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Review

Ribosome biogenesis in skeletal development and the pathogenesis of skeletal disorders☆

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ABSTRACT

The skeleton affords a framework and structural support for vertebrates, while also facilitating movement, protecting vital organs, and providing a reservoir of minerals and cells for immune system and vascular homeostasis. The mechanical and biological functions of the skeleton are inextricably linked to the size and shape of individual bones, the diversity of which is dependent in part upon differential growth and proliferation. Perturbation of bone development, growth and proliferation, can result in congenital skeletal anomalies, which affect approximately 1 in 3000 live births [1]. Ribosome biogenesis is integral to all cell growth and proliferation through its roles in translating mRNAs and building proteins. Disruption of any steps in the process of ribosome biogenesis can lead to congenital disorders termed ribosomopathies. In this review, we discuss the role of ribosome biogenesis in skeletal development and in the pathogenesis of congenital skeletal anomalies. This article is part of a Special Issue entitled: Role of the Nucleolus in Human Disease.

1. Introduction

1.1. Ribosome biogenesis

The ribosome is a large ribonucleoprotein machine that translates mRNA into protein to synthesize all protein within the cell. The eukaryotic ribosome catalyzes protein synthesis through distinct and yet collaborative roles of its two subunits during translation. The small 40S subunit decodes mRNA sequence while the large 60S subunit links amino acids through peptide bonds [2]. As ribosomes are universally responsible for the quality and quantity of proteins in all cells, ribosome production is highly regulated by, and integrated with, many cellular processes including growth, proliferation, differentiation, and hypertrophy.

Ribosome biogenesis, the process of making ribosomes, is a complex and metabolically expensive endeavor that involves coordination of all three RNA polymerases. Ribosomes are an assembly of 4 rRNAs transcribed by RNA polymerases I and III, in addition to approximately 80 ribosomal proteins, accessory proteins and about 70 small nucleolar RNAs (snoRNA) all transcribed by RNA polymerase II [2,3]. Ribosome biogenesis begins with transcription of both the 47S precursor ribosomal RNA (rRNA) by RNA polymerase I (RNA Pol I) in the nucleolus and the 5S rRNA by RNA polymerase III in the nucleus. Transcription of rRNA is the rate-limiting step in ribosome production and accounts for about 60% of the overall transcription in eukaryotic cells [4]. Additionally, a significant proportion of mRNA transcription by RNA polymerase II in the nucleus is also required for the production of the ribosomal proteins [5]. Following transcription, the 47S rRNA precursor is cleaved into a 45S rRNA which is modified covalently at nearly 200 nucleotides by snoRNPs, bound by ribosomal proteins, and further cleaved into 5.8S, 18S, and 28S rRNAs. Ultimately, the 18S rRNA and 32 small subunit ribosomal proteins (RPSs) are assembled into the 40S subunit while the 5S, 5.8S, and 28S with 47 large subunit ribosomal proteins (RPLs) are assembled into the 60S ribosomal subunit. These ribosomal subunits are then exported to the cytoplasm where they unite to form the translationally active mature 80S ribosome [2,6].

As ribosomes determine the capacity for protein production and their synthesis commandeers much of the cell's metabolic efforts, ribosome biogenesis determines growth, cell division rates, and survival [7]. Given the ribosome's universal importance in all cell types, it is remarkable that disruptions in ribosome biogenesis lead to congenital ribosomopathies with very specific clinical phenotypes that include defects in the craniofacial, axial and limb skeleton (Fig. 1). Here we discuss how the etiology and pathogenesis of ribosomopathies can reveal new information about the role of ribosome biogenesis in proliferation, growth, and differentiation in skeletal development.

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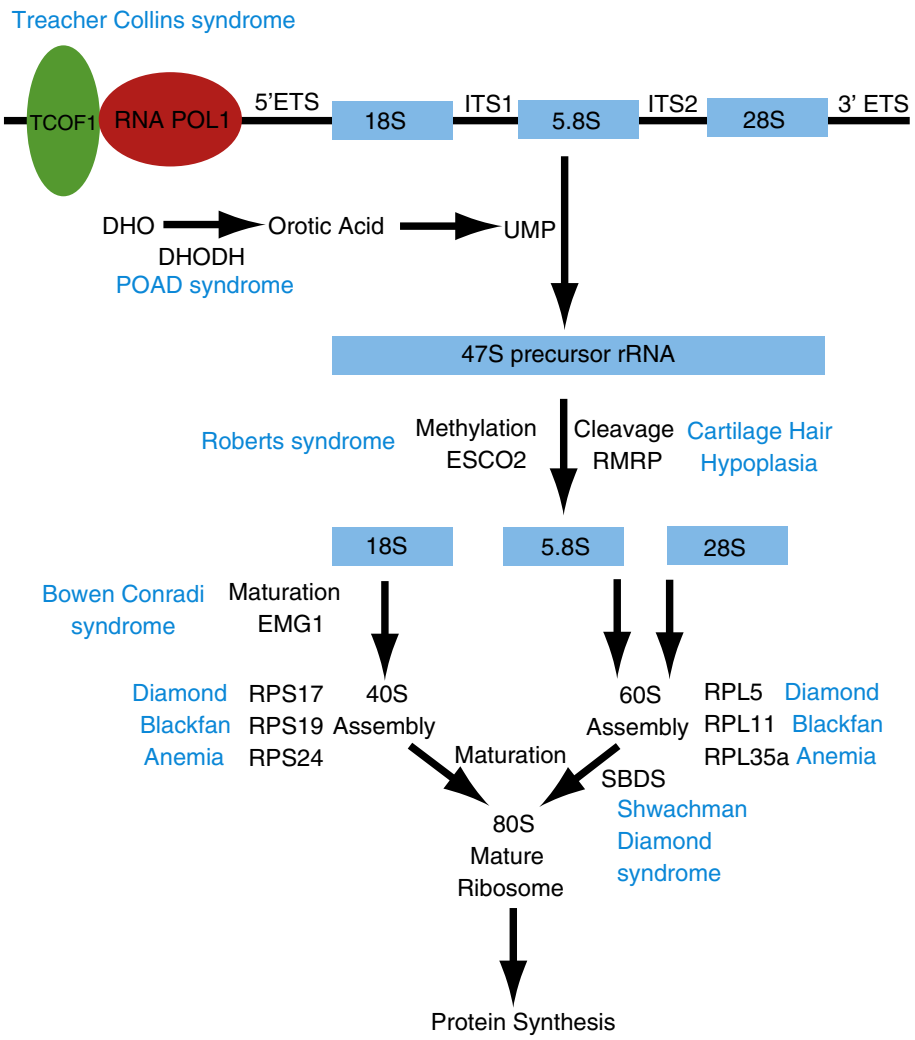

Fig. 1. Schematic diagram summarizing the major components of ribosome biogenesis and the intersection of specific genes that leads to the pathogenesis of distinct ribosomopathies.

