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Rare syndromes of the head and face: mandibulofacial and acrofacial dysostoses

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Craniofacial anomalies account for approximately one-third of all congenital birth defects reflecting the complexity of head and facial development. Craniofacial development is dependent upon a multipotent, migratory population of neural crest cells, which generate most of the bone and cartilage of the head and face. In this review, we discuss advances in our understanding of the pathogenesis of a specific array of craniofacial anomalies, termed facial dysostoses, which can be subdivided into mandibulofacial dysostosis, which present with craniofacial defects only, and acrofacial dysostosis, which encompasses both craniofacial and limb anomalies. In particular, we focus on Treacher Collins syndrome, Acrofacial Dysostosis-Cincinnati Type as well as Nager and Miller syndromes, and animal models that provide new insights into the molecular and cellular basis of these congenital syndromes. We emphasize the etiologic and pathogenic similarities between these birth defects, specifically their unique deficiencies in global processes including ribosome biogenesis, DNA damage repair, and pre-mRNA splicing, all of which affect neural crest cell development and result in similar tissue-specific defects. © 2016 Wiley Periodicals, Inc.

INTRODUCTION

The craniofacial complex houses and protects the brain and most of the body’s primary sense organs and is essential for feeding and respiration. Composed of nerves, muscles, cartilage, bone and connective tissue, head and facial development begins during early embryogenesis with formation of the frontonasal prominence and the pharyngeal arches, which are transient medial and lateral outgrowths of cranial tissue (Figure 1). The frontonasal prominence ultimately gives rise to the forehead and the nose, while the reiterated pattern of paired pharyngeal arches give rise to the jaws and parts of the neck.2 The basic structure of each prominence and arch is the same. Externally, they are composed of ectoderm, which with respect to the pharyngeal arches, forms the pharyngeal clefts or grooves. Internally, the frontonasal prominence and pharyngeal arches are lined with endoderm, which forms the pharyngeal pouches. At the junctions that separate the pharyngeal arches, the endoderm contacts the ectoderm by an active movement called out-pocketing.2–4 Between the ectoderm and endoderm epithelia is a mesenchymal core. In the frontonasal prominence the core is composed of neural crest cells (NCCs), while in the pharyngeal arches the mesenchymal core is composed of both NCC and mesoderm.5,6 NCC are a multipotent progenitor cell population that is derived from the neuroepithelium, undergoes an epithelial to mesenchymal transformation, delaminates and then migrates, colonizing the frontonasal prominence and pharyngeal arches2–3 (Figure 1(a)–(c)). Collectively, these four tissues, ectoderm, endoderm, NCC, and...
mesoderm, interact to give rise to the skeletal, muscular, vascular, and nervous tissue elements of the head and neck2,7,8 (Figure 1(d)–(f)). The complexity of craniofacial development renders it susceptible to developmental anomalies. Approximately one third of all congenital anomalies affect the head and face, and to date, more than 700 distinct craniofacial syndromes have been described.

The facial dysostoses describe a set of rare, clinically and etiologically heterogeneous anomalies of the craniofacial skeleton. Facial dysostoses arise as a consequence of abnormal development of the first and second pharyngeal arches and their derivatives, including the upper and lower jaw and their hyoid support structures. Facial dysostoses can be subdivided into mandibulofacial dysostosis and acrofacial dysostosis. Mandibulofacial dysostosis (OMIM610536)9 manifests at birth as maxillary, zygomatic, and mandibular hypoplasia (Figure 2), together with cleft palate, and/or ear defects. Many distinct mandibulofacial dysostosis syndromes have been described; however, clinically, the best understood is Treacher Collins syndrome (TCS; OMIM 154500).10–13 In contrast, acrofacial dysostoses present with craniofacial anomalies similar to those observed in mandibulofacial dysostosis but with the addition of limb defects. The acrofacial dysostoses include the well-characterized disorders of Miller syndrome (OMIM263750)14,15 and Nager syndrome (OMIM154400)16–18 as well as more recently identified conditions such as Acrofacial Dysostosis-Cincinnati type (OMIM616462).19

TREACHER COLLINS SYNDROME

TCS occurs with an incidence estimated at 1:50,000 live births.9,20 TCS is defined clinically by bilaterally symmetrical features that include hypoplasia (underdevelopment) of the facial bones, in particular the mandible (lower jaw) and zygomatic complex (cheek bones), coloboma (notching) of the lower eyelids; downward slanting of the palpebral fissures (opening...
between the eyelids); microtia or atresia (under-development or absence) of the external ears; narrowing of the ear canal, often resulting in conductive hearing loss (Figure 2); and micrognathia (small lower jaw) with or without cleft and/or high-arched palate. A considerable degree of interfamilial and, in multigeneration families, intrafamilial variation has been observed. In severely affected cases, TCS may result in perinatal death due to a compromised airway. In contrast, individuals may be so mildly affected that it can be difficult to establish an unequivocal diagnosis solely by clinical examination. Indeed, some patients are only diagnosed after the birth of a more severely affected child.

