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Dynamic regulation and requirement for ribosomal RNA transcription during mammalian development

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Ribosomal RNA (rRNA) transcription by RNA polymerase I (Pol I) is a critical rate-limiting step in ribosome biogenesis, which is essential for cell survival. Despite its global function, disruptions in ribosome biogenesis cause tissue-specific birth defects called ribosomopathies, which frequently affect craniofacial development. Here, we describe a cellular and molecular mechanism underlying the susceptibility of craniofacial development to disruptions in Pol I transcription. We show that Pol I subunits are highly expressed in the neuroepithelium and neural crest cells (NCCs), which generate most of the craniofacial skeleton. High expression of Pol I subunits sustains elevated rRNA transcription in NCC progenitors, which supports their high tissue-specific levels of protein translation, but also makes NCCs particularly sensitive to rRNA synthesis defects. Consistent with this model, NCC-specific deletion of Pol I subunits *Polr1a*, *Polr1c*, and associated factor *Tcof1* in mice cell-autonomously diminishes rRNA synthesis, which leads to p53 protein accumulation, resulting in NCC apoptosis and craniofacial anomalies. Furthermore, compound mutations in Pol I subunits and associated factors specifically exacerbate the craniofacial anomalies characteristic of the ribosomopathies Treacher Collins syndrome and Acrofacial Dysostosis–Cincinnati type. Mechanistically, we demonstrate that diminished rRNA synthesis causes an imbalance between rRNA and ribosomal proteins. This leads to increased binding of ribosomal proteins Rpl5 and Rpl11 to Mdm2 and concomitantly diminished binding between Mdm2 and p53. Altogether, our results demonstrate a dynamic spatiotemporal requirement for rRNA transcription during mammalian cranial NCC development and corresponding tissue-specific threshold sensitivities to disruptions in rRNA transcription in the pathogenesis of congenital craniofacial disorders.

RNA polymerase 1 | neural crest cells | rRNA transcription | ribosome biogenesis | Mdm2-p53

Ribosomal RNA (rRNA) transcription and ribosome biogenesis are critical for cell growth, proliferation, differentiation, and survival. Ribosomes translate cellular proteins and are responsible for the quality and quantity of proteins (1, 2). The ability to modulate translation rates and translation capacity to meet cell-specific needs is regulated in part by the number of ribosomes available to translate messenger RNAs (mRNAs) (2–5). A critical rate-limiting step in ribosome biogenesis is RNA polymerase (Pol) I–mediated rRNA transcription (6, 7), which accounts for about 60% of all cellular transcription (2, 8) and is integral to increased protein translation during cell growth and proliferation and in response to other metabolic needs. In mammals, Pol I consists of 10 core, 1 stalk, and 2 dissociable subunits (9) that transcribe the 47S precursor rRNA, which is then modified, processed, and cleaved into 5.8S, 18S, and 28S rRNAs. These rRNAs, together with 5S rRNAs transcribed by Pol III, associate with ribosomal proteins and form the catalytic core of the ribosome (10).

Considering the requirement for Pol I-mediated rRNA transcription and ribosome biogenesis in all cell types, it is surprising that defects in these processes result in cancers or tissue-specific developmental disorders known as ribosomopathies (11–14). For example, mutations in the Pol I catalytic subunit *POLR1A* result in Acrofacial Dysostosis–Cincinnati type (AFDCIN) (15), whereas mutations in *POLR1C* and *POLR1D*, shared subunits of Pol I and III, or Pol I-associated factor *TCOF1* cause Treacher Collins syndrome (TCS) (16–18). *TCOF1* encodes the nucleolar phosphoprotein TREACLE, which is involved in rRNA transcription and processing, as well as in DNA damage repair (19–21). AFDCIN and TCS present with a range of phenotypes, which primarily include abnormal craniofacial skeletal development, micrognathia, cleft palate, and malar hypoplasia (15, 17, 18). The majority of the craniofacial tissues affected in TCS and AFDCIN are derived from neural crest cells (NCCs). NCCs are a transient progenitor population, which arise from the neuroepithelium and then delaminate, proliferate, and migrate into