1.2. Ribosome biogenesis in skeletal development

Bones, in their many shapes and sizes, underlie form and function of the vertebrate skeleton. The 206 bones in the human skeleton protect our vital organs, provide a reservoir of minerals, facilitate movement, and underlie the basis for physical appearance. These mechanical and biological functions are possible because bones come in a multitude of shapes and sizes—long, short, flat, and irregular. Skeletal diversity arises in the developing embryo by a stepwise series of events that demarcate when, where, and how bone is built.

Skeletal development begins when loose networks of mesenchymal cells coalesce and condense, prefiguring mature cartilage and bone. Different mesenchymal populations give rise to anatomically distinct groups of bones. Neural crest-derived mesenchyme forms bones in the face, jaw and rostral calvarium whereas mesoderm-derived mesenchyme forms bones in the caudal calvarium, vertebral column, rib cage, and limbs. These mesenchymal populations build bone through either endochondral or intramembranous ossification. During endochondral ossification the mesenchyme initially forms a cartilage scaffold that is later replaced by bone. During intramembranous ossification, the mesenchyme differentiates directly into bone. Skeletal elements that develop through endochondral ossification are largely mesoderm-derived, while those that form by intramembranous ossification are almost exclusively neural crest-derived and localized to the craniofacial region [8,11].

The commencement of skeletal development is followed by a long period of growth that progresses throughout fetal development and continues postnatally through adolescence. The directionality of growth, which modifies the size and shape of bones, is differentially regulated by endochondral and intramembranous ossification. Intramembranous ossification leads to appositional growth, adding new bone at the leading edge or surface to increase thickness or length. During this process osteoblasts deposit osteoid matrix against the bone's surfaces or at the osteogenic fronts. Addition of bone at the leading edge, while useful for growing flat bones in the skull, is not compatible with bones whose ends articulate into a moving joint. This is solved by endochondral ossification, which promotes interstitial growth from the bone's center to increase its length [8,11].

A stratified cartilage structure known as the growth plate is responsible for interstitial growth within endochondral bones. The growth plate, a holdover of the precartilage condensation in the epiphysis, is arranged into four functional zones that represent the developmental progression of endochondral ossification (Fig. 2). The resting zone holds a reservoir of chondroprogenitor cells that when activated move into the proliferative zone where they rapidly divide to form columnar stacks parallel to the long axis of the bone. This transition is regulated by the Sox trio: Sox 5, 6 and 9 [12,13]. Proliferating chondrocytes then transition into the zone of hypertrophy were they swell several times their original volume and secrete copious amounts of matrix rich in collagen [14]. This process of chondrocyte maturation is regulated by the

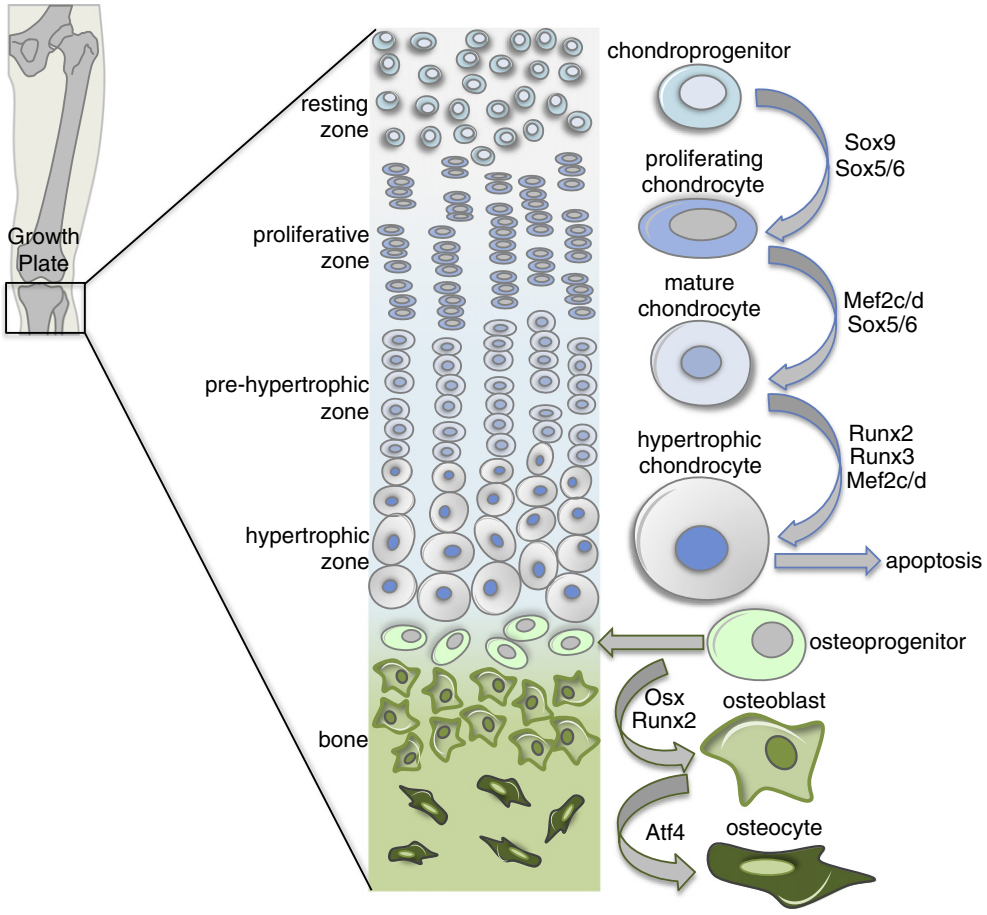

Fig. 2. Skeletal development proceeds either through endochondral or intramembranous ossification. This schema of the stratified growth plate within a long bone depicts the transition of the chondroprogenitor cell through the developmental progression of endochondral ossification, which includes proliferation, maturation, hypertrophy and ultimately cell death. Osteoprogenitor cells then invade the void left by hypertrophy, differentiate into osteoblasts, and maturate into osteocytes. Differentiation and maturation of osteoprogenitor cells is indistinguishable between endochondral and intramembranous ossification.