The Genetic Basis of TCS

A combination of genetic, physical, and transcript mapping led to the identification of causative mutations for TCS in the gene designated TCOF1 on chromosome 5q32 in humans. The major TCOF1 transcript was found to comprise an open-reading frame of 4233 bp encoded by 26 exons. However, two alternatively spliced exons, exon 6A and exon 16A, may also be present in the minor transcripts. Several hundred largely family-specific deletions, insertions, splicing, and nonsense mutations have subsequently been identified with partial gene deletions accounting for a small proportion of all mutations. The typical effect of the mutations is the introduction of a premature termination codon and the induction of nonsense-mediated mRNA degradation, leading to haploinsufficiency of TCOF1. This hypothesis is supported by the observation that cells derived from TCS patients exhibit significantly reduced levels of TCOF1, with the mutant allele being less abundant than its wild-type counterpart. To date, only a very small number of missense mutations have been identified and these all affect amino acid residues toward the N-terminus of the protein.

FIGURE 2 | Mandibulofacial dysostosis. (a) Schematic of the pharyngeal arches of a healthy human embryo. (b) Maxilla and mandible bone structures derived from neural crest cells that colonize the first pharyngeal arch. (c) Schematic of the pharyngeal arches of a human embryo with mandibulofacial dysostosis which arises as a consequence of hypoplastic first and second pharyngeal arches. (d) Hypoplastic maxilla and mandible bone structures observed in mandibulofacial dysostoses.
carrier parents were completely normal clinically. The mutation was likely previously missed by direct Sanger sequencing, due to the expectation of a heterozygous sequence chromatogram peak given the characteristic autosomal dominant nature of the disease.

Collectively, about 80% of TCS cases are thought to be caused by mutations in TCOFI, which encodes the nucleolar phosphoprotein, Treacle (Figure 3). As all the large, multigeneration TCS families analyzed exhibited linkage to polymorphic markers within human chromosome 5q32, TCS was originally considered to be genetically homogeneous. However, despite extensive searches, the causative mutation in a subset of patients exhibiting classic features of TCS remained unidentified. The use of genome-wide copy number analysis in a child with TCS who was negative for a TCOFI mutation, led to the identification of a de novo 156-kb deletion within human chromosome 13q12.2 that resulted in deletion of the entire POLR1D gene. POLR1D encodes a subunit of RNA polymerase I and III (Figure 3). Subsequently, a further 242 individuals with classic features of TCS, but who were negative...
The Biochemical Basis of TCS: The Role of Treacle in Ribosome Biogenesis

TCOF1 encodes the low complexity, nucleolar phosphoprotein Treacle which contains putative nuclear export and nuclear import signals at the N- and C-terminus, respectively, with a central repeat domain which is subject to a high degree of phosphorylation by casein kinase 2. Immuno-fluorescence studies indicated that Treacle exhibits nucleolar localization dependent upon C-terminal motifs, and subsequently, Treacle was shown to colocalize with UBF, one of two transcription factors required for accurate transcription of human ribosomal RNA genes by RNA polymerase I (PolI) (Figure 3). These observations suggested an association between Treacle and the ribosomal DNA transcription machinery. Immuno-precipitation and yeast two-hybrid analyses confirmed a direct interaction between Treacle and UBF, and siRNA-mediated knockdown of Treacle in vitro resulted in inhibition of rDNA transcription. Downregulation of Treacle expression also resulted in decreased methylation of 18S pre-rRNA, possibly via its interaction with NOP56 protein, a component of the pre-rRNA methylation complex.

Recent studies have demonstrated that Treacle functions as a stable constituent in the PolI complex independent of UBF by associating with PolI through its central repeat domain, whereas the C-terminus of Treacle interacts with UBF, human Nopp140, and the rDNA promoter. Importantly, depletion of Treacle drastically alters the localization of UBF and PolI indicating an essential role for Treacle in nucleolar retention of these two proteins, possibly by acting as a scaffold protein to maintain PolI in the nucleolus. Deletion constructs engineered to mimic mutations observed in TCS patients demonstrated that, in the presence of endogenous Treacle, C-terminal deletions did not alter PolI localization nor block pre-rRNA transcription. These data indicate that the C-terminal truncations of Treacle do not act in a dominant-negative manner and provide further evidence that the craniofacial features of TCS patients are the result of TCOF1 haploinsufficiency.

The Cellular Basis of TCS: The Role of Treacle in Neuroepithelial Survival and NCC Proliferation

The first insights into the developmental pathogenesis of TCS were derived from expression analyses in mouse embryos, which indicated that although the murine orthologue of TCOF1 was widely expressed, the highest levels were observed in the neuroepithelium and in NCC-derived facial mesenchyme. Subsequently, a mouse model of TCS was generated using gene targeting to replace exon 1 of Tcof1 with a neomycin-resistance cassette. Neonatal mice exhibit severe craniofacial anomalies exceeding the spectrum of those observed in TCS patients, including exencephaly, abnormal development of the maxilla, hypoplasia of the mandible, anophthalmia, and agenesis of the nasal passages, resulting in death shortly after birth due to asphyxia. The facial phenotype was subsequently found to be strongly dependent on the genetic background on which the mutation was placed, ranging from neonatal lethality in three strains of mice, including C57BL/6, to viable and fertile in two others, DBA and BALB/c, allowing the mutation to be maintained and evaluated in these backgrounds.

