REVIEW

New gene discoveries in skeletal diseases with short stature

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Abstract

In the last decade, the widespread use of massively parallel sequencing has considerably boosted the number of novel gene discoveries in monogenic skeletal diseases with short stature. Defects in genes playing a role in the maintenance and function of the growth plate, the site of longitudinal bone growth, are a well-known cause of skeletal diseases with short stature. However, several genes involved in extracellular matrix composition or maintenance as well as genes partaking in various biological processes have also been characterized. This review aims to describe the latest genetic findings in spondyloepiphyseal dysplasias, spondyloepimetaphyseal dysplasias, and some monogenic forms of isolated short stature. Some examples of novel genetic mechanisms leading to skeletal conditions with short stature will be described. Strategies on how to successfully characterize novel skeletal phenotypes with short stature and genetic approaches to detect and validate novel gene-disease correlations will be discussed in detail. In summary, we review the latest gene discoveries underlying skeletal diseases with short stature and emphasize the importance of characterizing novel molecular mechanisms for genetic counseling, for an optimal management of the disease, and for therapeutic innovations.

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Introduction

Genetic skeletal diseases, often called skeletal dysplasias, are a heterogeneous group of heritable conditions with generalized bone and cartilage impairment caused by pathogenic variants in genes primarily affecting skeletogenesis and/or bone homeostasis (1, 2). Although the overall incidence of genetic skeletal diseases is around one in 5000 births (3), some conditions are extremely rare and can only be found in a handful of families worldwide. In the last decade, the widespread use of massively parallel sequencing (MPS) has boosted the diagnostic rate of rare skeletal diseases and has led to the identification of several novel disease loci. According to the latest Nosology of Skeletal Disorders published in 2019, in total 461 skeletal diseases have been characterized so far. These diseases have been classified into 42 groups based on a combination of radiological findings, clinical features, and underlying molecular mechanisms (2).

Genetic skeletal diseases are characterized by a broad phenotypic heterogeneity. The clinical manifestations can vary greatly among patients with the same gene defect and significantly even among those harboring the exact same pathogenic variant. This broad phenotypic and genetic heterogeneity brings challenges in establishing efficient treatments. Presently, a pharmacological treatment is only available for a limited number of bone disorders, including various genetic forms of osteoporosis, FGF23-related hypophosphatemia (MIM 193100), hypophosphatasia (MIM 146300), and achondroplasia (4, 5, 6).

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Although genetic skeletal diseases most often feature short and disproportionate stature, sometimes normal or even tall stature, like in overgrowth syndromes, can be noticed. Moreover, short stature is sometimes part of a broader phenotypic spectrum that also includes other skeletal impairments, such as short extremities, skeletal deformities, high or low bone mineral density as well as extra-skeletal features, including impaired vision or hearing. The scope of this review is to describe the latest genetic discoveries underlying some severe skeletal diseases characterized by short stature.

Skeletal diseases with reduced linear growth

Longitudinal linear bone growth is determined by endochondral ossification, which is mediated by the growth plate, a complex cartilaginous structure located between the metaphysis and the epiphysis of the long bones (Fig. 1). From the epiphyseal end the growth plate can be subdivided into three different zones, each of which is composed of chondrocytes characterized by different size, shape, orientation, proliferative capacity, and function: (1) resting zone, (2) proliferative zone and (3) hypertrophic zone (7). The resting zone is a source of stem-like progenitor cells restoring the pool of proliferative chondrocytes (8). In the proliferative zone, the flattened chondrocytes divide longitudinally at a high rate and synthetize a large amount of ECM. Finally, in the hypertrophic zone chondrocytes stop dividing, become large in size, and start producing factors that trigger mineralization and the invasion of blood vessels, thus promoting chondrocyte apoptosis (9). Consequently, osteoblasts invade the hypertrophic zone and bone formation takes place. At the end of puberty, when linear growth no longer occurs, the growth plate fuses with the epiphysis.

Pathogenic variants in several genes encoding factors involved in chondrocyte proliferation and differentiation as well as defects in ECM components and cell-matrix interactions are known to be responsible for different monogenic skeletal conditions often accompanied by short stature (Fig. 1). Moreover, in some conditions growth plate defect can be secondary to abnormal bone metabolism and/or mineralization, such as in hypophosphatemic rickets (MIM 307800), in which hypophosphatemia leads to impaired chondrocyte apoptosis (10).

As mentioned previously, usually disproportionate short stature is not an isolated finding, but it is part of a wider spectrum of clinical features. From 2015 to 2019, pathogenic variants in 45 novel genes have been linked to skeletal diseases (2, 11). Among these, 15 novel gene defects, which have also been included in the Online Mendelian Inheritance in Man (OMIM) database, have been linked to either spondylometaphyseal dysplasia (SMD) or spondyloepimetaphyseal dysplasia (SEMD), two diseases that are mainly characterized by severe short stature and skeletal impairments affecting the spine, metaphyses and epiphyses (only SEMD) (Table 1) (2). In addition, pathogenic variants in the gene encoding the ribosomal protein eL13 (*RPL13*) have been recently linked to a novel form of SEMD (Table 1) (12, 13). These newly characterized conditions are caused by pathogenic variants in genes involved in several different biological and molecular processes (Fig. 2). Below, some examples of these conditions will be described in more detail.

Defects in fibronectin-1 cause SMD-corner fracture type

Fibronectin-1 (FN) is a dimeric glycoprotein that is abundant in several tissues and can be found either in a soluble form, like in the plasma, or as an insoluble multimeric fibrillar component of the ECM (14). FN is important for mesenchymal stem cell (MSC) differentiation and for deposition of collagen type I in the ECM (15, 16). Monoallelic pathogenic mutations in the fibronectin-1 gene (*FN1*) were first identified in 2017 in patients with a subtype of SMD, namely SMD with corner fractures (SMD-CF) (MIM 1842559) (17). Corner fractures are not considered as real fractures but they are key radiological findings appearing as lucent areas in the proximal metaphyses (growth plate) of tubular bones (18). SMD-CF was first described by Sutcliffe in 1966 and it was recognized as a separate entity in 1990 (19). Since then, approximately 30 families have been reported and while pathogenic variants in *COL2A1* were identified in some subjects with SMD-CF, most of the patients lacked a genetic diagnosis for several decades (17, 20, 21). Nowadays, 11 different disease-causing missense variants and a single amino acid deletion in *FN1* have been reported in 13 families with SMD-CF (17, 20, 21). In addition to corner fractures, patients typically feature short stature, developmental coxa vara, scoliosis, and abnormal ossification at the growth plate and secondary ossification sites.