Significance

RNA polymerase I (Pol I)–mediated ribosomal RNA (rRNA) transcription is required for protein synthesis in all cells for normal growth and survival, as well as for proper embryonic development. Interestingly, disruptions in Pol I-mediated transcription perturb ribosome biogenesis and lead to tissue-specific birth defects, which commonly affect the head and face. Our results show that during mouse development, Pol I-mediated rRNA transcription and protein translation are tissue specifically elevated in neural crest cells, which give rise to bone, cartilage, and ganglia in the head and face. Using mouse models, we further show that neural crest cells are highly sensitive to disruptions in Pol I and that when rRNA synthesis is genetically down-regulated, it results in craniofacial anomalies.

The authors declare no competing interest.

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the frontonasal prominences and pharyngeal arches, where they differentiate into most of the craniofacial bone and cartilage, among other tissues (22). TCS and AFDCIN are therefore considered both ribosomopathies and neurocristopathies due to deficits in ribosome biogenesis and NCC development. Previous work has elucidated the basic functions of proteins involved in rRNA transcription and ribosome biogenesis in various organisms, including yeast and human cell lines (23–25), and demonstrated that mutations in *Tcof1*, *polr1a*, *polr1c*, and *polr1d* disrupt NCC development (15, 26–29). However, the mechanisms by which global disruptions in Pol I–mediated transcription result in tissue-specific phenotypes remain poorly understood. In particular, it has not yet been determined 1) why cranioskeletal development is highly susceptible to defects in rRNA transcription and 2) if rRNA transcription is tissue-specifically required during mammalian craniofacial development.

We hypothesized that different cells and tissues have distinct threshold requirements for rRNA transcription, ribosome biogenesis, and protein synthesis to meet their cell-specific needs and that this leads to distinct cell- and tissue-specific threshold sensitivities to deficiencies in rRNA transcription. Given the high incidence of cranioskeletal defects in ribosomopathies, we posited that NCCs are one of the cell types that require high levels of rRNA and protein synthesis. We therefore examined the role of Pol I and rRNA transcription in NCCs during craniofacial development. Through lineage tracing, expression, and translation analyses, we discovered that neuroepithelial cells and NCCs exhibit elevated levels of rRNA transcription that correlate with high levels of protein translation compared with surrounding cells during early embryogenesis.

To understand the intrinsic function of Pol I-mediated transcription in NCCs, we generated models of Pol I disruption via null and conditional tissue-specific deletion of a catalytic subunit (*Polr1a*), noncatalytic subunits (*Polr1c* and *Polr1d*), and associated factor *(Tcof1)* of Pol I in mice. We discovered that Pol I-mediated transcription is essential for cell survival and that cranial NCCs are particularly sensitive to decreased rRNA transcription during early craniofacial development. Pol I subunit and associated factor loss-of-function results in rRNA deficiency, which perturbs the stoichiometric balance between rRNA and ribosomal proteins. This imbalance leads to ribosomal stress and increased binding of ribosomal proteins RPL5 (uL18) and RPL11 (uL5) to Murine double minute 2 (Mdm2), a major regulator of p53 activity. Concomitantly, Mdm2 binding to p53 is reduced, which results in p53 accumulation in the nucleus and, consequently, NCC apoptosis and craniofacial anomalies. Thus, global perturbation of rRNA transcription leads to tissue-specific posttranslational accumulation of p53 protein, which contributes to the tissue specificity of developmental ribosomopathy phenotypes. Taken together, our work demonstrates the dynamic tissue-specific regulation and requirement for rRNA transcription during craniofacial development that mechanistically accounts for tissue-specific threshold sensitivities to perturbation of rRNA transcription. Finally, our data show that ubiquitously expressed genes thought to play fundamental housekeeping functions exhibit cell-type-specific functions, providing insights into the roles of rRNA transcription in regulating embryonic development and disease.

