (45%)	13/16 (81%)	32/58 (55%)	4/15 (27%)	n/a	4/15 (27%)
Limb contractures	HP:0003121	14/37 (38%)	-(0/3)	14/40 (35%)	1/14 (7%)	n/a	1/14 (7%)
Dyskinetic movements	HP:0100022	10/38 (26%)	2/3 (67%)	12/41 (29%)	8/15 (53%)	n/a	8/15 (53%)
Brain MRI abnormalities	HP:0410263	28/38 (73%)	24/25 (96%)	52/63 (83%)	10/13 (77%)	3/7 (43%)	13/20 (65%)
Miscellaneous							
Dysmorphic features	HP:0001999	30/39 (77%)	8/8 (100%)	38/47 (81%)	9/15 (60%)	6/9 (67%)	15/24 (63%)
Abnormal spine curvatures	HP:0010674	15/42 (36%)	+(3/3)	18/45 (40%)	6/16 (38%)	n/a	6/16 (38%)
Lethality	HP:0003819	12/32 (38%)	2/29 (6%)	14/61 (23%)	-	-	-

F: females. M: males. IQR: Interquartile Range. H: Homozygous. CH: Compound Heterozygous. DN: De Novo. GDD: Global Developmental Delay. ID: Intellectual Disability.



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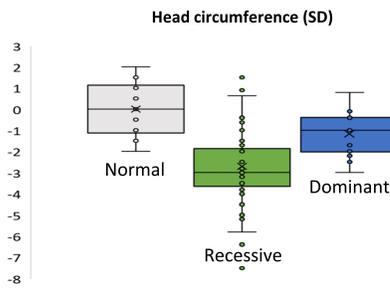
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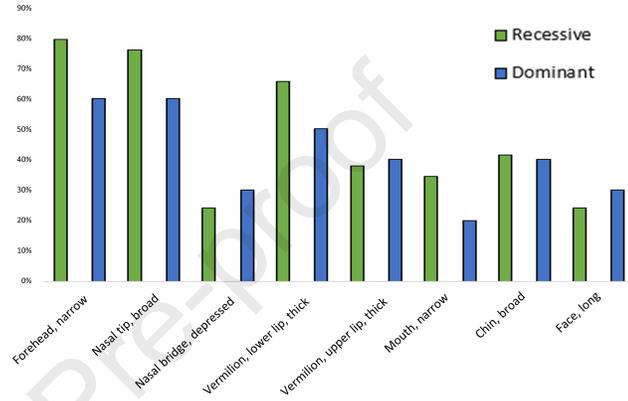
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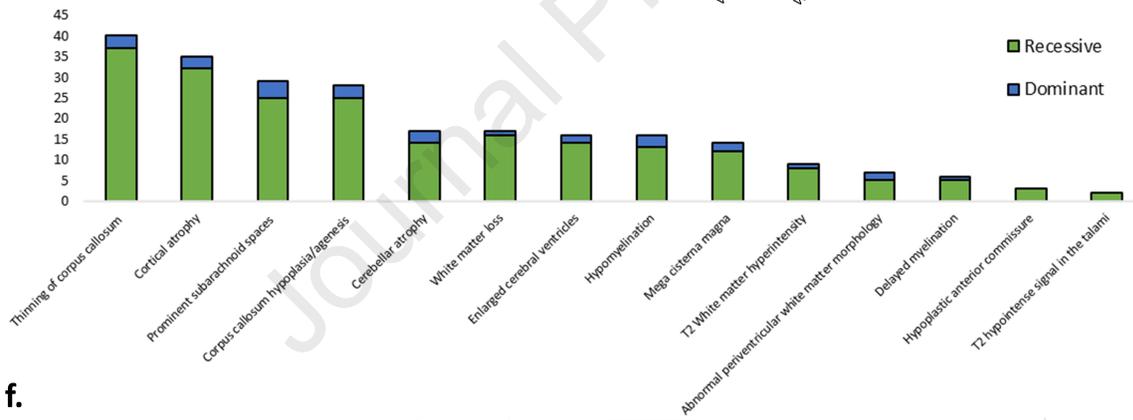
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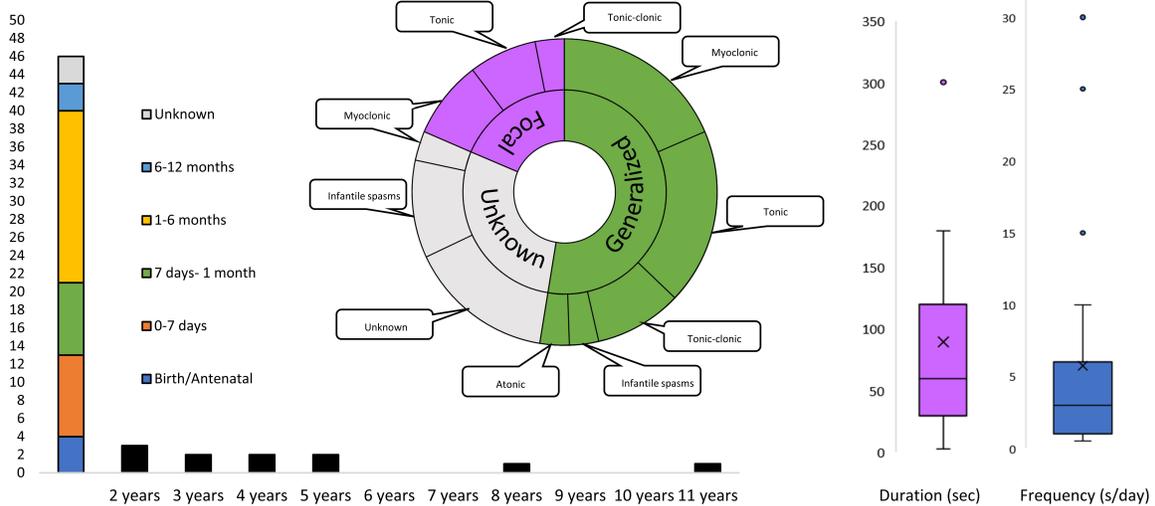
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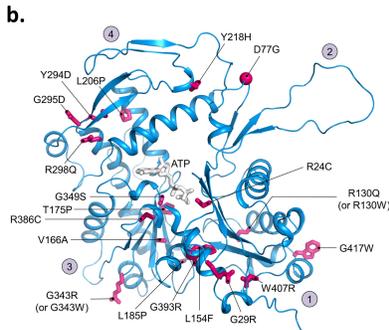
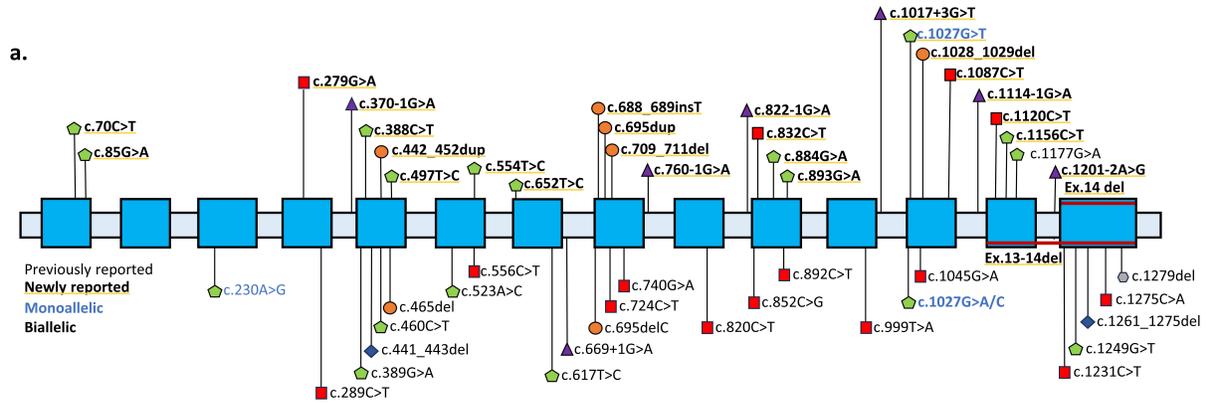


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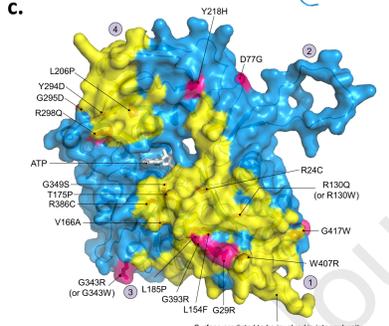
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d.