FN is a dimer constituted by three types of modules, domains types I–III, which bind other components of the ECM, such as collagen, integrins and glycosaminoglycans as well as signaling and cell adhesion molecules (14, 22).

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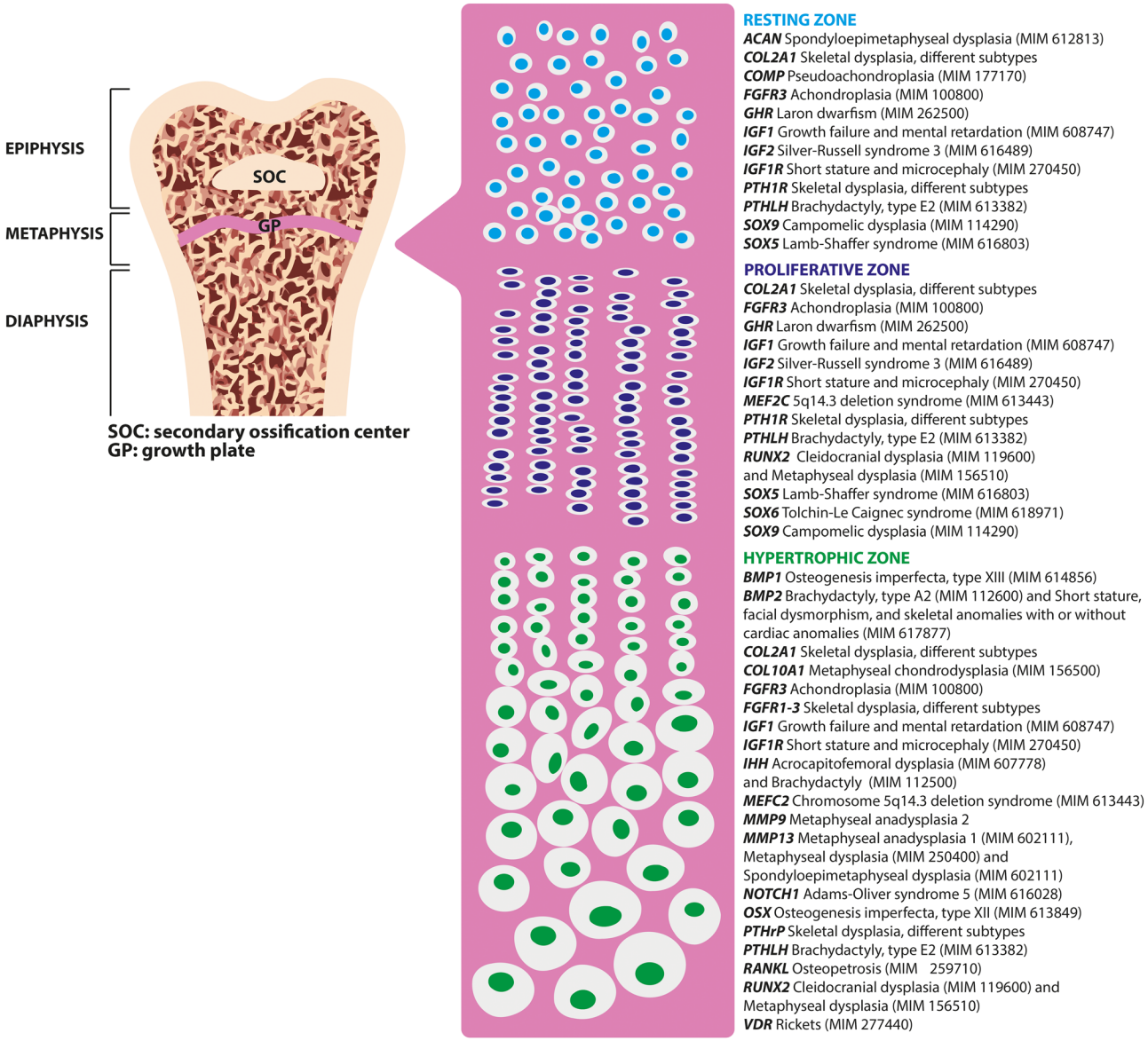

Figure 1
Structure of a long bone with major focus on the growth plate and the key genes regulating longitudinal bone growth. On the left, structure of a long bone. Pink panel: schematic representation of the chondrocytes within the three different zones of the growth plate. On the right, some key genes regulating longitudinal bone growth and list of monogenic skeletal conditions caused by pathogenic mutations in each of these genes (96, 97, 98). *ACAN*, aggrecan; *BMP1*, bone morphogenetic protein 1; *COL2A1*, collagen type II alpha 1 chain; *COL10A1*, collagen type X alpha 1 chain; *FGFR1-3*, fibroblast growth factor receptor 1-3; *GHR*, growth hormone receptor; *IGF1*, insulin-like growth factor 1; *IGF1R*, insulin-like growth factor 1 receptor; *IHH*, Indian hedgehog signaling molecule; *MEF2C*, myocyte enhancer factor 2C; *MMP9/13*, matrix metallopeptidase 9/13; *NOTCH1*, Notch receptor 1; *OSX*, Sp7 transcription factor; *PTH1R*, parathyroid hormone 1 receptor 2; *PTHLH*, parathyroid hormone-like hormone; *RUNX2*, Runt-related transcription factor 2; *SOX9/5*, SRY-Box transcription factor 9/5; *MIM*, phenotype OMIM number.