Although Tcof1+/−/DBA mice appear grossly normal, they exhibit abnormalities in middle ear cavitation and growth of the auditory bullae resulting in profound conductive hearing loss. Notably, Tcof1−/−/DBA mice also exhibit significant hypoplasia of the brain compared with their wild-type littermates even though there is no difference in the body weight between the different genotypes. The microcephaly observed in the mutant mice arises from an anomaly in neural progenitor maintenance. In this context, Treacle localizes to the centrosomes and kinetochores in mitotic cells, and its interaction with Polo-like kinase 1 (Plk1) is essential for the control of spindle orientation, mitotic progression, and subsequent maintenance of neural progenitor cells during brain development.
Crucially, intercrossing Tcof1<sup>−/−</sup> DBA mice onto the C57BL/6 background yielded heterozygous embryos that displayed a consistent phenotype with features highly similar to those observed in TCS patients. These features include deficiencies of the nasal and frontal bones, the premaxilla, maxillary, and palateine bones, as well as cleft palate. More detailed investigation of the palatal anomalies observed in the Tcof1<sup>−/−</sup> mice indicated that while 46% exhibited either a complete cleft of the secondary palate (6%) or a cleft affecting the soft palate only (40%), the remainder displayed high-arched palate with reductions in palatal length and width. Analysis of embryos on a mixed DBA:C57BL/6 background revealed no differences in the patterns of NCC migration between Tcof1<sup>−/−</sup> and wild-type embryos, thus disproving the long-held hypothesis of perturbed NCC migration as a cause of TCS. What was apparent, both from lineage tracing and molecular markers of NCC, such as Sox10, was that fewer crest cells appeared to migrate from the neural folds into the developing facial complex. Flow cytometry analyses of GFP-labeled NCC indicated that there were 22% fewer migrating NCC in Tcof1<sup>−/−</sup> embryos compared to their wild-type littermates, resulting in hypoplastic cranial sensory ganglia and skeletal elements. TUNEL staining in combination with DiI tracing in vivo to delineate the earliest waves of migrating crest cells at E8.0 to E8.5, demonstrated conclusively that migrating NCC were viable in Tcof1<sup>−/−</sup> embryos. However, elevated levels of cell death were observed throughout the neuroepithelium, suggesting that Treacle was essential for the viability of neuroepithelial cells and progenitor NCC. Furthermore, BrdU labeling of E8.5–E9.0 embryos revealed a significant reduction of proliferation in neuroepithelial cells and in neural crest-derived cranial mesenchyme, effects that correlated with the spatiotemporal expression pattern of Tcof1. Importantly, Tcof1 haploinsufficient mouse embryos displayed a significant reduction in pre-rRNA levels, confirming Treacle plays a crucial role in ribosomal RNA production and subsequent ribosome biogenesis, which is essential for cell growth and proliferation. Treacle’s role in ribosome biogenesis has also been documented using the Y10B antibody, which recognizes epitopes of rRNA and is used as a marker of mature ribosomes. Homotypic transplantation of Di-labeled midbrain and hindbrain tissue demonstrated that Treacle functions cell-autonomously to promote neuroepithelial and progenitor NCC proliferation and survival through dynamic regulation of the spatiotemporal production of mature ribosomes in neuroepithelial cells and NCC.

Consistent with these observations, a recent study also proposed a link between Treacle and ribosome biogenesis associated factors in NCC development. More specifically, the cullin-RING ligase proteins comprise the largest class of ubiquitination enzymes, and the vertebrate-specific CUL3 adapter protein KBTBD8 was shown to be an essential regulator of NCC specification through ubiquitination of Treacle and the nucleolar and coiled-body phosphoprotein 1 (NOLC1). Formation of a Treacle-NOLC1 complex connects RNA PolII with enzymes responsible for ribosomal processing and modification. Ultimately, the KBTBD8-dependent assembly of a ribosome modification platform remodels the translational program of differentiating cells undergoing neural conversion in favor of NCC specification. However, these studies, which were performed in Xenopus embryos, were based solely on gene expression which is not an indicator of lineage and need to be evaluated by rigorous lineage tracing for the presence of NCC as well as be repeated in other model systems.

**Prevention of TCS through Inhibition of p53 Function**

A key breakthrough in our understanding of the role of Treacle came from microarray analyses of Tcof1<sup>−/−</sup> embryos and their wild-type littermates which revealed that many well-recognized targets and mediators of p53-dependent transcription were upregulated in Tcof1<sup>−/−</sup> embryos. These included Ccg1, Trp53inp1, Pmaip1, Perp, and Wig1 which have been linked to diverse cellular processes such as cell-cycle regulation, apoptosis, senescence, and DNA repair. This observation suggested a strong correlation between Tcof1 haploinsufficiency and p53-dependent cell-cycle arrest and apoptosis. p53 protein is rapidly degraded under normal physiological conditions (Figure 3); however, immunohistochemistry using an anti-p53 antibody revealed elevated levels of p53 in the neuroepithelium of Tcof1<sup>−/−</sup> embryos, providing a conclusive link between p53 stabilization, neuroepithelial cell death, and the subsequent deficiency of migrating crest cells. Moreover, this points to the hypothesis that inhibition of p53 function might ameliorate, or even prevent, the key features of TCS in Tcof1<sup>−/−</sup> embryos.