Results

Cranial NCCs Have High Levels of rRNA and Protein Synthesis. Mutations in Pol I subunits result in tissue-specific craniofacial anomalies in humans (15, 16, 30). We hypothesized that the underlying cause for these tissue-specific defects is differential transcription of rRNA in NCCs during early embryogenesis. We therefore performed ViewRNA in situ hybridization (31) for the 47S preribosomal RNA (prerRNA) 5′ External Transcribed Spacer (ETS) (Fig. 1*A*) as a measure of nascent rRNA transcription (32, 33) in *Wnt1-Cre;ROSAeYFP* mouse embryos. This transgenic combination lineage labels the dorsal neuroepithelium, including NCC progenitors and their descendants, with yellow fluorescent protein (YFP) (34). At embryonic day (E) 8.5, during NCC formation and early migration, 5′ETS was globally expressed. However, 5′ETS expression was significantly higher in NCCs (YFP+ cells) relative to surrounding non-NCCs (YFP− cells) (Fig. 1 *B* and *C*). At E9.5, during later migration and the onset of differentiation, 5′ETS expression remained higher in NCCs versus non-NCCs (Fig. 1 *D* and *E*), although quantitatively, the difference was less than observed at E8.5. This indicates that NCC have endogenously high levels of rRNA transcription at early stages of development while they are in a more progenitor and highly proliferative state compared with surrounding tissues.

Given its importance as a rate-limiting step in ribosome biogenesis, which leads to translation of all cellular protein, we hypothesized that high rRNA transcription in NCCs would correlate with elevated protein synthesis. To test this idea, we performed *O*-propargyl-puromycin (OPP) labeling as a measure of nascent translation (35) in *Wnt1-Cre;ROSAeYFP* embryos, which revealed that cranial NCCs have significantly higher protein synthesis compared with other surrounding cells at E8.5 (Fig. 1 *F* and *G*) and slightly higher, although not statistically significant, levels of protein synthesis at E9.5 (Fig. 1 *H* and *I*). To determine whether increased ribosomal DNA (rDNA) transcription and translation correlates with tissue-specific proliferative capacity, we performed 5-bromo-2′-deoxyuridine (BrdU) incorporation in E8.5 wild-type embryos and immunostained transverse sections for BrdU and the mitotic marker phospho-Histone H3 (pHH3). We observed that the neuroepithelium, which includes premigratory NCCs, is more proliferative than the surrounding mesoderm and endoderm (*SI Appendix*, Fig. S1 *A* and *B*). Together, these observations reveal a correlation between high proliferation with elevated rRNA transcription and protein synthesis at E8.5 in neuroepithelial cells and NCC progenitors. Consequently, we posited that the neuroepithelium and NCCs would be particularly sensitive to disruptions in Pol I–mediated rRNA transcription during embryonic development. Indeed, culturing E8.5 wild-type mouse embryos for as short as 8 h with a Pol I inhibitor, BMH-21 (36), resulted in apoptosis specifically in neuroepithelial cells and NCC progenitors (Fig. 1 *J*–*L* and *SI Appendix*, Fig. S2). Our data therefore demonstrate that endogenously high rRNA transcription and protein translation in the neuroepithelium and NCC progenitors underpins their cell survival-specific threshold sensitivity to disruptions in Pol I.

***Polr1a*, *Polr1c*, *Polr1d*, and *Tcof1* Are Broadly Expressed with Elevated Levels in the Neuroepithelium and Pharyngeal Arches.** To understand if tissue-specific differences in rRNA transcription correlate with differential expression of Pol I subunits during embryogenesis, we examined the expression of *Polr1a*, *Polr1c*, *Polr1d*, and the associated factor Treacle, which is encoded by *Tcof1*, during early embryogenesis. *Polr1a*$^{+/-}$, *Polr1c*$^{+/-}$, and *Polr1d*$^{+/-}$ mice carrying a gene trap vector with a βGeo cassette in the endogenous locus of each gene were generated (*SI Appendix*, Fig. S3*A*), allowing for evaluation of *Polr1a*, *Polr1c*, and *Polr1d* spatiotemporal gene expression by LacZ