Interestingly, the majority of the pathogenic variants that have so far been linked to SMD-CF locate within the fibronectin type-I domains. In particular, nine out of the 11 reported variants associated with SMD-CF affect cysteine residues partaking in disulfide bonds, which maintain the highly organized structure of FN. Pathogenic variants in *FN1* impair the secretion of FN into the ECM in patient-derived fibroblasts as well as in HEK293 cells transfected with plasmid expressing mutant FN (17, 20). Surprisingly, pathogenic variants in FN domain type III cause another disease, named Glomerulopathy with fibronectin deposits 2 (phenotype MIM 601894). Renal dysfunction has not been reported in any patient with SMD-CF thus suggesting that the location of the pathogenic variant determines the development of either one or the other disease.

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Table 1 Novel genetic defects underlying SMD and SEMD.

Gene	Protein	Function	Disease	IP	OMIM phenotype MIM #	Reference
AIFM1	Apoptosis inducing factor mitochondria associated 1	Mitochondrial protein involved in oxidative phosphorylation and redox control in healthy cells	SEMD	XLR	300232	Mierzewska *et al*. 2017 (99)
BGN	Biglycan	Structural component of articular cartilage. It participates in the assembly of the chondrocyte extracellular matrix and it also plays a role in cell signaling	SEMD	XLR	300106	Cho *et al*. 2016 (100)
COL27A1	Collagen type XXVII alpha 1 chain	Plays a role during the calcification of cartilage and in the transition of cartilage to bone	SEMD	AR	615155	Gonzaga *et al*. 2015 (101)
EXTL3	Exostosin-like glycosyltransferase 3	Glycosyltransferase that catalyzes the transfer of N-acetylglucosamine to glycosaminoglycan chains. This reaction is important in heparin and heparan sulfate synthesis	SEMD	AR	617425	Volpi *et al*. 2017 (102)
FN1	Fibronectin 1	Glycoprotein binding cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin	SMD	AD	184255	Lee *et al*. 2017 (17)
HSPA9	Heat shock protein family A (Hsp70) member 9	Chaperone in the mitochondria, cytoplasm, and centrosome	SEMD	AR	616854	Royer-Bertrand *et al*. 2015 (103)
IARS2	Isoleucyl-TRNA synthetase 2, mitochondrial	Nuclear-encoded mitochondrial isoleucine-tRNA synthetase that catalyzes the attachment of an isoleucine residue to a cognate mt-tRNA	SEMD	AR	616007	Schwartzentruber *et al*. 2014 (104)
LTBP3	Latent transforming growth factor beta binding protein 3	Involved in TGF-beta signaling pathway	SEMD	AR	601216	Huckert *et al*. 2015 (105)
NANS	N-acetylneuraminate synthase	Enzyme that functions in the biosynthetic pathways of sialic acids	SEMD	AR	610442	van Karnebeek *et al*. 2016 (106)
PISD	Phosphatidylserine decarboxylase	Enzyme that catalyzes the conversion of phosphatidylserine to phosphatidylethanolamine in the inner mitochondrial membrane	SEMD	AR	NA	Girisha *et al*. 2019 (107)
RPL13	Ribosomal protein eL13	Component of the large ribosomal subunit 60S	SEMD	AD	618728	La Caignec *et al*. 2019 (12)
RSPRY1	Ring finger and SPRY domain containing 1	Glycoprotein with unknown function	SEMD	AR	616723	Faden *et al*. 2015 (108)
SGMS2	Sphingomyelin synthase 2	A major component of cell and Golgi membranes involved in the synthesis of sphingomyelin	SMD	AD	126550	Pekkinen *et al*. 2019 (13)
TONSL	Tonsoku like, DNA repair protein	Involved in DNA replication	SEMD	AR	271510	Burrage *et al*. 2019 (109)
TRIP11	Thyroid hormone receptor interactor 11	Predicted to play a role in the assembly and maintenance of the Golgi ribbon structure around the centrosome	SMD	AR	184260	Wehrle *et al*. 2019 (110)
UFSP2	UFM1 specific peptidase 2	Involved in protein ubiquitination	SEMD	AD	617974; 142669	Di Rocco *et al*. 2018 (111)

AD, autosomal dominant; AR, autosomal recessive; IP, inheritance pattern; NA, not available; SMD, spondylometaphyseal dysplasia; SEMD, spondyloepimetaphyseal dysplasia; XLR, X-linked recessive.

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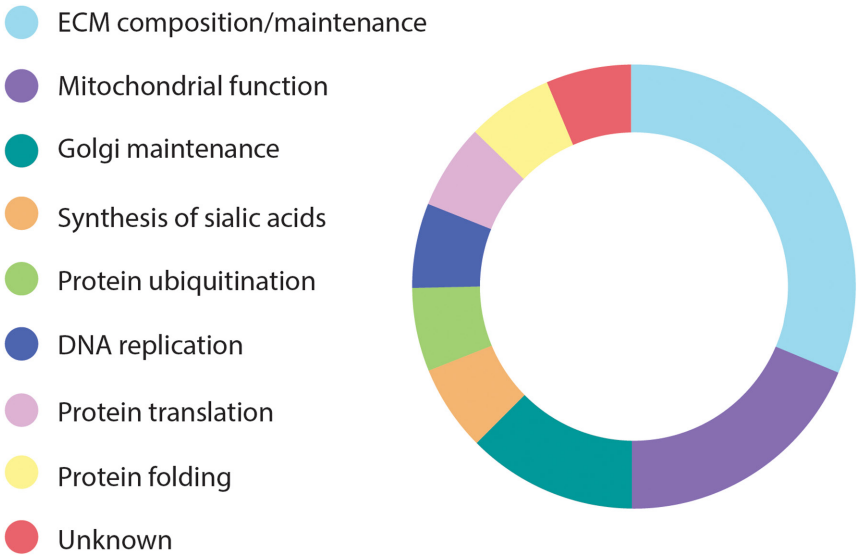

Figure 2
Function of the 16 genes recently linked to SMD and SEMD. Most of the genes recently reported as causing SMD and SEMD play a role in the ECM (5/16), in the mitochondria (3/16) and in the Golgi apparatus (2/15). Each of the remaining genes is involved in a different biological mechanism.