Subsequently, daily administration of pifithrin-α, a chemical inhibitor of p53-dependent transcription and apoptosis, from E6.5 to E8.5, was shown to substantially reduce neuroepithelial apoptosis in...
The Role of Treacle in the DNA Damage Response

Recent research has shown that Treacle also plays a fundamental role in the DNA damage response pathway which is activated to maintain genome integrity. Treacle colocalizes with P-ATM (phosphorylated -ataxia telangiectasia mutated protein) and Rad50 (a protein involved in DNA double-strand break repair) to DNA lesions in association with DNA damage. More specifically, Treacle interacts physically with the MRN complex (a protein complex consisting of Mre11, Rad50, and Nbs1), which recruits ATM to DNA double-strand breaks where it phosphorylates H2AX (H2A histone family, member X), in response to DNA damage. This newly phosphorylated histone is then responsible for recruiting DNA repair proteins to the damage sites. Based on these observations, it was hypothesized that the neuroepithelial cell death observed in Tcof1+/− embryos might be associated with DNA damage in vivo. Consistent with this idea, immunostaining indicated that γH2AX, which occurs via phosphorylation of H2AX by ATM at double-strand breaks in response to DNA damage, was present in neuroepithelial cells of E8.5 Tcof1+/− embryos. Furthermore, γH2AX-positive neuroepithelial cells were labeled with phosphorylated Chk2 (cell cycle checkpoint kinase 2), a protein that transmits the DNA damage response signal to the apoptotic pathway and caspase 3 (a marker of apoptosis). Interestingly, treating wild-type embryos with 3-nitropipionic acid, a potent inducer of reactive oxygen species (ROS), results in a substantial increase in neuroepithelial cell death. Thus, compared with cells of the nonneural ectoderm, mesoderm and endoderm, the neuroepithelium exists in a highly oxidative state and is very sensitive to exogenous oxidative stress. Consequently, it was proposed that Tcof1 loss of function or haploinsufficiency could increase a cell or tissue’s sensitivity to oxidative stress-induced DNA damage. Consistent with this idea, dietary supplementation of Tcof1+/− embryos with N-acetylcysteine, a strong antioxidant, reduced the number and size of DNA damage-induced foci, diminished the level of neuroepithelial apoptosis, and substantially ameliorated the craniofacial anomalies observed in Tcof1+/− mice. Together these results suggest that antioxidant supplementation may provide a therapeutic avenue for the prevention of TCS.

In parallel studies, DNA damage was shown to induce the recruitment of Nijmegen breakage syndrome protein 1 (NBS1) into the dense fibrillar component of the nucleoli where it silences ribosomal RNA transcription. Furthermore, NBS1 was shown to bind Treacle directly and that an Nbs1-Treacle complex controls rRNA transcription in response to DNA damage. Thus, Treacle, in addition to its role in ribosome biogenesis, facilitates the preservation of genomic stability after DNA damage and importantly links these two critical processes together.

The Biochemical and Molecular Basis of TCS; The Role of POLR1C and POLR1D in Ribosome Biogenesis

Although the effects of TCOF1 mutations in the pathogenesis of TCS have been studied extensively, less is known about the molecular basis behind POLR1C or POLR1D mutations in the context of TCS. However, the first insights into the roles of both polr1c and polr1d in vertebrate development were recently described in zebrafish. Similar to Tcof1 in the mouse embryo, polr1c is ubiquitously expressed throughout the zebrafish embryo with elevated levels in specific tissues including the eye,
midbrain, central nervous system, and the pharyngeal arches and their derivatives, such as Meckel’s cartilage and the lower jaw.\textsuperscript{65,66} A comparable expression pattern is also observed for \textit{polr1d}.\textsuperscript{55} Consistent with these observations, alcan blue staining revealed that in both \textit{polr1c}\textsuperscript{+/−}\textit{(polr1c}\textsuperscript{hi1124Tg})\textsuperscript{65,66} and \textit{polr1d}\textsuperscript{+/−}\textit{(polr1d}\textsuperscript{hi2393Tg})\textsuperscript{65} zebrafish, cartilage elements such as the palatoquadrate and Meckel’s cartilage were hypoplastic, mimicking defects observed in TCS patients. Since the majority of the craniofacial cartilage is NCC-derived, a variety of markers for different phases of NCC development were tested via \textit{in situ} hybridization to understand the roles of \textit{polr1c} and \textit{polr1d} in NCC development. Analysis of \textit{sox2}, a marker for NCC specification and formation, and \textit{sox10} and \textit{foxd3}, as markers of premigratory and migratory NCC, revealed that NCC specification, formation and migration occurred appropriately in \textit{polr1c}\textsuperscript{−/−} and \textit{polr1d}\textsuperscript{−/−} mutant embryos.\textsuperscript{65,66} However, diminished domains of \textit{dlx2} expression indicated a reduction in the number of mature NCC populating the pharyngeal arches. In agreement with this observation, Fli1a:egfp which labels postmigratory NCC, also revealed a significant reduction in the size of the pharyngeal arches in mutant embryos compared to that of controls.\textsuperscript{65}