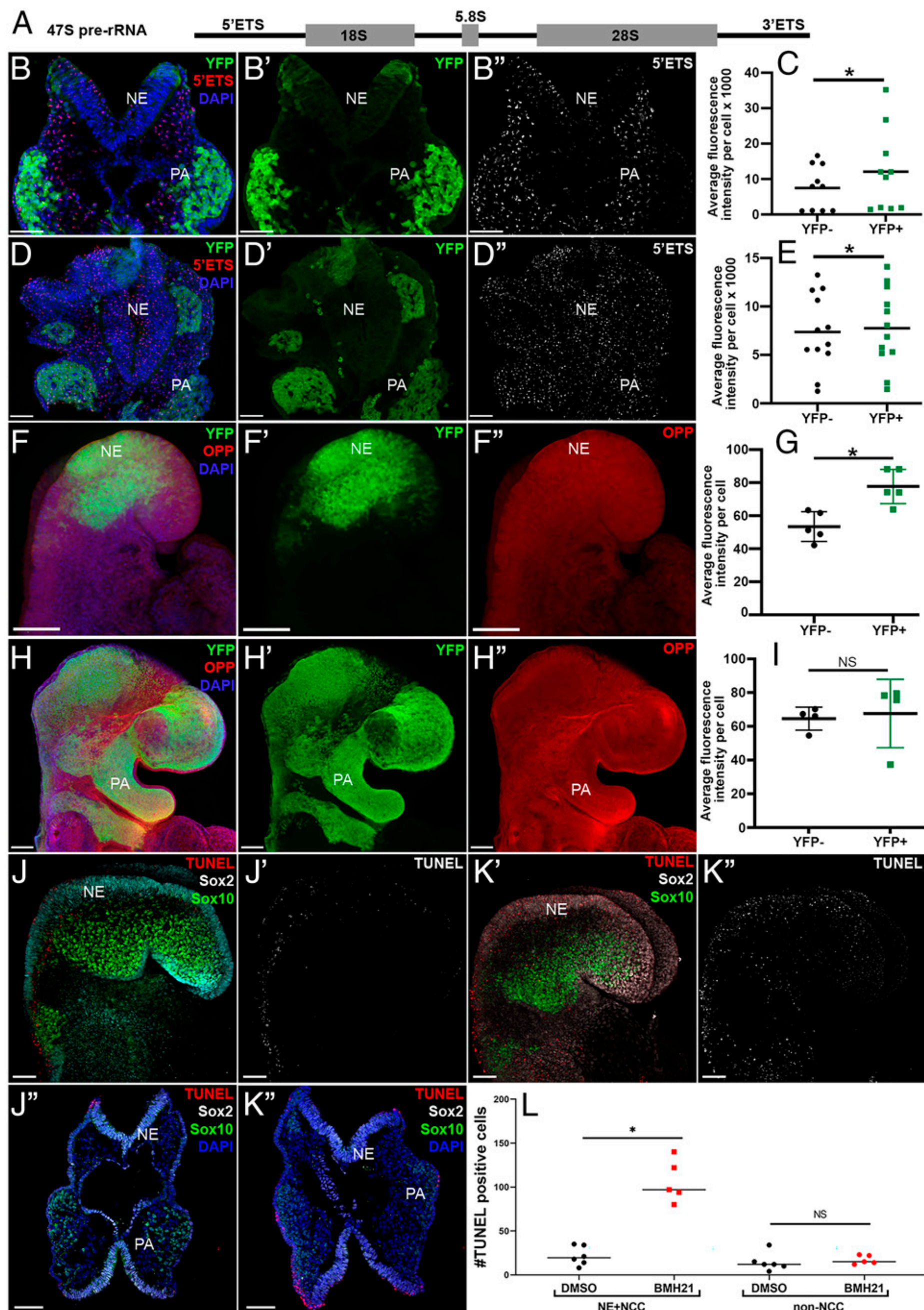


Fig. 1. Elevated levels of rRNA synthesis in NCCs results in high sensitivity to disruptions in Pol I. (*A*) Diagram of 47S prerRNA showing the 5′ETS, 18S, 5.8S, and 28S rRNA components and 3′ETS. (*B–E*) Fluorescence in situ hybridization using ViewRNA for the 5′ETS of the 47S prerRNA in transverse sections of wild-type *Wnt1-Cre*;*ROSAeYFP* embryos. At E8.5, 5′ETS expression (red in *B*, white in *B″*) is significantly higher in NCCs (YFP+; *B′*) compared with non-NCCs (YFP−); quantification is in *C*. At E9.5, 5′ETS expression (*D* and *D″*) remains slightly higher in NCCs (*D′*) compared with non-NCCs (*D″*); quantification is in *E*. (*F–I*) Nascent protein synthesis was analyzed via OPP incorporation in wild-type *Wnt1-Cre*; *ROSAeYFP* embryos. (*F*) NCCs (YFP+) have elevated OPP staining at E8.5 relative to non-NCCs (YFP−); quantification is in *G*. (*H*) OPP staining is comparable between NCCs and non-NCCs by E9.5; quantification is in *I*. (*J* and *K*) Disruption of Pol I transcription in wild-type embryos at E8.5 with Pol I inhibitor BMH-21 (*K*, *K′*, and *K″*) results in increased TUNEL-positive cells (red in *J*, *J″*, *K*, and *K″* and white in *J′* and *K′*) in the neuroepithelium (labeled with Sox2), including the dorsal neuroepithelium, where NCC progenitors (labeled with Sox10) are located compared with DMSO-treated embryos (*J*, *J′*, and *J″*). Transverse sections confirm increased TUNEL staining in BMH-21–treated embryos (*K″*) relative to controls (*J″*). (*L*) Quantification of TUNEL-positive cells in DMSO-treated and BMH-21–treated embryos in NCCs and non-NCCs. *$P < 0.05$ using the paired *t* test. NE, neuroepithelium; NS, not significant; PA, pharyngeal arches. (Scale bars, 100 μm).