Although additional experiments are required to investigate the consequences of pathogenic variants in *FN1* on the bone ECM, the critical role of FN in skeletal development and tissue maintenance is confirmed by the lethality of mice lacking FN (23).

Defects in sphingomyelin synthase 2 cause SMD

Sphingomyelin is a sphingolipid of the plasma membrane and the Golgi membranes. Monoallelic pathogenic variants in the gene encoding sphingomyelin synthase 2 (*SGMS2*), an enzyme that catalyzes the synthesis of sphingomyelin, have recently been identified in patients affected by a rare form of genetic osteoporosis, named Calvarial doughnut lesions with bone fragility with or without SMD (MIM 126550) (24). Thus far, only three different *SGMS2* pathogenic variants in eight unrelated families have been described in the literature (24, 25). While some patients only have early-onset osteoporosis, the most severely affected patients feature severe short stature, neonatal fractures, and SMD. The variable severity of the disease is related to the type of pathogenic variant: subjects harboring the nonsense variant c.148C>T (p.Arg50*) are either healthy (25) or more mildly affected than patients with the missense changes c.185T>G (p.Ile62Ser) or c.191T>G (p.Met64Arg) (24). The absence of a bone phenotype in some patients harboring the nonsense variant suggests incomplete penetrance. The p.Arg50* change generates a truncated protein that lacks the whole membrane-spanning core domain and leads to a catalytically inactive enzyme (24). On the other hand, in cell and yeast models, the two missense variants produce a catalytically active enzyme that accumulates in the endoplasmic reticulum and synthesizes sphingomyelin at a remarkably higher rate than the wildtype enzyme. Since SMS2 is a homodimer the missense pathogenic variants might lead to a dominant-negative effect induced by the mutated SMS2 forming a heterodimer with the wildtype enzyme. Patient-derived bone biopsies reveal significant tissue-level pathology, thin cortical bone, and disturbed bone mineralization (24, 25). Pathogenic variants in two genes encoding two other enzymes involved in sphingomyelin metabolism, phosphatidylserine synthase 1 (*PTDSS1*) and choline-phosphate cytidylyltransferase A (*PCYT1A*), cause two other types of skeletal dysplasias, named Lenz-Majewski syndrome (MIM 151050) (26) and spondylometaphyseal dysplasia with cone-rod dystrophy (MIM 608940) (27).

Defects in ribosomal protein eL13 cause SEMD

In some cases, short stature and skeletal abnormalities can arise from pathogenic mutations in genes encoding ribosomal proteins (RPs), transcribing ribosomal RNAs (rRNAs) or required for ribosome biosynthesis (28). Ribosomes are vital organelles of the cells that are responsible for synthetizing proteins. Eukaryotic ribosomes (80S) are composed of two subunits: the large 60S subunit and the small 40S subunit. Human ribosomes are mainly located in the cytoplasm (either bound to the endoplasmic reticulum or floating in the aqueous part) and are formed by a complex of around 80 RPs and four rRNAs (29, 30). Recently, we and others identified inherited or *de novo* pathogenic variants in the gene encoding the ribosomal

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protein eL13 (*RPL13*) in altogether nine index patients with skeletal dysplasia without extra-skeletal features (12, 13, 31). The phenotype of the so far reported patients with pathogenic *RPL13* variants varies greatly and it ranges from normal stature with multiple epiphyseal dysplasia in one patient (31) to disproportionate short stature, growth deficiency, broad metaphyses, and delayed ossification of the epiphyses in the majority of the other patients. Interestingly, in two families variable clinical expressivity and incomplete penetrance of the disease have been identified (13). Surprisingly, in one family the index patient died during early childhood while his mother is healthy, despite harboring the same *RPL13* mutation as her child (13). Polysome profiling, carried out in both patient-derived lymphoblasts and dermal fibroblasts, shows a reduced 80S peak, thus suggesting a change in translation efficiency (12, 13). Moreover, a zebrafish model harboring a frameshift deletion within *rpl13* partly recapitulates the human phenotype by featuring cartilage deformities during early stages of development (13). Although the molecular mechanisms underlying skeletal dysplasia RPL13-type are yet unknown, tissue specificity with skeletal involvement and incomplete penetrance of the disease have been found in other conditions caused by ribosomal dysfunction, collectively named ribosomopathies (28, 32). Tissue specificity suggests that ribosomes could be involved in other mechanisms beside translation (33) and *RPL13* might potentially play a role in skeletogenesis.

Concerning the presence of short stature and skeletal abnormalities due to defects in ribosomal components, other congenital conditions with these clinical features have been described previously, including CHH, Diamond-Blackfan anemia 1 (MIM 105650), and Shwachman-Diamond syndrome (MIM 260400) (34). Moreover, common findings among ribosomopathies are bone marrow failure and anemia. Although the patients that have so far been reported as being affected by skeletal dysplasia RPL13-type do not feature hematological impairments, bone marrow dysfunction could lead to defective mesenchymal stem cell production, consequently affecting chondrogenic/osteogenic differentiation. Moreover, patients with ribosomopathies often have increased propensity to develop cancer (35, 36). Since only nine families with skeletal dysplasia due to *RPL13* variants have been described so far, it is not possible to estimate the cancer risk for this type of skeletal dysplasia. Certainly, more patients need to be identified and characterized to better delineate the clinical and genetic scenario of skeletal dysplasia RPL13-type and additional functional work, possibly using chondrogenic/osteogenic cell lines and *in vivo* models, is required to fully explore the pathogenesis of the disease.

Mildest forms of skeletal dysplasia in patients diagnosed with idiopathic short stature

Idiopathic short stature (ISS) is defined as stature more than 2 S.D. below the mean for age and sex, when no cause for the short stature has been identified despite standard clinical and laboratory evaluation (37, 38). For diagnosis of ISS, systemic or endocrine diseases, dysmorphic syndromes, small birth size (small for gestational age, SGA), and skeletal dysplasias should be excluded (38). Height is a polygenic trait and several hits associated to height variation have been identified by genome-wide association studies so far (39, 40). However, monogenic forms of short stature have also been identified. In cohorts of ISS children, some children have pathogenic variants in genes that are associated with skeletal dysplasias and ISS phenotype, thus represents the mildest end of the spectrum of these disorders.