transcription factors Mef2c, Mef2d, Runx2, and Runx3 [14,16]. Hypertrophic chondrocytes ultimately undergo cell death in the zone of ossification and osteoblasts invade the void where the calcified cartilage matrix has been eroded by matrix metalloproteinases and osteoclasts. In adulthood, when the long bones have reached their full length, the growth plate cartilage becomes fully replaced by bone.

Chondrocytes require high translational capacity to meet the demands of proliferation, collagen matrix production, and then cellular hypertrophy within the stratified growth plate. Although a specific role for ribosome biogenesis in chondrocyte proliferation remains to be determined, the skeletal phenotypes of specific ribosomopathies infer such a connection. Moreover, the importance of ribosome biogenesis to general cell growth is well known. The role of ribosome biogenesis in chondrocyte maturation however is clearer due to the molecular link between rRNA transcription and transcriptional regulators of chondrocyte hypotrophy [15,16]. Runx2 and Runx3 associate with rDNA and regulate transcription through interactions with UBF1, an architectural factor that promotes transcription by RNA Pol I [17,19]. However, it is not known whether Runx2 and/or Runx3 regulate rRNA transcription in chondrocytes. Nonetheless, UBF1 is an ideal candidate to control chondrocyte hypertrophy as regulation of UBF1 is the key to the increased ribosome biogenesis and protein accumulation observed in association with cardiomyocyte hypertrophy [20].

While bone can be built through direct or indirect ossification, the role of the osteoblasts in these developmentally distinct processes is indistinguishable. Osteoprogenitor cells, whether located within the periosteum of endochondral bones or the mesenchymal condensation and osteogenic front of intramembranous bones, experience the same process of osteoblast differentiation (Fig. 2). Osteoprogenitors are induced to differentiate by growth factors including FGF and BMP (reviewed in [21,22]). Acquisition of osteoblast cell fate requires the activity of the transcription factors Runx2, Osx/SP7, and Atf4 to promote expression of osteoblast specific genes including those that encode osteoid matrix proteins such as type I collagen, osteocalcin, and osteopontin [23,25]. Once secreted, this collagen-rich osteoid matrix mineralizes upon binding to calcium salts brought to the region through neighboring vasculature. A subpopulation of osteoblasts become entrapped by the mineralized matrix and matures into osteocytes that actively regulate the lifelong process of bone turnover.

Regulators of osteoblast differentiation are unquestionably integrated with ribosome biogenesis. For example NO66, a nucleolar demethylase implicated in ribosome assembly and maturation, interacts with OSX/SP7 and inhibits OSX-mediated promoter activation in preosteoblasts thereby negatively regulating osteoblast differentiation [26,27]. The most well established molecular connector between ribosome biogenesis and osteoblast differentiation is Runx2, the master regulator of bone formation. Runx2 performs two essential functions in osteoprogenitor commitment. Firstly, it promotes osteoblast differentiation by transcriptionally activating bone-specific genes, and secondly it restricts cell division through inhibition of UBF1 and attenuation of rDNA transcription [17,28,31]. Integrating ribosome biogenesis with regulators of bone formation is a means to couple and coordinate osteoprogenitor cell proliferation and differentiation during skeletal development.

Thus ribosome biogenesis plays a critical role in intramembranous ossification of the neural crest-derived craniofacial skeleton and endochondral ossification of the mesoderm-derived limb skeleton. The

incidence of skeletal dysplasia is 1:3000 [1], and mutations in ribosomal proteins and regulators of ribosome biogenesis can cause hypoplasia of the craniofacial bones, shortening of the long bones, and anomalies of the digits. This is exemplified in congenital ribosomopathies with skeletal defects, the etiology and pathogenesis of which are discussed in this review.

1.3. Treacher Collins syndrome

Treacher Collins syndrome (TCS1, MIM 154500; TCS2, MIM 613717), also known as Franschetti–Zwahlen–Klein syndrome and mandibulofacial dysostosis, is a congenital birth defect that affects head and facial development [32,33]. TCS is most notably characterized by midface hypoplasia encompassing the maxilla, dentary and zygomatic complex bones [34]. Individuals with TCS also often exhibit underdeveloped external ears, which together with atresia of the external auditory canals and anomalies of the middle ear ossicles can lead to conductive hearing loss [35]. Abnormalities in brain development including microcephaly, mental retardation and psychomotor delay can present as part of the clinical spectrum of TCS, however, these features present in fewer than 5% of affected individuals [36,39].

Treacher Collins syndrome occurs with a frequency of 1 in 10,000–50,000 births and arises primarily due to autosomal dominant mutations in the *TCOF1*, gene [40]. Changes identified in *TCOF1* include splice site, missense and nonsense mutations as well as insertions and deletions. Of the more than 200 family specific mutations in the *TCOF1* gene that have been recorded to date, deletions ranging in size from 1 to 40 nucleotides appear to be the most common. Interestingly, a reoccurring 5 bp deletion in exon 24 accounts for nearly 20% of TCS cases. (http://genoma.ib.usp.br/TCOF1_database/). Recently however, individuals with TCS but without *TCOF1* mutations were found through whole exome sequencing to carry mutations in *POLR1C* or *POLR1D* [41]. Therefore to date, the genetic causes of TCS are attributable to autosomal dominant mutations in *TCOF1*, autosomal dominant mutations in *POLR1D* or autosomal recessive mutations in *POLR1C* [41].