staining. *Polr1a*, *Polr1c*, and *Polr1d* were broadly expressed at E8.5 (Fig. 2 *A–C*) and E9.5 (Fig. 2 *I–K*), although *Polr1d* expression was more restricted at this later stage, with high levels of expression in the neuroepithelium and pharyngeal arches, which are the bilateral structures that develop into the jaw and neck (Fig. 2 *E–G* and *M–O*). Similarly, Treacle immunostaining of wild-type embryos revealed broad expression in E8.5 and E9.5 embryos, with elevated levels in the neuroepithelium and pharyngeal arches (Fig. 2 *D*, *H*, *L*, and *P*). Furthermore, single-cell RNA sequencing of E8.5 embryos revealed that all the Pol I subunits, including *Polr1a*, *Polr1c*, and *Polr1d*, as well as *Tcof1*, were expressed broadly in progenitor craniofacial cells and tissues (*SI Appendix*, Fig. S4), but each exhibited enriched expression in the neuroepithelium and NCCs. Altogether, this suggests that elevated expression of Pol I subunits and associated factor *Tcof1* in the neuroepithelium and NCCs contributes to their high levels of rRNA transcription.

To determine the function of individual Pol I subunits during development, heterozygous and homozygous *Polr1a*, *Polr1c*, and *Polr1d* mutant mice were generated (*SI Appendix*, Fig. S3). $Polr1a^{+/-}$, $Polr1c^{+/-}$, and $Polr1d^{+/-}$ embryos were morphologically indistinguishable from their wild-type littermates at E18.5 and survived to adulthood, indicating that a single copy of each gene is sufficient for proper development in mice (*SI Appendix*, Fig. S3*B*). However, $Polr1a^{-/-}$ ($n = 23$), $Polr1c^{-/-}$ ($n = 22$), and $Polr1d^{-/-}$ ($n = 20$) embryos were embryonic lethal by E3.5 (*SI Appendix*, Fig. S3*C*). Their arrest at the morula stage and failure to develop into blastocysts and implant demonstrate that these genes are necessary for survival during preimplantation mammalian development.