One well-known example is short stature homeobox (*SHOX*) haploinsufficiency, which causes short stature in 2–15% of children previously diagnosed with ISS (MIM 300582) (41). In addition to isolated short stature, heterozygous *SHOX* pathogenic variants cause Leri-Weill dyschondrosteosis (MIM 127300), a skeletal dysplasia characterized by disproportionate short stature, mesomelic limb shortening, and the Madelung deformity (42). On the other hand, biallelic pathogenic variants in *SHOX* cause the more severe Langer mesomelic dysplasia (MIM 249700) (43). The clinical severity of *SHOX* pathogenic variants varies even within family members carrying the same variant and damaging mutations of the gene encoding the retinoic acid catabolizing enzyme (*CYP26C1*) have been reported to act as genetic modifiers of SHOX deficiency (44). Recently, it was reported that *CYP26C1* damaging mutations without SHOX deficiency can also lead to short stature (45).

Pathogenic variants in natriuretic peptide receptor 2 gene (*NPR2*) also cause a large variability in phenotype, ranging from acromesomelic dysplasia (MIM 602875), caused by biallelic mutations, to isolated short stature (MIM 616555). Family members who were mutation carriers were found to have significantly lower height than non-carrier family members and the general population, suggesting that heterozygous variants in *NPR2* are associated with short stature (46). Subsequent studies in ISS cohorts have found heterozygous *NPR2*

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variants in 2–6% of the patients (47, 48, 49). Variable phenotype was observed, with cases of both proportional and disproportional short stature. NPR2 is the principal receptor of natriuretic peptide C, encoded by the natriuretic peptide precursor C (*NPPC*). Heterozygous *NPPC* pathogenic variants have been identified as a cause of short stature and small hands phenotype in 0.6% of patients with short stature (50).

Pathogenic variants in the fibroblast-growth factor receptor 3 (*FGFR3*) are responsible for a wide range of clinical severity, from lethal thanatophoric dysplasia types I and II (MIM 187600-187601) to nonlethal achondroplasia (MIM 100800) and hypochondroplasia (MIM 146000) (51). In 2015, a *FGFR3* variant was determined as a cause of proportionate short stature in one family (52), further widening the clinical spectrum. Interestingly, an analog of C-type natriuretic peptide, which promotes bone growth by inhibiting fibroblast-growth factor-mediated mitogen activated protein kinase (MAPK) activation, is presently used in phase III trials to treat achondroplasia (6).

Aggrecan (ACAN) is a proteoglycan in the extracellular matrix and its major function is to resist compression in cartilage. Homozygous or compound heterozygous pathogenic variants in *ACAN* cause SEMD aggrecan type (MIM 612813), while heterozygous pathogenic variants can cause either spondyloepiphyseal dysplasia Kimberley type (MIM 608361) or short stature with advanced bone age (MIM 165800) (2). Many patients with short stature and advanced bone age caused by pathogenic variants in *ACAN* develop early-onset osteoarthritis and degenerative disc disease (53). Midface hypoplasia, joint problems, and broad great toes have also been reported (54). Recently, subjects with *ACAN* haploinsufficiency were reported to have an elevated mean arm span to height ratio in childhood and adolescence, and a slightly elevated ratio until age 50 (53, 55).

Indian hedgehog signaling molecule (IHH) is known to cause acrocapitofemoral dysplasia (MIM 607778), brachydactyly type A1 (MIM 112500), syndactyly (Lueken type) and syndactyly with craniosynostosis (Fig. 1) (2). Additionally, heterozygous pathogenic *IHH* variants causing short stature have been identified in a cohort of patients with growth disorders (56). Short stature was mildly disproportionate in most cases and many had shortening of the middle phalanx of second and/or fifth finger. Some of the patients were born small for gestational age (SGA). Recently, heterozygous pathogenic *IHH* variants were identified in another cohort with short stature and/or brachydactyly, including the first patient with a complete deletion of *IHH*, who presented with both short stature and brachydactyly (57).

Novel molecular mechanisms underlying skeletal diseases

For a long time, it has been thought that the non-coding genome (approximately 98% of the whole genome), also named junk DNA, does not play any vital function. However, this concept has been disproved by the discovery of skeletal diseases caused by variants in the non-coding regulatory genome and two examples are discussed here.

Aberrant microRNA (miRNA) expression and processing, miRNA deletions as well as point mutations in miRNA have been associated with congenital conditions (58). In 2019, the first skeletal dysplasia caused by gain-of-function pathogenic variants in a miRNA, miRNA-140, were characterized by Grigelioniene *et al.* (59). The three patients from two families affected by this disease, named spondyloepiphyseal dysplasia Nishimura type (MIM 618618), mainly feature dwarfism, short limbs, and small hands and feet. All affected subjects had the same heterozygous missense variant, MIR140:NR_029681.1:n.24A>G, in the seed sequence of miRNA-140, which is highly expressed in chondrocytes and it was found to be associated with a chondrocyte-specific super-enhancer both in humans and mice (59). Mice lacking this miRNA are short and have craniofacial deformities (60). Heterozygous and homozygous knock-in mice harboring the same pathogenic variant as detected in the patients showed a more severe phenotype, including also delayed secondary ossification of tubular and carpal bones, delayed cartilage development in the larynx, trachea, anterior ribs, and decreased expression of *Col10a1* (59).

In recent years, the discovery of topological associated domains (TADs), megabase-scale 3D rearrangements of the chromatin, has demonstrated that regulatory regions might be located far away (in terms of linear DNA sequence) from the gene they regulate (61). Acropectorovertebral dysplasia (MIM 102510), characterized by carpal and tarsal synostoses, syndactyly, hypodactyly and polydactyly of feet as well as spina bifida occulta arises from structural variants (SVs) disrupting a TAD domain spanning the genes *WNT6*, *IHH, EPHA4*, and *PAX3* (62). This TAD disruption is likely to rewire gene-enhancer interactions, leading to abnormal gene expression. Disruption of other TAD domains might then be the cause of other skeletal diseases with unknown genetic basis.