Despite the identification and high penetrance of causative gene mutations underlying TCS, there is considerable inter- and intrafamilial variation in the severity of the disorder [42,43]. However, there is no apparent correlation between parental origin or type of pathogenic mutation, whether sporadic or familial, male or female, with the severity of Treacher Collins phenotype. This lack of a genotype–phenotype correlation in TCS [38,44,47], suggests that modifiers may be involved. Therefore, environmental factors or genetic background, may be contributing factors to the clinical variability of developmental anomalies observed in TCS patients [48].

TCOF1 encodes a putative nucleolar phosphoprotein termed Treacle [40], that has been observed to co-localize with upstream binding factor 1 (UBF1) and RNA Pol I in nucleolar organizing regions within the nucleolus, where it plays an essential role in rDNA transcription [49,50]. Treacle has also been shown to participate in ribosomal RNA processing as an integral component of the human Nop56-associated pre-ribosomal ribonucleoprotein complexes, which 2′O-methylates pre-ribosomal RNA in the nucleolus during the early stages of pre-RNA processing [49,50]. Collectively, this implies that Treacle specifically regulates multiple steps during the process of ribosome biogenesis and that deficient ribosome biogenesis may underlie the etiology of the characteristic features of TCS.

Consistent with this idea, a *Tcof1* loss-of-function mouse model of TCS [48], exhibits decreased levels of 28S rRNA generation and defective maturation of the 60S ribosomal subunit. Inadequate ribosome biogenesis is known to cause nucleolar stress activation of the p53 pathway, resulting in p53-mediated cell death. Correlating with this phenomenon, extensive p53 dependent apoptosis was observed throughout the neuroepithelium and including pre-migratory neural crest cells in E8.0-10.5 *Tcof1*$^{+/-}$ embryos. While *Tcof1* is widely expressed throughout the embryo during these developmental stages, *Tcof1* expression becomes specifically elevated in the neuroepithelium and neural crest cell derived facial mesenchyme [51]. Consequently, extensive cell death in the neuroepithelium reduces the neural crest cell population by nearly 25% in *Tcof1*$^{+/-}$ embryos compared to wild-type controls [51,52]. This is significant to the TCS phenotype, because neural crest cells are the skeletal precursors of the face and their deficiency can lead to incomplete formation of the craniofacial bones, cartilage and connective tissues. Deficiencies of neural crest cell development are not unique to TCS. In fact, many craniofacial anomalies are attributed to deficiencies in neural crest cell development. Together, these results mechanistically illustrate how *Tcof1* can influence neural crest cell formation and survival through the regulation of ribosome biogenesis.

Interestingly, pharmacological and genetic suppression of p53 has been shown to prevent neuroepithelial apoptosis in *Tcof1*$^{+/-}$ mouse embryos, preserving normal formation and survival of neural crest cells. Consequently, inhibition of p53-dependent cell death, prevents cranioskeletal abnormalities in a mouse model of TCS [52]. These results in animal models demonstrate that Treacher Collins syndrome may be clinically preventable and provide hope for future therapy. Thus the TCS animal models that phenocopy the salient features and variability observed in humans with TCS have been invaluable in uncovering the mechanisms underlying the etiology and pathogenesis of this severe congenital craniofacial disorder [51,52].

The identification of causative mutations in *POLR1D* and *POLR1C* in association with TCS is consistent with the classification of TCS as a ribosomopathy disorder. POLR1C and POLR1D constitute subunits within the catalytic core of RNA Pol I and III. Similar to the role of TCOF1 as an RNA Pol I binding factor and regulator or rDNA transcription and pre-ribosomal RNA processing, POLR1C and POLR1D may also play critical roles in rDNA transcription. Therefore, in the future it will be critical to develop new animal models for TCS through mutations in *POLR1C* and *POLR1D*, determine the precise function of POLR1C and POLR1D, and examine any degree of overlapping function with TCOF1. The identification of mutations in these three distinct genes involved in ribosome biogenesis, a process which underpins cell growth and proliferation, strongly indicates that TCS is a true ribosomopathy disorder.

1.4. Postaxial acrofacial dysostosis

Postaxial Acrofacial dysostosis (POADS, MIM 263750) also known as Miller, Genee–Wiedemann, or Wildervanck–Smith syndromes, is an acrofacial dysostosis syndrome that presents with abnormalities in the craniofacial and limb skeleton. The spectrum of craniofacial anomalies includes micrognathia, orofacial clefts, malar hypoplasia and cup-shaped ears. Limb defects are confined to the postaxial limb skeleton and include absence of either the fifth or both the fourth and fifth rays of the hands and feet, and ulnar and fibular hypoplasia [53,55]. The craniofacial features of POADS share considerable similarity to the key features of TCS.

POADS is caused by compound heterozygous mutations in the gene coding for dihydroorotate dehydrogenase (DHODH) [56,57], an enzyme that participates in the mitochondrial electron transport chain and is required for de novo pyrimidine synthesis. DHODH catalyzes the oxidation of dihydroorotate (DHO) to orotic acid, which is subsequently converted to uracil monophosphate. Uracil is a constituent base of RNA and is therefore integral to ribosome biogenesis. In vivo and in vitro assays have shown that DHODH activity from POADS-associated alleles is diminished. This implies that affected individuals have a deficiency of de novo pyrimidine synthesis, which is consistent with the need for a threshold level of DHODH activity. Furthermore it suggests that a deficiency in ribosome biogenesis may be an underlying feature of POADS.

Similar to the etiology of TCS, it is surprising that mutations in *DHODH* would lead to such tissue specific phenotypes since uracil and RNA synthesis are global processes. However, analyses of *Dhodh* expression in mouse embryos have uncovered spatiotemporally specific activity in the pharyngeal arches, forelimbs, hindlimbs and somites [57]. It is

important to note that these tissues are the embryonic precursors of the structures affected in POADS syndrome and that *DHODH* loss-of-function may therefore result in spatiotemporally dynamic rate-limiting effects on cell growth and proliferation. Consistent with this idea, zebrafish treated with leflunomide, an inhibitor of DHODH show an almost complete abrogation of neural crest cell development due to blocked transcriptional elongation of genes critical for neural crest cell function [58]. This suggests that deficient numbers of neural crest cells can account for the cellular basis of POADS syndrome in a mechanism highly analogous to that underlying the pathogenesis of TCS.