Polr1a, Polr1c, and Polr1d Genetically Interact with Tcof1 during Craniofacial Development. Given their largely overlapping expression patterns with elevated levels in the neuroepithelium

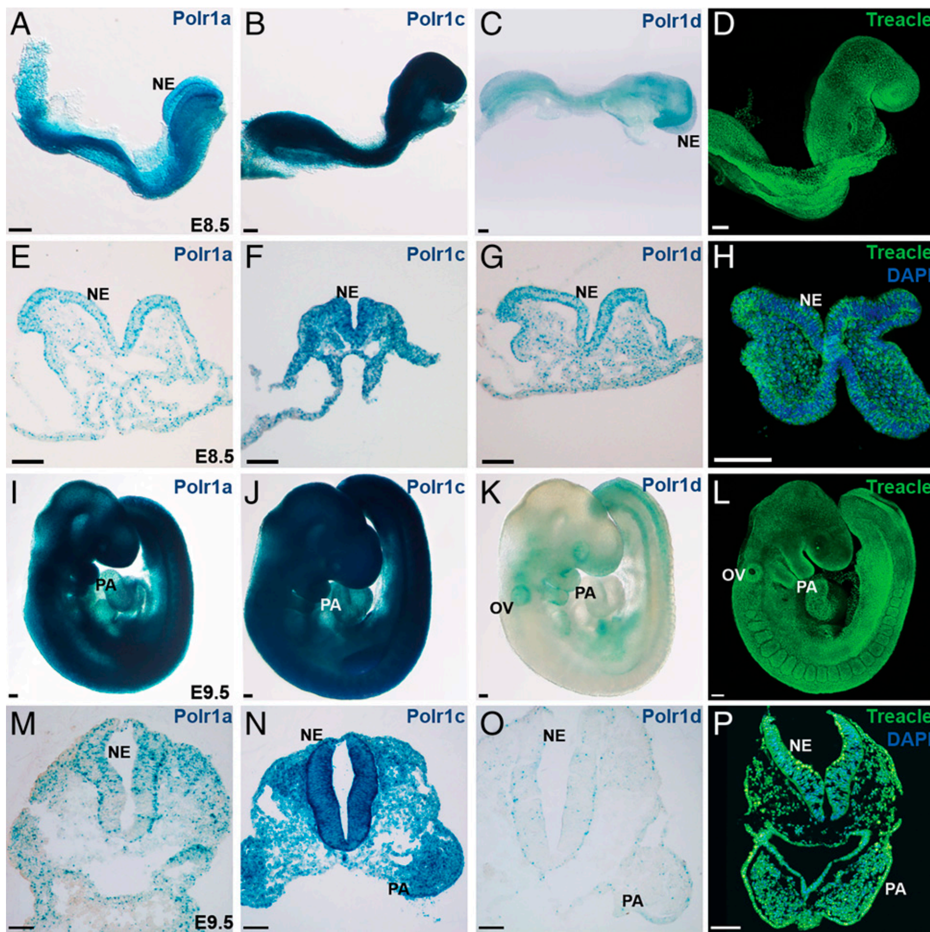


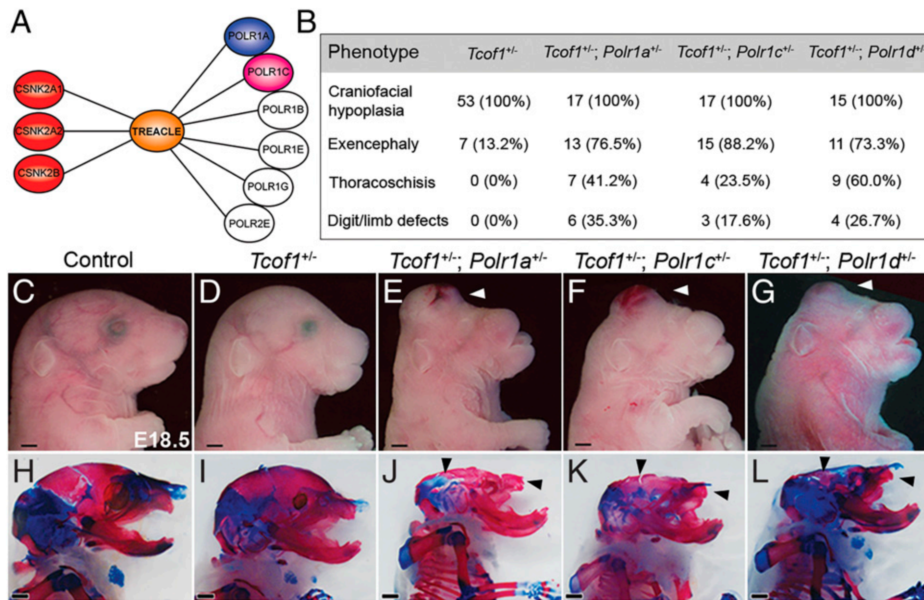
Fig. 2. Pol I subunits and associated factor Treacle are broadly expressed during mouse embryogenesis. (*A*–*C*) Broad expression of Pol I subunits *Polr1a*, *Polr1c*, and *Polr1d*, as observed by LacZ staining in E8.5 embryos. (*E*–*G*) Transverse sections through the cranial region indicate high levels of *Polr1a* (*E*), *Polr1c* (*F*), and *Polr1d* (*G*) expression in the neuroepithelium. (*I*–*K*) At E9.5, *Polr1a* and *Polr1c* remain broadly expressed (*I* and *J*), while *Polr1d* is expressed specifically in the neuroepithelium, pharyngeal arches, otic vesicle, and somites (*K*). Transverse sections through the cranial region at E9.5 indicate higher expression of *Polr1a* (*M*), *Polr1c* (*N*), and *Polr1d* (*O*) in the neuroepithelium and pharyngeal arches compared with surrounding tissues. (*D*, *H*, *L*, and *P*) Immunostaining for Treacle reveals broad expression in whole-embryo and transverse sections of E8.5 and E9.5 embryos, with dynamic elevated levels in the neuroepithelium (*H* and *P*). NE, neuroepithelium; OV, otic vesicle; PA, pharyngeal arches. (Scale bars, 100 μm).

and NCCs, together with their shared Pol I-associated function in rRNA transcription, we hypothesized that *Polr1a*, *Polr1c*, and *Polr1d* genetically interact with *Tcof1* during mouse craniofacial development. In support of this idea, we performed Multi-Dimensional Protein Identification Technology (MudPIT) analysis (37, 38) of HEK293T-derived cell lines stably expressing FLAG-tagged