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How to identify novel phenotypes

Novel skeletal disorders and their genetic etiology can be studied by combining careful phenotyping with selected genetic strategies. Assessment includes detailed family history and information about possible consanguinity, in order to determine the possible inheritance pattern. As skeletal dysplasias may present in different family members with a varying degree of severity, a clinical evaluation of family members may be necessary to determine whether they might have a mild form of the same disease.

In a patient with short stature, accurate and multiple growth chart measurements, including SDS for height, weight, and head circumference are essential for assessing growth. It should be determined whether the patient was born with short stature and whether macrocephaly is present. Disproportionate short stature is typical in skeletal dysplasia and it can be assessed by measuring arm span, sitting height, and sitting height/height ratio. Alternatively, the upper/lower body segment ratio can be calculated and compared to references (38). Disproportionate short stature affecting the limbs can be either rhizomelic, mesomelic or acromelic depending on whether proximal (humerus and femur), middle (radius, ulna, tibia, or fibula), or distal (hand and foot) segments are shortened (63). Spinal involvement may be difficult to evaluate clinically and often requires radiographic assessment.

Evaluation of facial dysmorphic features may include evaluation of the fontanels, nasal bridge, midface, philtrum, mandible, palate, and ears (1). One should also assess upper and lower limb lengths and possible asymmetry, carrying angle of the elbow, and any anomalies in the digits (oligo-, poly-, brachy- or syndactyly). Frontal bossing, cleft palate, dental defects, clavicle aplasia or hypoplasia, scoliosis, joint hyperlaxity/contractures/dislocations, genu varum or valgum, fractures, and abnormalities in nails, hair, and skin should be examined.

Skeletal dysplasias may also be associated with various extra-skeletal manifestations, such as problems in hearing, vision, renal or respiratory system, heart defects, genital abnormalities, immune deficiency, anemia, and intestinal problems such as Hirschprung disease (1, 63, 64, 65). Further evaluation of these systems may be needed.

Skeletal survey is an essential tool in assessing skeletal dysplasias. A skeletal survey may include radiographs of the skull (posteroanterior and lateral), spine (anteroposterior and lateral), thorax (anteroposterior), pelvis (anteroposterior), upper limb (anteroposterior), left hand and wrist (posteroanterior) and lower limb (anteroposterior) (38). Based on localization of radiological findings, the dysplasias can be characterized as spondylo, epiphyseal, metaphyseal or diaphyseal dysplasia, or a combination of these.

Genetic approaches to variant and gene discoveries

In addition to an in-depth clinical investigation, choosing the correct genetic approach to investigate a family with a rare skeletal disease with short stature is crucial for a successful diagnosis (Fig. 3).

In last decades, the field of clinical genetics has greatly evolved thanks to the development of novel sequencing technologies and the advances in molecular biology. Before the MPS era, novel genes underlying monogenic conditions were mapped by positional cloning and linkage analysis. These methods, which allowed the identification of large regions to be prioritized by Sanger sequencing, were not only time consuming but they also required large multi-case pedigrees. Nowadays, Sanger sequencing is only used to sequence one or a couple of candidate genes/exons when a disease-gene correlation is suspected.

Since the majority of skeletal diseases is characterized by genetic heterogeneity, methods that allow to screen several loci at the same time are often required (Fig. 3). Among the different MPS methods, gene panels are designed for capturing and sequencing a certain number of candidate genes. Running a gene panel has become a faster and less expensive alternative to Sanger sequencing (66).

Alternatively, when the investigated phenotype overlaps with more than one condition and/or a long list of genes needs to be screened, exome sequencing (ES) is a more cost-effective option. ES gives the possibility not only to screen for variants in known disease-causing genes but also to search for variants in the rest of the protein-coding genes.

Finally, genome sequencing (GS), currently the most expensive method in the field, enables the sequencing of the entire genome. Some advantages of using GS instead of ES include: (1) sequencing of variants in regulatory regions (67) as well as deep intronic variants that could affect gene expression and the splicing mechanism (68), respectively, (2) sequencing of non-coding RNAs, such as miRNAs, that could regulate the expression of key genes in skeletogenesis, (3) more even coverage to improve the detection of intragenic deletions and duplications, and (4) possibility of detecting SVs, which are large chromosomal