It is important to note however that mutations in *Uridine Monophosphate Synthetase* (*UMPS*), which functions immediately downstream of DHODH, result in the pathogenesis of orotic aciduria. Orotic aciduria presents with the classic feature of reduced pyrimidine availability which is megaloblastic anemia. Furthermore, orotic aciduria can be effectively treated with dietary uridine supplementation, demonstrating that the lack of pyrimidines underlies the orotic aciduria disease state. This raises the question as to why individuals with POADS do not have megaloblastic anemia or conversely, why individuals with orotic aciduria do not exhibit skeletal malformations. While it is possible that phenotypic overlap between these disorders has gone unrecognized, it seems more plausible that the underlying basis of POADS may not be restricted to deficient pyrimidine synthesis. Furthermore, there is no clear evidence to date for nuclear localization of DHODH. It will be critical therefore in the future, to characterize the dynamic localization of DHODH, as well precisely determine the biochemical functions of DHODH and the cellular basis of POADS syndrome in vivo, through mammalian animal models of *Dhodh* loss-of-function.

1.5. Diamond–Blackfan anemia

Diamond–Blackfan anemia or Inherited Erythroblastopenia (DBA, MIM 105650) is a congenital erythroid dysplasia, where affected individuals typically present with anemia (deficiency of red blood cells). In addition, DBA is primarily characterized by a selective decrease or absence of erythroid precursors as well as reticulocytopenia and macrocytosis [59]. However, individuals with DBA often (40–62%) present with a range of craniofacial and cardiac defects as well as thumb abnormalities [59]. Interestingly, the craniofacial phenotype of DBA has considerable overlap with TCS and can include cleft palate and microtia. Taken together with the fact that the three genes mutated in association with TCS all influence ribosome biogenesis, this raised the possibility of a similar role for ribosome biogenesis in neural crest cell formation in the pathogenesis of ribosomopathies such as DBA. In contrast to TCS, which results from the deficient transcription of rRNA, DBA is caused by mutations in genes encoding ribosomal proteins including *RPS17*, *RPS19*, *RPS24*, *RPL5*, *RPL11*, and *RPL35A* (reviewed in [59,60]). Currently however, these mutations account for only about 50% of individuals with DBA, illustrating there is still much to be learned about the etiology of DBA.

DBA patients that exhibit craniofacial and cardiac anomalies, more often have mutations in *RPL5* than any other RP gene linked to DBA [61]. Although this suggests that there may be spatiotemporally specific roles for individual ribosomal proteins, there is no clear evidence to date of a role for RPL5 in cranial or cardiac neural crest cell development. Interestingly, zebrafish *rpl11* morphants exhibit hematopoietic defects [62], as well as diminished activity of neurogenic markers [63]. Although not formally investigated, the substantial apoptosis observed in these embryos could disrupt neural crest cell formation and account for diminished expression of neurogenic markers. Consistent with this idea, inhibition of p53 prevented DBA in *rpl11* morphants [63]. Furthermore p53 inhibition was also successful in preventing developmental anomalies in mice and zebrafish with mutations in *Rps19*/*rps19* [64,65]. These results are very similar in principle to the prevention of TCS in *Tcof1*$^{+/-}$ mice [52] implying some commonality to their pathogenesis.

Remarkably, dietary supplementation with L-leucine considerably improves the phenotype of mouse and zebrafish models of DBA [66,67]. For example, when *rps14* or *rps19* morphant zebrafish were grown in L-leucine supplemented media, their craniofacial abnormalities were substantially ameliorated [66]. Furthermore, it has also been reported, albeit on a very limited scale, that dietary L-leucine supplementation successfully treated the anemia in some individuals with DBA [68,69]. Mechanistically it is thought that TORC1 (target of rapamycin complex 1, also known as mTOR) which is an important regulator of ribosome biogenesis, cell growth and cell proliferation, is activated by amino acids such as L-leucine. Leucyl-tRNA sythetase (LeuRS), which charges tRNA with leucine facilitates TORC1 activity [66,70] leading to phosphorylation of the RNA Pol I initiation complex and consequently stimulation of rDNA transcription [71,72]. More specifically, phosphorylation of 4E-BP1 by TORC1 releases it from the translation initiation complex, allowing for translation of ribosomal proteins [73]. Furthermore, TORC1 also governs the phosphorylation of Maf1, which is an important regulator of RNA Pol III [74,75].

Although the impact of L-leucine on neural crest cell development has not been thoroughly investigated, dietary supplementation with L-leucine could provide a therapeutic option for the prevention of other neurocristopathy disorders in which deficient ribosome biogenesis underlies their manifestation. Deficient ribosome biogenesis can cause nucleolar stress activation of p53 and cell death. Hence the craniofacial anomalies observed in DBA are likely caused by p53 dependent neuroepithelial cell death, diminished NCC formation and proliferation in a similar fashion to the pathogenesis of TCS. In support of this idea *Rps19*-deficient mice, administered L-leucine in their drinking water exhibited a down-regulation of p53 and subsequently, improved hematopoiesis [67]. A thorough exploration of the dynamics of neural crest cell development in specific animal models of DBA is still needed in the future to facilitate a better understanding of the functions of ribosomal proteins in the pathogenesis of DBA.

1.6. Roberts syndrome

Roberts syndrome (RBS, MIM 268300) is characterized by pre- and post-natal growth retardation, bilateral symmetric limb reduction, and craniofacial abnormalities including hypertelorism, cleft lip and palate, and hypoplastic nasal alae [76]. RBS is inherited in an autosomal recessive manner and is caused by mutations in the *ESCO2* gene [77], which have been recently shown to influence production of rRNA [78]. ESCO2, Establishment of Cohesion 1 Homolog 2 (Eco1 in yeast), is an acetyl transferase important in assembly of Cohesin. Cohesin is a complex of proteins which binds chromosomes and holds sister chromatids together from DNA replication to cell division [79]. However, Cohesin also has other cellular roles including binding genes with paused RNA Polymerase, facilitating DNA looping to bring together enhancers and promoters, and DNA repair (reviewed in [80]).