Mutation	Surface	Buried	Near BAF contact surface	Near ATP pocket	Inter-domain motions	Potential misfolding
R24C						
G29R	✓					
D77G	✓		✓			
R130Q		✓			✓	✓
R130W		✓			✓	✓
L154F		✓			✓	✓
V166A		✓			✓	✓
T175P		✓			✓	✓
L185P	✓		✓	✓		✓
L206P						✓
Y218H						✓
Y294D	✓		✓			✓
G295D	✓		✓			✓
R298Q	✓		✓			✓
G343R	✓		✓			✓
G343W	✓		✓			✓
G349S		✓		✓		✓
R386C		✓		✓	✓	✓
G393R		✓		✓	✓	✓
W407R		✓		✓	✓	✓
G417W	✓		✓			✓



e.

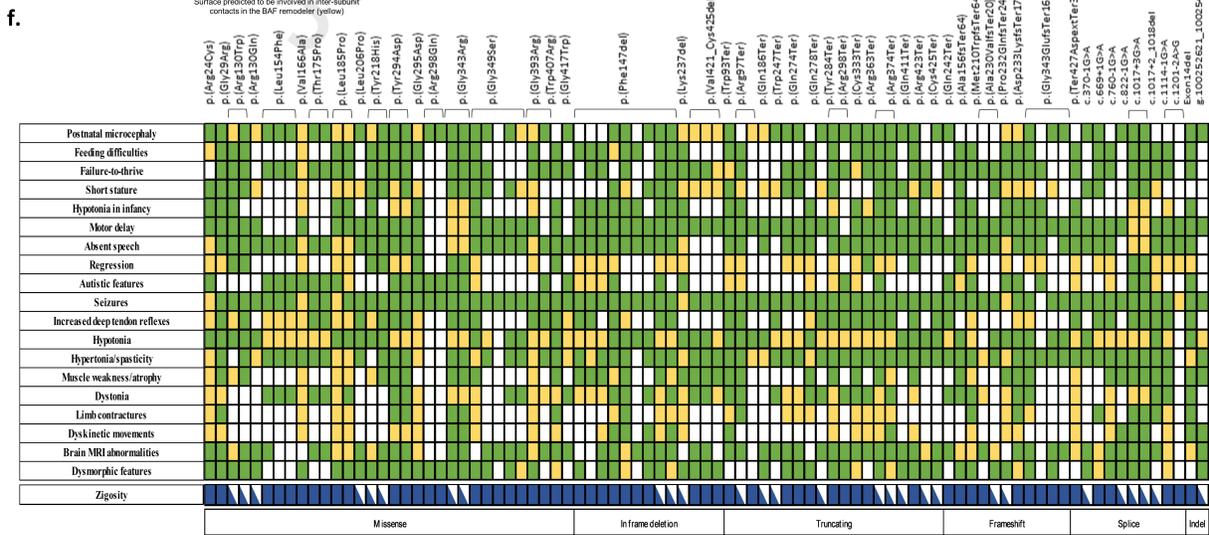
Legend:

- Newly-reported biallelic missense variants (Yellow)
- Previously-reported monoallelic missense variants (Light Blue)
- Previously-reported biallelic missense variants (Light Green)
- Newly-reported monoallelic missense variants (Light Purple)

Protein sequence (residues 1-400):

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10 20 30 40 50 60 70 80 90 100
MSGVYGGDE V GALVFDIGS FSVRAGTAGE DCFKADPTT VGLLAEEGG GLELEGDKREK KGIKFIH DYN ALIVRPAE VMSPLRNGMI EDWECFRALL
110 120 130 140 150 160 170 180 190 200
DHTYSKIVKES EPNLHPVIMS EAPNTRAKR EKILTELMFEQ YNI PFFLCK TAVITAFANG RSTGLDSDG ATHHTAIPVH DGVVQQGIV KSPLAGDFIS
210 220 230 240 250 260 270 280 290 300
MQCREPQEM AIDLIIPPAMI AAKLPVREGA FENWKKREKL PQVSKSWHNY MCNEVIQEPQ ASVLQVSDSP YDEVAAQMP TVHYEMPNQY NTUFAEELR
310 320 330 340 350 360 370 380 390 400
IPEGLFDFSN VKGLSGNTML GVGHVVTTSI GMCDIDIRPG LYR SVIVTSG NTLIQGPTDR LNRELSQKPT PSMRLKLIAS NSTNERRFPST RTGCGSIIAQL
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