Collectively, these results raised the question of whether increased cell death or decreased cell proliferation was the underlying cause of the diminished number of NCC colonizing the pharyngeal arches in \textit{polr1c} or \textit{polr1d} mutant zebrafish. TUNEL staining revealed a significant increase in cell death in the neuroepithelium, affecting premigratory NCC progenitors, but no cell death was observed in migratory NCC in \textit{polr1c} or \textit{polr1d} mutants.\textsuperscript{65} Interestingly, however, apoptosis was detected in the lower jaw and pharyngeal region of 5 dpf \textit{polr1c} mutant zebrafish, suggesting a late embryonic role for \textit{polr1c} in cell survival during skeletal differentiation in addition to its earlier role in the neuroepithelium.\textsuperscript{66} Moreover, the cell death observed in \textit{polr1c} and \textit{polr1d} zebrafish was p53 dependent and genetic inhibition of p53 was able to suppress neuroepithelial apoptosis and ameliorate the craniofacial anomalies in \textit{polr1c} and \textit{polr1d} mutants.\textsuperscript{65}

\textbf{ACROFACIAL DYSOSTOSIS-CINCINNATI TYPE}

Acrofacial dysostosis describes a congenital syndrome which presents with craniofacial defects similar to those observed in mandibulofacial dysostosis (Figure 2) but with the addition of limb defects.\textsuperscript{19,67} Acrofacial Dysostosis-Cincinnati type was recently defined in three affected individuals with variable phenotypes ranging from mild mandibulofacial dysostosis to more severe acrofacial dysostosis.\textsuperscript{19} All three patients presented with variable craniofacial phenotypes similar to those observed in TCS, including hypoplasia of the zygomatic arches, maxilla and mandible; severe micrognathia; downslanting palpebral fissures; coloboma or inferiorly displaced orbits; bilateral anotia; and conductive hearing loss. Additionally, similar to other acrofacial dysostoses, two out of three patients presented with limb anomalies, including short bowed femurs; delayed epiphyseal ossification; flared metaphysis and dysplastic acetabula, while the other patient presented with short and broad fingers and toes.\textsuperscript{19} Interestingly, all three individuals were found to carry a heterozygous mutation in \textit{POLR1A}, which encodes the largest subunit of RNA polymerase I, which is responsible for transcribing rRNA.\textsuperscript{19}

\textit{Polr1a} is initially expressed ubiquitously in zebrafish embryos before becoming enriched in cranial tissues including the brain, eyes and otic vesicles, as well as the somites and presumptive fins.\textsuperscript{19} Similar to the domains of activity and functional roles of \textit{Tcof1}, \textit{polr1c} and \textit{polr1d}, it was hypothesized that mutations in \textit{POLR1A} would also perturb rRNA transcription and ribosome biogenesis thereby disrupting NCC and craniofacial development during embryogenesis. Consistent with this idea, homozygous \textit{polr1a}\textsuperscript{−/−} (\textit{polr1a}\textsuperscript{hi3639Tg}) zebrafish embryos exhibit craniofacial defects mimicking the phenotypes observed in individuals with Acrofacial Dysostosis-Cincinnati type.\textsuperscript{19} Reduced expression of the NCC markers: \textit{sox10}, \textit{sox9} and \textit{dlx2}, indicated that deficiencies in NCC are the cause of the craniofacial malformations in \textit{polr1a} mutant zebrafish. TUNEL assays subsequently revealed that \textit{polr1a} is required for neuroepithelial cell survival and the generation of NCC but is not required for the survival of migrating NCC,\textsuperscript{19} which is similar to the established roles of \textit{Tcof1}, \textit{polr1c} and \textit{polr1d}.

Analyses of \textit{Tcof1}\textsuperscript{+/−} mice, and \textit{polr1c}\textsuperscript{+/−} and \textit{polr1d}\textsuperscript{+/−} zebrafish, demonstrated that neuroepithelial...
Mandibulofacial Dysostosis with Microcephaly