The specific developmental anomalies associated with mutations in *ESCO2* have begun to be investigated in animal models. Antisense morpholino oligonucleotide knockdown of *esco2* in zebrafish, results in embryos with a smaller head and eyes, and incorrectly shaped somites at 24 hpf [81]. Later in development, additional features such as abnormal pigmentation, reduced craniofacial cartilage and pectoral fin growth, and cardiac defects became apparent. Distorted spindles and disorganized chromosomes were seen in the mitotic cells of *esco2* morphants, suggesting that these cells are subsequently unable to proceed through mitosis and undergo apoptosis. Therefore, similar to TCS and DBA, it seems likely that the RBS phenotype may be related, at least in part, to increased cell death and death of neural crest precursors. However, in contrast to TCS and DBA, the cell death in *esco2* morphants is p53 independent [81].

Studies in yeast and human cell lines revealed that *eco1* mutants exhibited defects in ribosome biogenesis including reduced protein translation [78]. Specifically, production of the methylated 25S and

18S rRNA transcripts was diminished and nucleolar morphology was disrupted in *eco1* mutants [78]. Consistent with this, fibroblasts from individuals with RBS showed diminished rRNA production and protein synthesis. This suggests a similar disregulation of ribosome biogenesis underlies the pathogenesis of TCS, DBA and RBS.

Cohesin proteins may therefore normally facilitate production of ribosomal RNA and protein translation. Although RBS is classically considered a cohesinopathy [82], RBS can also be classified as a ribosomopathy given the features of diminished ribosome biogenesis, nucleolar disruption, and apoptosis. It remains to be determined what impact these cohesinopathy mutations have on neural crest cell development, however, future work with RBS models may reveal novel roles for *esco2* in neural crest formation, migration, or differentiation and provide new information for the development of therapies to improve the quality of life for RBS patients.

1.7. Shwachman–Diamond syndrome

Shwachman–Diamond syndrome (SDS, MIM 260400) is another ribosomopathy disorder that encompasses skeletal abnormalities and bone marrow failure [83,84]. In fact, abnormal dermal bone formation and abnormal endochondral bone formation are characteristic features of SDS. This initially manifests as delayed bone aging and maturation in association with abnormal growth plate and metaphysis development, resulting in short stature, but it also leads to progressive deleterious pathological conditions including osteopenia, osteoporotic vertebral anomalies and fractures [83,85]. Similar to TCS, there is considerable variability in the severity and extent of skeletal phenotypes in individuals with SDS, despite patients within the same family carrying identical mutations [86].

SDS occurs with an estimated incidence of 1 in 50,000 live births and approximately 90% of individuals with SDS carry biallelic mutations in *SBDS* [87]. Thus SDS is an autosomal recessive disorder arising from mutations in the *SBDS* gene [87]. These mutations are thought to be likely hypomorphic, since by comparison, mice that are homozygous null for mutant alleles of *SBDS* exhibit early embryonic lethality, illustrating that SBDS is essential for normal development [88].

Analyses in mammalian cells imply that SBDS performs multiple functions in distinct processes during development. These include ribosome biogenesis [89], cell motility [90,91], and the regulation of oxidative stress [92] and mitotic spindle stabilization [93]. However, analyses in yeast and human cells support the notion that SDS is a disorder of ribosome biogenesis. *Sdo1*, the yeast ortholog of *SBDS*, has been shown to localize to the nucleolus and function in ribosome biogenesis [94,95]. Furthermore, cells derived from individuals with SDS exhibit perturbed activity of ribosome biogenesis associated genes, particularly those that govern the processing of tRNA and mRNA [96]. Moreover, the activity of ribosomal proteins known to influence cell survival and growth was also similarly decreased [97]. SBDS however, influences not only early steps in the ribosome biogenesis process, but also late steps in ribosomal subunit maturation and function. The eukaryotic initiation factor 6 (eIF6), typically keeps the 60S subunit functionally inactive. However, a pre-requisite for the initiation of 80S ribosome translational activity is the release of eIF6 from the 60S ribosome and this release is facilitated by SBDS [98].

The varied clinical phenotypes observed in SDS have been hypothesized to reflect the level of residual SBDS protein expression coupled with critical threshold requirements for SBDS function in meeting translational demands in different tissues at various times during development [99,100]. Although, the expression and role of SBDS in cartilage and bone remains unknown, chondrocytes and osteoblasts of the skeletal system are all characterized by high protein secretory capacity. Impaired SBDS function may therefore mediate its effects through perturbation of global protein translation or more likely through defective translation of critical proteins involved in axial and limb skeletal development. Either way, SDS is a ribosomopathy, caused primarily by impaired release of eIF6 and deficient 80S translational activity as a consequence of mutations in the function of the ribosome assembly factor SBDS.

1.8. Cartilage hair hypoplasia

Cartilage hair hypoplasia (CHH, MIM 250250), which is also known as metaphyseal chondrodysplasia McKusick type [101], is characterized by short-limb dwarfism with adults ranging in height from 104 to 149 cm. Metaphyseal or spondyloepiphyseal dysplasia underlie CHH and similar short-limb dwarfism conditions. The limbs and ribs are most affected, with sparing of the spine and skull. Radiographic studies reveal short and thick tubular bones, with splaying and irregular metaphyseal borders of the growth plates that are widened, scalloped and irregularly sclerotic [102,103]. The costochondral junctions are similarly affected. These metaphyseal changes are present at birth and are distinguished from other metaphyseal chondrodysplasias by more severe involvement of the knee region than in the proximal femora [104]. CHH is a variant of short-limb dwarfism in which fine, thin and sparse hair is also present, beginning in the newborn period. Other common features include ligamentous laxity, defective T-cell and/or B-cell mediated immunity, hypoplastic anemia, and variable aganglionosis of the intestine, which leads to gastrointestinal anomalies such as Hirschsprung disease in some individuals [103,105,107].