mouse Treacle and found that Treacle pulled down human Pol I subunits, including POLR1A and POLR1C, together with previously known direct targets, such as Casein kinase II subunits (Fig. 3*A* and *SI Appendix*, Fig. S5 and Table S1) (39). Thus, POLR1A, POLR1C, and TREACLE interact at a protein level either directly or possibly through a protein–RNA intermediate consistent with being components and associated factors of Pol I.

To functionally test whether these factors interact at a genetic level, we generated $Tcof1^{+/-}$;$Polr1a^{+/-}$, $Tcof1^{+/-}$;$Polr1c^{+/-}$, and $Tcof1^{+/-}$;$Polr1d^{+/-}$ double heterozygous mutants. As described (26, 40), and compared with controls (Fig. 3 *C* and *H*), E18.5 $Tcof1^{+/-}$ mouse embryos displayed craniofacial malformations, including dome-shaped heads and hypoplasia of the skull, nasal, premaxillary and maxillary bones, together with partially penetrant cleft palate and ear and eye abnormalities (Fig. 3 *B*, *D*, and *I* and *SI Appendix*, Fig. S6 *A* and *B*), which phenocopies TCS in humans. By comparison, each of the E18.5 double heterozygote mutants exhibited craniofacial defects considerably more severe than found in $Tcof1^{+/-}$ embryos (Fig. 3 *B* and *D*–*G*). Double heterozygotes display exacerbated craniofacial malformations, including fully penetrant cleft palate, together with exencephaly and microphthalmia (Fig. 3 *B* and *D*–*L*). Furthermore, Alcian blue and Alizarin red staining revealed the comparatively more severe hypoplasia and malformation of craniofacial cartilage and bone, particularly of the skull, maxilla, and mandible (Fig. 3 *I*–*L* and *SI Appendix*, Fig. S6 *K*–*W*), illustrating the particular sensitivity of craniofacial tissues to perturbations in Pol I function.