Mandibulofacial dysostosis with microcephaly (MFDM), which is also known as mandibulofacial dysostosis (Figure 2), Guion-Almeida type (MFDM; MFDGA; MIM 610536) represents a subgroup of individuals with acrofacial dysostosis that also present with microcephaly.67 MFDM is characterized primarily by midface hypoplasia, downward slanting of the palpebral fissures, unusually small jaw (Figure 2), abnormalities of the external ears, which can lead to conductive hearing loss, and occasional abnormalities of the thumbs.67,68 The similar phenotypes observed in patients with MFDM, including downward slanting of the palpebral fissures, unusually small jaw (Figure 2), abnormalities of the external ears, which can lead to conductive hearing loss, and occasional abnormalities of the thumbs,67,68 This syndrome was described as the first multiple-malformation syndrome primarily attributed to a defect in the major spliceosome, which is responsible for removing introns from pre-mRNA.67,68 Recently, whole-exome sequencing studies revealed causative mutations in EFTUD2, which encodes a subunit of two complexes termed the major and the minor spliceosomes.69 Similar to TCS, a wide variety of mutation types have been identified in patients with MFDM, including nonsense and missense mutations, large deletions, frame-shifts and splice-site mutations, all of which are consistent with haploinsufficiency.67,70 Interestingly, eftud2 is ubiquitously expressed with enriched levels in the head, brain, tectum, eye and pharyngeal arches of zebrafish embryos.71 Unfortunately, zebrafish embryos homozygous for eftud2 mutations die prior to 2 dpf while heterozygotes do not show any noticeable phenotypes compared to their wild-type siblings.71 The phenotypes present in MFDM patients have been proposed to arise due to the aberrant splicing of genes specifically involved in NCC and/or bone development.69

Recently, a novel mutation in EFTUD2 was identified in a patient with microphthalmia, anophthalmia and coloboma (MAC).71 To date, no known EFTUD2-positive MFDM patients develop any abnormalities of the eye and it is possible that this is because individuals with MAC carry a secondary mutation that has yet to be identified.71 However, this discrepancy could also simply reflect the small number of reported cases with MFDM. Alternatively, the eye anomalies characteristic of MAC could be caused by an interaction between mutations in EFTUD2 and other genes involved in eye development.71 To understand these mechanisms, screening for eye anomalies in MFDM patients and for craniofacial anomalies in MAC patients is needed. It will also be critical to understand the mechanisms and developmental roles of EFTUD2 in mammalian development. Therefore, in the future, it will be necessary to generate conditional loss-of-function animal models and also recapitulate the human mutations to better understand the role of EFTUD2 and splicing during development and in the pathogenesis of MFDM and MAC.

Nager Syndrome

Nager syndrome (OMIM #154400) is the most frequent and well-studied type of acrofacial dysostosis. In addition to the overlapping craniofacial phenotypes with MFDM and TCS, including downward slanting of the palpebral fissures (Figure 2), Nager syndrome was identified as an acrofacial dysostosis condition due to the presence of preaxial limb defects, most commonly hypoplasia or absence of the thumbs.72,73 The similar phenotypes observed in Nager syndrome in comparison to other facial dysostoses, plus the small number of reported cases (n = 100), makes diagnosis and identification of common mutations in Nager syndrome challenging.67 Despite these limitations, recent studies identified mutations in SF3B4 in about 60% of Nager syndrome cases.74 Similar to TCS, Nager syndrome is rare and is primarily associated with de novo mutations, although both autosomal dominant and autosomal recessive mutations have also been reported.16,18,75 SF3B4 (Splicing factor 3b, subunit 4), encodes spliceosome-associated protein 49, which is a component of the pre-mRNA spliceosome complex that removes introns from pre-mRNAs during the production of mature mRNAs.74,76 SF3B4 expression in Xenopus embryos was reported to be ubiquitous with elevated levels in the pharyngeal arches and developing eyes.72 Similar to ribosome biogenesis in
the context of TCS, the spliceosome complex may act tissue-specifically in the context of Nager syndrome. Consistent with this idea, translation blocking morpholino knockdown of SF3B4 in Xenopus embryos resulted in a decrease in NCC progenitors as a consequence of cell death.\(^7\)\(^2\) The specificity of the defects suggests that aberrant splicing of genes involved in NCC, craniofacial and limb development may be an underlying cause of the Nager syndrome phenotypes of the craniofacial and preaxial skeleton.

It has also been proposed that SF3B4 mutations might cause Nager syndrome via mechanisms unrelated to a role in splicing.\(^6\)\(^7\)\(^7\)\(^2\) In support of this idea, a yeast two-hybrid screen using SFSB4 as bait identified downstream targets of BMP signaling which play important roles in craniofacial and limb development.\(^7\)\(^7\) SF3B4 inhibits BMP-2 (bone morphogenetic protein-2)-mediated osteogenic and chondrogenic differentiation, which suggests that in addition to its roles in mRNA splicing, SF3B4 may also inhibit BMP-mediated osteochondral cell differentiation.\(^7\)\(^8\)

Therefore, it will be important to study noncanonical roles of SF3B4, as it is possible that similar to the roles of Tcof1 in DNA damage repair in the context of TCS, SF3B4 acts tissue specifically through yet undetermined mechanisms.