First described in Amish people [101] the incidence of CHH in the Amish is 1 in 1340 births, with a carrier frequency of 1:19. In contrast, in Finland, CCH presents with a frequency of 1 in 18,000–23,000 live births with an estimated carrier rate of 1:76 [103]. CHH is an autosomal recessive disorder with equal male-to-female frequency that arises from mutations in the gene *RMRP*, which maps to 9p12 [108]. The founder mutation 70ArG contributes to 92% of Finnish and 48% of non-Finnish patients with CHH [109].

The RMRP gene encodes the untranslated RNA subunit of the ribonucleoprotein endoribonuclease processing complex, RNase MRP. RMRP is therefore a snoRNA that localizes to the nucleolus and mitochondria of cells. Within the nucleolus, snoRNAs form small nucleolar ribonucleoprotein complexes (snoRNPs) that regulate the synthesis of ribosomal RNA [110]. The yeast ortholog of *RMRP* is *nme1*, and it functions in (i) ribosome synthesis, via nucleolar cleavage of preribosomal RNA (pre-rRNA) and rRNA production; (ii) the generation of RNA primers for mitochondrial DNA replication, via cleavage of RNA [110]; and (iii) in governing cell growth via degradation of cell cycle-regulated mRNA [111]. Mutation of *nme1* affects endonuclease cleavage of 5.8S rRNA at the ITS-1 A3 site, which consequently disrupts 60S ribosomal assembly [112,113]. The magnitude of the effect on *nme1* mutant yeast cell growth, is directly proportional to the severity of the defect in 5.8S rRNA-processing [114]. Furthermore, deficient production or incorporation of a single subunit of the 60S ribosome, can lead not only to the disintergration of the entire 60S ribosome, but can also negatively impact regulatory feedback signals from the secretory machinery on ribosome biogenesis [115].

Overexpressing RMRP in human fibroblasts substantially elevates production of the cleaved or processed 5.8S rRNA and consequently accelerates cellular growth rates [116]. In contrast the mutated form of RMRP found in individuals with CHH elicits minimal cleavage or processing of the 5.8S rRNA. Furthermore, cells overexpressing the CCH variant of RMRP showed significantly decreased cyclin A2 levels and significantly increased cyclin *B2* mRNA levels. This is indicative of decreased mRNA degradation, which leads to mitotic delay. Thus *RMRP* gene mutations affect cell growth through their perturbation of both ribosomal assembly and cyclin-dependent cell–cycle regulation. These alterations are consistent with slower cell cycle rates that can account for the decreased rates of growth in the clinical phenotype of CHH.

Mutations in *RMRP* have also been found responsible for two other autosomal recessive skeletal dysplasias. Metaphyseal dysplasia without hypotrichosis (MIM 250460) is an allelic variant of CHH that exhibits only skeletal manifestations [117,118]. The *RMRP* mutations

in Anauxetic dysplasia (MIM 607095) disrupt its endonucleolytic activity, specifically leading to poor processing of rRNA while allowing for normal mRNA processing of B-cyclin [116]. This disorder is characterized by midface hypoplasia and extremely short stature with defects in the growth plate including marked hypocellularity of the resting cartilage, severely reduced numbers of proliferating chondrocytes, and diminished columnization of the hypertrophic zone [119,120].

1.9. Bowen–Conradi syndrome

Bowen–Conradi syndrome (BWCNS; MIM 211180) is characterized by micrognathia, a distinct, prominent nose, pre-natal and post-natal growth retardation together with joint anomalies such as camptodactyly. Other clinical features of BWCNS can include microcephaly and psychomotor delay [121,122]. This spectrum of anomalies overlap with those associated with cerebro-oculo-facial-skeletal syndrome and trisomy 18 disorders. Nearly all individuals born with BWCNS are from Hutterite families of the Canadian Prairies and die within the first year of life. Recently a c.400A/G, p.D86G mutation in the *EMG1* gene was identified in association with BWCNS illustrating that BWCNS is an autosomal recessive disorder [123] and one that occurs with an incidence of about 1 in 355 [122].

EMG1 can function as a dimer or multidimer and the D86G mutation is thought to alter the structure of the EMG1 protein. Although this results in diminished levels of EMG1 protein in fibroblasts derived from BWCNS patients, the effect of the D86G mutation is to increase the interaction of EMG1 subunits, 10-fold [123]. The D86 residue in EMG1, which is consistently mutated in association with BWCNS is conserved across all orthologs analyzed to date [123]. Known as Nep1 in yeast, Emg1 is a constituent of the small subunit (SSU) processome, a ribonucleoprotein complex required for 18S rRNA biogenesis [124]. During the complex process of ribosome biogenesis, precursor 35S rRNA undergoes considerable modification and processing to generate mature 5.8S, 25S, and 18S rRNA [125]. EMG1 is a SPOUT methyltransferase, which methylates the ribose 2′–OH group, guanine N1, or uridine N3 of rRNA or tRNA as part of this maturation process [126]. More specifically, EMG1 is intimately involved in regulating the snoRNP complex that 2′–OH ribose methylates 18S rRNA at G1570. Furthermore, EMG1 plays a critical role in recruiting the SSU processome protein, Rps19 [127]. Interestingly, the depletion of individual components of the SSU processome result in G1 cell-cycle arrest in yeast [128]. A G1/S checkpoint helps to ensure that prior to cell division, sufficient levels of mature ribosomes are achieved to support cell division and proliferation. This supports the concept that cell cycle progression is intimately connected to ribosome biogenesis [129,130].

Thus through its participation in the SSU processome, EMG1 influences ribosome biogenesis maturation and processing in multiple ways. Specifically, EMG1 appears to play critical roles in the maturation of 18S rRNA and subsequently 40S ribosome biogenesis through the process of methylation [131,132]. EMG1 is broadly expressed in most embryonic and adult tissues with elevated levels in organs such as the brain, heart, liver, stomach and kidney. EMG1 loss-of-function might therefore render cells incapable of generating enough 40S ribosomes to support normal rates of cell division and proliferation during both embryonic development and adult homeostasis.