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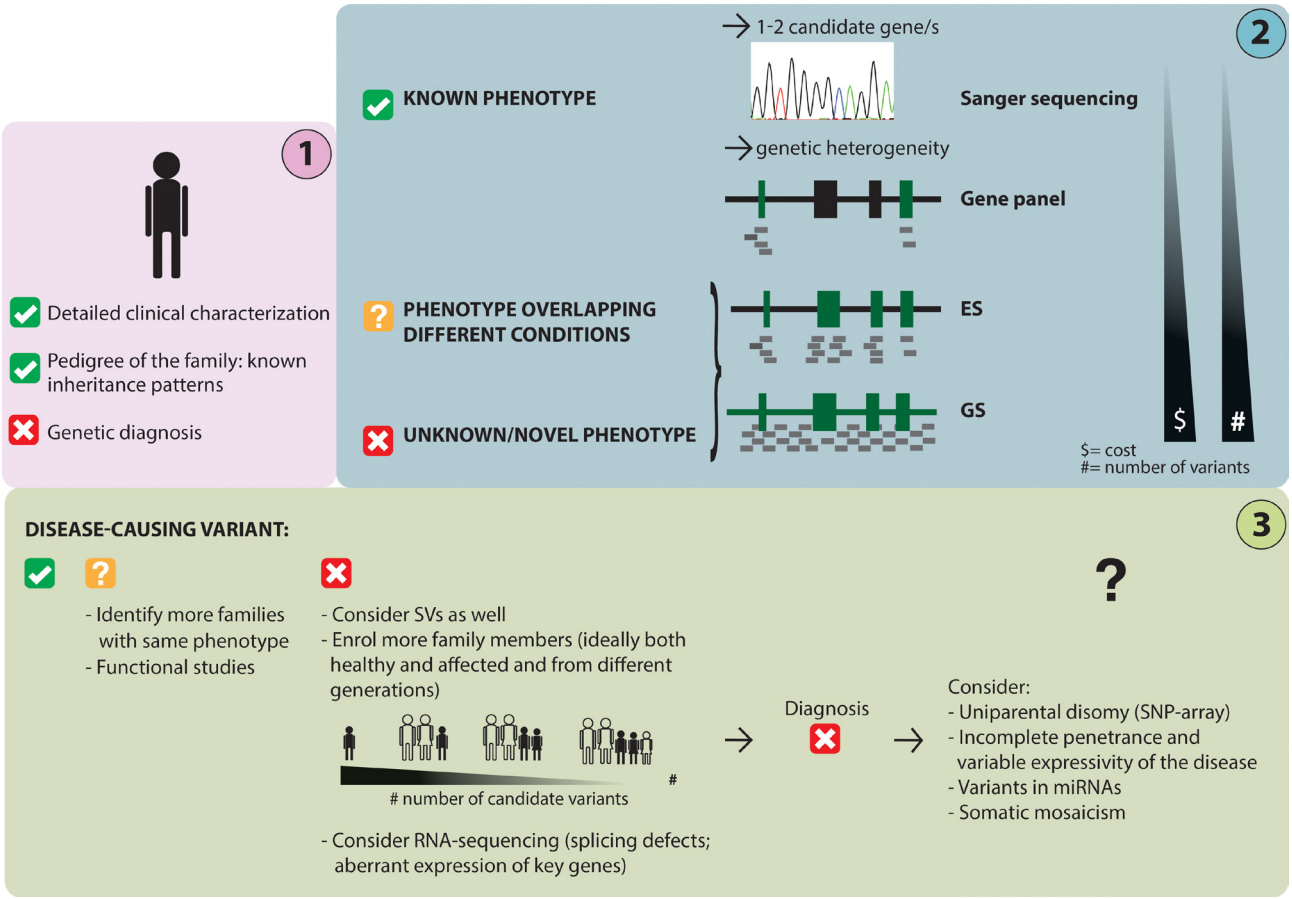

Figure 3
Schematic workflow of the genetic approach to identify the disease-causing variant in a patient with a skeletal disease. After an in-depth clinical characterization (step 1), the phenotype of the studied patient might overlap a known condition, several conditions or be a novel/unknown phenotype; this will determine the genetic approach/method to be chosen (step 2). Data analysis could directly lead to a genetic diagnosis, but often further investigations are needed to validate a genetic finding or to pinpoint the genetic defect (step 3). ES, exome sequencing; GS, genome sequencing; SVs, structural variants.

rearrangements (>50 base pairs) (69) that could disrupt known disease-causing genes. Although most of the pathogenic variants that have so far been reported to cause skeletal dysplasias are small-scale variants, sometimes SVs, including copy number variants (CNVs), inversions and translocations, can give rise to skeletal diseases (70). CNVs can also be detected using SNP and comparative genomic hybridization (CGH)-arrays, two older methods that can pinpoint large changes in DNA copies.

SNP-array, ES, or GS data can also be used to detect uniparental disomy (UPD) (71), a genetic phenomenon where both copies of a chromosome are inherited from the same parent. UPD can lead to imprinting disorders by disrupting the expression pattern of imprinted genes. For example, paternal UPD of chromosome 20 has been reported in up to 18% of the patients with pseudohypoparathyroidism type 1b (PHP1b) (72) and maternal UPD of chromosome 7 is found in approximately 5–10% of patients with Silver–Russell syndrome (SRS) (MIM 18060) (73). In addition to UPD, imprinting disorders are also caused by methylation defects, such as loss of methylation at the *GNAS* imprinted region in PHP1b (74) and loss of methylation at 11p15 in SRS (73).

Despite the fact that nowadays large multi-case families are not needed to pinpoint a novel disease locus, it is often advantageous to perform MPS on at least a family trio (patient and his/her parents) in order to narrow down the number of rare candidate variants.

Finally, to improve the knowledge on non-coding variants and to increase the diagnostic yield in skeletal dysplasias, a combination of ES/GS and RNA-sequencing might be needed. Transcriptome analysis in monogenic diseases does not only allow to pinpoint deep intronic variants and synonymous changes that affect the splicing

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mechanism but also to identify variants in regulatory regions (promoters, enhancers and UTRs) that result in aberrant gene expression (68). Since the transcriptome signatures differ in different tissues, it is crucial to perform the investigations on the affected tissue or to identify a good proxy tissue for the disease, which in the case of skeletal conditions would be bone and/or cartilage.

Recently, transcriptome analysis performed on RNA extracted from primary skin fibroblasts of patients with CHH revealed differential expression of several genes regulating the cell cycle (75). These results suggest that if the investigated gene plays a pivotal role not only for the skeleton but also for the ectoderm, skin biopsies could be used as a source material to carry out RNA-sequencing.

To summarize, it is important to choose the most appropriate genetic approach based on clinical evaluation of the patients, available resources as well as cost-effectiveness/limitations of each method.

Validation of novel gene-disease associations

In vitro studies

Once a candidate variant in a gene that has not been previously linked to disease is identified, functional studies are needed to validate this finding. As previously mentioned, an important aspect to consider when investigating genetic findings is the source of material chosen/available to investigate the disease pathogenesis. Often, bone biopsies would be the most appropriate tissue and source of cells for investigating the pathogenesis of the disease. However, obtaining a bone biopsy is an invasive procedure that is not performed unless it is required for diagnostic purposes or the patient needs to undergo elective surgery. Bone biopsies are valuable for skeletal research since they can be used to assess several parameters, such as bone microarchitecture, mineralization, and the morphology and organization of the different types of bone cells (24, 76). Moreover, bone marrow aspirates are a source of MSCs. Although several protocols for osteogenic cell line differentiation from MSCs are available, one limitation of this method is related to the limited availability of these cells from healthy donors.