Miller Syndrome

Miller syndrome (OMIM263750), also termed postacrofacial dysostosis (POADS), Genée-Wiedemann, and Wildervanck-Smith syndromes, is classified as an acrofacial dysostosis disorder.\(^7\)\(^9\) Similar to TCS and Nager syndromes, Miller syndrome is characterized by the craniofacial abnormalities such as downward slanting of the palpebral fissures, coloboma of the lower eyelid, hypoplasia of the zygomatic complex (Figure 2), micrognathia, and microtia which can lead to conductive hearing loss.\(^7\)\(^9\) Signifying Miller syndrome as a form of acrofacial dysostosis is the presence of postaxial limb defects, which contrasts with the preaxial defects presented by Nager syndrome.\(^7\)\(^9\)

Miller syndrome was the first Mendelian syndrome whose molecular basis was identified via whole-exome sequencing, and was found to correlate with autosomal recessive or compound heterozygous mutations in dihydroorotate dehydrogenase (DHODH).\(^8\)\(^0\) DHODH encodes a key enzyme in the de novo pyrimidine synthesis pathway and mitochondrial electron transport chain. Until recently, it remained unclear how DHODH gene mutations led to the defects characteristic of Miller syndrome. Furthermore, similar to the facial dysostoses described above, the mechanisms by which global processes, such as pyrimidine synthesis and the mitochondrial electron transport chain cause tissue-specific defects remained a mystery. However, treating zebrafish with lefunomide, a DHODH inhibitor, led to almost complete abrogation of NCC and a reduction of NCC self-renewal in association with inhibition of transcriptional elongation of neural crest genes.\(^8\)\(^1\) Analogous to TCS, this suggests that mutations in DHODH lead to apoptosis of NCC progenitors, which results in a decrease in the number of migratory NCC and consequently defects in the craniofacial skeleton. Additionally, similar to the role of Tcof1,\(^6\)\(^1\) DHODH has also been implicated in oxidative stress.\(^8\)\(^2\) Inhibition or depletion of DHODH leads to an increase in ROS production,\(^8\)\(^3\) which has been shown to trigger cell death.\(^6\)\(^1\) Thus, DHODH depletion may induce NCC progenitor cell death through mitochondrial dysfunction or increased ROS, underpinning the analogous craniofacial anomalies observed in both mandibulofacial and acrofacial dysostosis. Dietary supplementation with antioxidants such as N-acetyl-L-cysteine could potentially ameliorate the phenotypes observed in Miller syndrome similar to that recently shown in mouse models of TCS,\(^6\)\(^1\) but this remains to be tested.

SUMMARY, CONCLUSIONS, AND PERSPECTIVES

Facial dysostoses comprise a group of rare clinically and etiologically heterogeneous craniofacial anomalies that arise due to defects in NCC development and their derivatives. TCS, is caused by mutations in three ribosome biogenesis-associated genes, TCOFI, POLR1C, and POLR1D (Figure 3).\(^1\)\(^0\),\(^3\)\(^7\) Work in both mouse and zebrafish has shown that TCOFI, POLR1C, and POLR1D loss-of-function leads to diminished ribosome biogenesis resulting in p53-dependent apoptosis of NCC progenitors and craniofacial anomalies.\(^8\)\(^8\),\(^6\)\(^5\),\(^6\)\(^6\) One of the key regulators of p53 is MDM2,\(^8\)\(^4\) which inhibits p53, and targets it to proteasome degradation.\(^8\)\(^5\) Studies have shown that ribosomal proteins such as Rpl5, Rpl11, and Rpl23, for example, have a binding affinity for MDM2.\(^8\)\(^6\)\(^\)\(^8\)\(^7\)\(^9\)\(^1\) Thus, it is possible that when TCOFI, POLR1C, or POLR1D are mutated causing deficient rRNA transcription, this leads to binding of ribosomal proteins to MDM2, blocking its ligase activity, which promotes p53 activation and stabilization.\(^8\)\(^6\) Ultimately this results in p53-dependent cell death (Figure 3).
Notably, inhibition of p53-dependent apoptosis can successfully rescue the phenotypes exhibited by Tcof1+/- mice as well as polr1c and polr1d mutant zebrafish. However, p53 functions as a tumor suppressor, and inhibiting p53 could potentially lead to tumorigenic side effects emphasizing the need to explore additional avenues for preventing TCS and other ribosomopathies that affect head and facial development. Interestingly, an unexpected role for Tcof1 in DNA damage and oxidative stress was recently identified. Consistent with a role for Tcof1 in protection against ROS-induced DNA damage, dietary supplementation with N-acetyl cysteine, reduced the levels of cell death and partially rescued the craniofacial phenotypes observed in Tcof1−/− mouse embryos. This suggests that antioxidant supplementation could potentially be a promising therapy to prevent or ameliorate TCS in humans and possibly other ribosomopathies.

An association between deficient ribosome biogenesis, p53-dependent apoptosis, reduced numbers of NCC, and craniofacial anomalies appears to be a common mechanism underlying many ribosomopathies. This is true for the recently characterized ribosomopathy acrofacial dysostosis-cincinnati type, which exhibits a similar mandibulofacial phenotype as observed in TCS, but with or without the addition of limb defects. Acrofacial dysostosis-cincinnati is caused by mutations in POLR1A, which encodes the catalytic subunit of RNA polymerase I. POLR1A loss-of-function results in diminished ribosomal RNA transcription which leads to p53-dependent apoptosis of NCC progenitors and craniofacial anomalies. It is surprising that ribosome biogenesis is globally required in every cell and yet defects in this process result in tissue-specific phenotypes. However, the tissue-specific expression of ribosomal genes, as described above, could potentially account for the tissue-specific roles of ribosome biogenesis during embryogenesis. The elevated expression of ribosome-associated genes in neuroepithelial cells and progenitor NCC may make them more susceptible to perturbations in ribosome biogenesis. The development of NCC as well as bone and cartilage are dynamic processes that require high levels of proliferation and high levels of protein synthesis. Therefore, highly proliferative tissues, such as those affected in the facial dysostoses described in this review, may require higher threshold levels of rRNA transcription, making them more sensitive to any disruption in the ribosome biogenesis pathway. Alternatively, specialized ribosomes may also play a role in the pathogenesis of ribosomopathies. In this scenario, core ribosome components, could act differently in specific tissues due to their interaction with transient proteins, cis-regulatory elements or other cofactors that are present within specific subsets of mRNAs.