2. Conclusions

The demonstration that perturbation of ribosome biogenesis underlies many skeletal disorders in humans is perhaps not surprising given the importance of proliferation and the dramatic changes in cells size that occur during cartilage and bone development. However, protein synthesis is a ubiquitous requirement in cells, tissues and organs, and furthermore ribosomal proteins are very widely expressed. Thus the fact that there is considerable variability in the phenotypic spectrum of individual ribosomopathy skeletal disorders, and also that the severity of individual phenotypes varies combined with distinct modes of inheritance, presents complex challenge for the field to mechanistically understand. For example, some syndromes specifically affect the craniofacial skeleton (Treacher Collins, syndrome), while other syndromes encompass combinatorial malformation of the craniofacial, axial and/or limb skeletal systems (Diamond–Blackfan anemia; Postacrofacial dysostosis; Roberts syndrome; Schwachman–Diamond syndrome; Cartilage hair hypoplasia; Bowen–Conradi syndrome). Similarly bone marrow failure may or may not present as part of the clinical spectrum of skeletal anomalies. Bone marrow failure is not a recognized component of Treacher Collins syndrome or Postacrofacial dysostosis, but is a defining feature of Diamond–Blackfan anemia, Shwachman–Diamond syndrome and Cartilage hair hypoplasia. This raises the fundamental question of how mutations in genes critical for ribosome biogenesis, which might normally have global or widespread roles during organism development, can lead to such selective defects.

The characteristic of broadly expressed genes exhibiting cell or tissue specific functions is not unique or exclusive to ribosomal genes. However at present there does not appear to be a single unifying factor that collectively links ribosomal genes and proteins except for their roles in various aspects of ribosome biogenesis and the clinical pathogenesis of ribosomopathies. One possibility however, is that the clinical differences and their variability may be explained by the types of alteration and the magnitude of their effect on ribosome biogenesis. For example, distinct mutations in *RMRP* give rise to the allelic conditions of Cartilage hair hypoplasia and Anauxetic dysplasia. Mutations in *RMRP* that reduce ribosomal RNA cleavage are associated with bone dysplasia, whereas mutations that affect mRNA cleavage are associated with hair hypoplasia, immunodeficiency, and dermatologic abnormalities [133]. This also raises a number of issues concerning the spatiotemporal dynamics of ribosome biogenesis and whether different threshold levels of activity are required in one tissue versus another at different times of development. Consistent with this idea, such a cell type specific proliferation requirement has been postulated in the pathogenesis of TCS [51].

While tremendous inroads have been made in understanding the many levels of regulation controlling gene expression, the regulatory control of protein production has garnered much less attention. The historic idea that ribosomes function constitutively to translate the genetic code has recently been challenged. An emerging idea is that ribosomes are specialized, built diversely of different rRNAs and combinations of ribosomal proteins and associated factors with variable post-translational modifications. This diversity has a considerable impact on how an mRNA template is translated into functional protein. Even core ribosome components are now thought to exert selective activity by virtue of their interactions with specific cis-acting regulatory elements that are present within subsets of mRNAs [134]. Thus ribosome activity appears to be highly regulated and this may provide an important new mode for governing spatiotemporal gene expression during normal embryonic development and in the pathogenesis of congenital skeletal anomalies.

A common feature shared by TCS, POADS and DBA, is craniofacial skeletal malformations caused by perturbations of neural crest cell development. This phenotypic overlap likely reflects a common molecular function for these proteins in ribosome biogenesis during craniofacial development. Thus, it will be important to understand how TCOF1, POLR1C, POLR1D, ribosomal proteins, and DHODH are functionally integrated in neural crest cell-derived bone. However, TCS, POADS and DBA exhibit distinct abnormalities of the skeleton and this reflects the need to better understand the individual roles of these proteins in skeletal development. Understanding how the mechanisms of disease overlap and diverge in ribosomopathies with skeletal defects will be instrumental in designing realistic avenues for their therapeutic prevention.

Under normal cellular growth conditions, Mdm2 targets p53 for degradation through polyubiquitination. In contrast, under conditions of perturbed ribosome biogenesis and nucleolar stress, unincorporated

ribosomal proteins bind to Mdm2 inhibiting its polyubiquitination capacity. This leads to activation and stabilization of p53 and ultimately cell death [135,136]. As a case in point, direct inhibition of p53 dependent apoptosis can successfully prevent the manifestation of ribosomopathy disorders such as TCS (*Tcof1*) and DBA (*Rps19*) [52,65]. However, p53 functions as a tumor suppressor and any inhibition of p53 would therefore carry a substantial risk of cancer or tumorigenesis side effects. This highlights the need to explore other avenues for ribosomopathy prevention. Interestingly, L-leucine supplementation has recently been used to successfully treat DBA in humans [68,69] and animal models [66,67]. Embryonic zebrafish and mice which model DBA showed considerably improved craniofacial and hematopoietic development when their diets were supplemented with L-leucine [66,67]. The mechanistic basis for this is in the craniofacial region, is that L-leucine stimulates ribosome biogenesis through the mTOR pathway and thus counters the p53 dependent apoptotic loss of neural crest cells. Thus L-Leucine supplementation may be a possible treatment option for other disorders of ribosome biogenesis. Consistent with this idea, L-leucine was recently shown to ameliorate the development of Roberts syndrome-like abnormalities in zebrafish and patient specific cell based models of the disorder [137].

The disorders described in this review arise due to deficient ribosome biogenesis. However, the converse is also likely to be true, that excessive ribosome biogenesis can also lead to skeletal anomalies. It might be expected that perturbations in signals that lie upstream to regulate levels of ribosome biogenesis in bone may be able to elicit such effects. Consistent with this idea, it has long been known that signaling molecules such as FGF, BMP, Wnt, and Hedgehog spatiotemporally regulate growth, proliferation and differentiation in the craniofacial, limb and axial skeleton. Yet, how these signals are integrated with ribosome biogenesis as a means to adapt to changing requirements for protein synthesis during bone formation and homeostasis is unclear. Resolving this issue will provide a better understanding of why bone is particularly sensitive to levels of ribosome biogenesis. The convergence of developmental signaling pathways with ribosome biogenesis can provide additional levels of translational specificity to influence fundamental aspects of cell growth and proliferation in the context of embryonic development, evolution and congenital disease.

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