If the studied gene is expressed in the ectoderm, most often skin punch biopsies obtained from patients are used to investigate the pathomolecular mechanisms leading to genetic skeletal diseases. From skin biopsies primary fibroblasts are derived and can be studied from different angles. A lot of information can be obtained for example by comparing cell proliferation, differentiation, and apoptosis as well as gene/protein expression in patients vs sex- and age-matched controls. However, it is always important to know that a defect in these cells might not necessarily reflect the true nature of the disease and, vice versa, lack of any experimental evidence in fibroblasts might not exclude that the pinpointed genetic finding has an adverse effect on bone. Despite these limitations, patient-derived fibroblasts are widely used in the field of skeletal research and have led to important discoveries. Moreover, dermal fibroblasts could be potentially reprogrammed into induced pluripotent stem cells (iPSCs) and re-differentiated into chondrocytes to study subtypes of skeletal dysplasias with short stature due to an intrinsic defect in the growth plate. However, the culture conditions allowing to differentiate iPSCs and MSCs into chondrocytes are not yet well understood (77).

Since 2012, the CRISPR-Cas9 technology (78) has transformed the field of genetic engineering by introducing the possibility of efficiently silencing a gene of interest (or introducing a specific pathogenic mutation) in commercially available cell lines (79). While this approach overcomes the problem of obtaining patient-derived osteogenic cells, the use of immortalized cell lines might introduce changes in the cells that might be incorrectly assessed as part of the phenotype induced by the introduced pathogenic variant/gene defect. Furthermore, patient-derived cells have the advantage of maintaining the whole genetic signature of each patient, thus allowing to detect the effects of potential interactions between a set of variants in the genome.

In vivo models

In order to explore the systemic effects of genetic pathogenic variants in an organism and to investigate cartilage and skeletal development, animal models are required. Genetically engineered mice (Mus Musculus) have been widely used for mimicking human skeletal diseases since this species shares a high percentage of coding DNA (~85%) with humans (80) and bone development and the skeletal elements are highly conserved between these two mammalian species. Mice also undergo longitudinal bone growth and the growth plate is the structure determining cartilage production and bone apposition (81). Unlike in humans, the murine growth plate does not undergo epiphyseal fusion with sexual maturation (82). Concerning bone remodeling, it takes place in the cancellous bone, as in humans.

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The most striking difference between mice and humans is that murine bone lacks osteons, the structural and functional units of cortical bone (81).

Since generating and handling transgenic mice is expensive and time consuming, zebrafish (Danio Rerio) has recently emerged as a model for studying rare skeletal diseases (83, 84, 85), primarily osteoporosis and OI (86, 87). Approximately 71% of protein-coding genes in the human genome have an ortholog in zebrafish (88). Every week zebrafish produces hundreds of eggs, which are externally fertilized, thus allowing fast and easy genetic manipulation. Moreover, zebrafish larvae are transparent, and they can be stained in whole-mount to study skeletal development and cartilage/bone mineralization. As in humans, bone development in zebrafish occurs by both intramembranous and endochondral ossification (89) and several key genes playing a role in skeletogenesis are conserved across the two species (79, 90). Despite the fact that there are also dissimilarities between human and zebrafish skeletogenesis (e.g. in zebrafish bone is not vascularized and the growth plate develops differently), several zebrafish models mimicking human skeletal disease have been generated in the last decade. Furthermore, it has been recently shown that zebrafish have structures resembling the human growth plate (91). This finding will open the possibility to better investigate diseases characterized by short stature due to impaired growth plate in this species.

In vivo models are also largely used for drug testing before a candidate undergoes clinical testing in humans. Recently, an antibody against TGF-β was tested in two different mouse models of OI (92, 93) and two chemical chaperones were proven to improve the skeletal phenotype of a zebrafish model of autosomal dominant OI due to a missense pathogenic variant in *col1a1a* (94).

Significance of gene discoveries

Identifying novel pathogenic mutations and novel gene defects underlying skeletal diseases is important in order to be able to provide an accurate diagnosis to the patients and to offer genetic counseling and optimized management of the disease to the families. Moreover, providing the risk of having another affected child to the parents also influences their reproductive choices. Since most of the skeletal diseases still lack a pharmacological treatment, there is necessity for exploring novel gene defects to find common disease mechanisms that can eventually lead to the development of novel effective drugs. Although orthopedic surgeries will always be required to correct skeletal deformities and severe scoliosis, pharmacotherapies might help in preventing or ameliorating some skeletal features, in particular if a diagnosis is made at an early stage. Growth hormone therapy has been shown to help in gaining height in patients with short stature due to *SHOX* defects, but in patients affected by other diseases, such as spondyloepiphyseal dysplasia, the skeletal impairments (e.g. scoliosis) may even worsen with treatment (95). Therefore, it is crucial to distinguish between different causes for short stature and use personalized treatment approaches. Due to the mechanistic differences in disorders leading to impaired growth, it is important, in research settings, that novel genetic findings are carefully investigated using *in vitro* and *in vivo* models to understand the pathomolecular mechanisms leading to disease.

Conclusions and future perspectives

During the past 5 years, 16 novel genetic forms of SMD and SEMD have been characterized. These successful results have been achieved not only because of the possibility to use MPS but also because of joint efforts of several experts in the field aiming to identify and carefully delineate novel skeletal phenotypes.

Although nowadays 92% of the thus far characterized skeletal conditions have a known genetic basis, it can be anticipated that in the near future the genetic defects underlying the remaining conditions will be identified. In addition, novel extremely rare skeletal phenotypes most likely continue to be characterized also in the coming years. A better knowledge about the non-coding genome and further attention on the genetic mechanisms escaping regular Mendelian inheritance, such as incomplete penetrance and somatic mosaicism, might be needed to solve the remaining cases of skeletal dysplasia with severe short stature.

Finally, the large increase in gene-disease associations that has characterized the last decade has lagged behind in in-depth characterization of the pathogenesis of these diseases. Additional work is, thus, required to explore the molecular mechanisms leading to impaired bone development and/or homeostasis and to pinpoint novel drug targets. This knowledge will eventually be applied to develop efficient therapeutic strategies to treat patients with skeletal diseases.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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