Acrofacial dysostoses, such as MFDM and Nager syndrome, can arise through perturbations of global processes other than ribosome biogenesis and yet still exhibit similar defects in cranial NCC and bone and cartilage development. Interestingly, these defects are attributed to disruptions in EFTUD2 in MFDM and in SF3B4 in Nager syndrome, both proteins components of the ubiquitous pre-mRNA spliceosome complex. Despite the putative widespread need for mRNA splicing, facial dysostoses exhibit malformation of the craniofacial skeleton. The aberrant splicing of genes involved in NCC development and possibly bone and cartilage differentiation may be the cause for the tissue-specific defects observed in MDFM and Nager syndrome. Additionally, different rates of transcription and translation in different cells may also play a role. Similar to deficient ribosome biogenesis, it is possible that the highly proliferative nature of NCC, as well as bone and cartilage progenitors makes them more susceptible to defects in mRNA splicing. However, it is also possible that similar to the noncanonical function of TCOFI/Treacle in DNA damage and oxidative stress, these spliceosomal proteins may be involved in other tissue-specific complex systems; for example, SF3B4 may regulate BMP signaling, which is known to play an important role in osteogenic and chondrogenic differentiation.

Miller syndrome, which is characterized by defects in the craniofacial and postaxial limb skeleton, is caused by mutations in DHODH. DHODH encodes a key enzyme in de novo pyrimidine synthesis and the mitochondrial electron transport chain. Unfortunately, the mechanistic roles of these pathways in craniofacial and limb development or Miller syndrome have not been well studied. However, similar to the facial dysostoses described above, DHODH loss-of-function was found to cause apoptosis of NCC progenitors which leads to defects in craniofacial bone and cartilage development. Interestingly, DHODH has been found to play roles in a variety of processes including, mitochondrial membrane potential, cell proliferation, ROS production and apoptosis in specific cell types. One possibility is that DHODH loss-of-function disrupts the transcriptional elongation of genes specifically required for NCC, bone and cartilage development. Alternatively, inhibition of DHODH may lead to increased ROS production in the neuroepithelium, leading to cell death. Therefore, it would be interesting to test if antioxidant supplementation can ameliorate the
phenotypes observed in DHODH-deficient zebrafish and mammalian models similar to that for TCS.

In conclusion, although acrofacial dysostoses can affect both craniofacial and limb formation, all of the syndromes described in this review primarily affect NCC-derived craniofacial bone and cartilage development. The sensitivity of NCC and skeletal development to disruptions in global process is perhaps not surprising. In fact, this seems to be a common phenomenon in ribosomopathies such as Nager syndrome and TCS as well as Diamond-Blackfan Anemia (DBA). DBA patients exhibit craniofacial defects similar to those observed in other ribosomopathies, while also exhibiting specific defects affecting bone marrow function. Differential regulation of gene expression, transcription factors or posttranslational modifications as well as the type and location of the mutation could each contribute to the underlying cause of the tissue-specific phenotypes observed in ribosomopathies and other mandibulofacial and acrofacial dysostoses. Alternatively, these tissues are highly proliferative and perhaps require relatively high levels and rates of ribosome biogenesis, mRNA splicing or other global processes. It is crucial that as we continue to investigate the developmental and disease roles for these genes and processes using animal models that we explore the possibility for interactions between each of these genes with other genes and pathways important for NCC, bone and cartilage development. There is much that remains to be learned about the spatiotemporal functional specificity of individual RNA polymerase and spliceosome complex subunits, as well as individual cell and tissue sensitivity to disruptions in ribosome biogenesis, pre-mRNA splicing and other global processes. Understanding this is especially important for the proper treatment and care of these patients. Careful phenotypic and genotypic analysis is clinically necessary in advancing treatment, personalized care, and most importantly prevention of these congenital diseases. Although many facial dysostoses present with similar and overlapping phenotypes, their etiology, developmental history, and genetics may require different treatment regimes. For example, although mutations in either Tcof1, Polr1c, or Polr1d lead to TCS, successful treatment and/or prevention may depend on the specific underlying genetic mutation. A thorough understanding of the distinct signals, switches and mechanisms which regulate both, normal development and disease is still needed.

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Craniofacial anomalies account for approximately one-third of all congenital birth defects. Here, we discuss the pathogenesis of a specific array of craniofacial anomalies, termed facial dysostoses, which affect development of the maxilla, mandible, and zygomatic arch.
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