Interestingly, the double heterozygous mutant embryos also exhibited variably penetrant developmental anomalies outside of the craniofacial region, which were not observed in $Tcof1^{+/-}$ embryos. These included thoracoschisis or omphalocele (fissure of the thoracic or abdominal wall) (Fig. 3*B* and *SI Appendix*, Fig. S6 *A*–*E*), as well as limb and digit anomalies, such as long-bone hypoplasia and an abnormal number or short and broad digits (*SI Appendix*, Fig. S6 *F*–*J*). While the penetrance of these phenotypes was slightly variable across the double heterozygous mice, we hypothesize that the maternal environment, as well as the background of the *Tcof1* mouse strain, contributes to some of the phenotypic variability (41). Nonetheless, the exacerbated and complete penetrance of cranioskeletal malformations compared with partial penetrance of other tissue anomalies demonstrates the different threshold sensitivities of distinct tissues to global disruptions in Pol I function. These protein and genetic interactions and the additive effects of their loss-of-function reiterate the importance of tissue-specific levels of rRNA transcription and illustrate that *Polr1a*, *Polr1c*, *Polr1d*, and *Tcof1* function together in rRNA transcription in mammalian NCCs during craniofacial development.

NCC-Specific Deletion of *Polr1a*, *Polr1c*, and *Tcof1* Results in Craniofacial Defects. Elevated rRNA transcription in NCC progenitors and NCCs and the high sensitivity of neuroepithelial and craniofacial tissues to defects in rRNA transcription



Phenotype	$Tcof1^{+/-}$	$Tcof1^{+/-}$; $Polr1a^{+/-}$	$Tcof1^{+/-}$; $Polr1c^{+/-}$	$Tcof1^{+/-}$; $Polr1d^{+/-}$
Craniofacial hypoplasia	53 (100%)	17 (100%)	17 (100%)	15 (100%)
Exencephaly	7 (13.2%)	13 (76.5%)	15 (88.2%)	11 (73.3%)
Thoracoschisis	0 (0%)	7 (41.2%)	4 (23.5%)	9 (60.0%)
Digit/limb defects	0 (0%)	6 (35.3%)	3 (17.6%)	4 (26.7%)

Fig. 3. *Tcof1* and Pol I subunits genetically interact, affecting craniofacial development. (*A*) MudPIT analysis for Treacle-binding proteins recognizes known binding proteins, such as Casein kinase II proteins, as well as Pol I protein subunits, including POLR1A and POLR1C. (*B*) $Tcof1^{+/-}$, $Tcof1^{+/-}$;$Polr1a^{+/-}$, $Tcof1^{+/-}$;$Polr1c^{+/-}$, and $Tcof1^{+/-}$;$Polr1d^{+/-}$ double mutants present with developmental defects with variable penetrance. Table indicates the number of embryos observed with a phenotype. The percentages of the total number of mutants observed are indicated in parentheses. (*C–G*) Brightfield images of $Tcof1^{+/-}$;$Polr1a^{+/-}$, $Tcof1^{+/-}$;$Polr1c^{+/-}$, and $Tcof1^{+/-}$;$Polr1d^{+/-}$ embryos indicate that these double heterozygous mutants exhibit more severe craniofacial defects compared with $Tcof1^{+/-}$ mutants alone, including exencephaly (white arrowheads). (*H–L*) Alizarin red and Alcian blue staining for bone and cartilage, respectively, reveals hypoplastic cartilage and/or bone and craniofacial anomalies, including smaller maxilla and flattened skulls (black arrowheads) in double-mutant embryos (*J–L*) compared with $Tcof1^{+/-}$ mutants (*I*) alone. (Scale bars, 500 μm).

suggest a cell-autonomous role for *Polr1a*, *Polr1c*, and *Tcof1* in Pol I transcription in NCCs during early development. We therefore conditionally deleted these factors in NCCs during their formation using *Wnt1-Cre* transgenic mice. *Wnt1-Cre* recombinase is expressed in the dorsal neuroepithelium, which includes NCC progenitors beginning at E8.5 (34, 42). We crossed *Wnt1-Cre* mice with $Polr1a^{flx/flx}$, $Polr1c^{flx/flx}$, or $Tcof1^{flx/flx}$ mice to generate NCC-specific knockouts (NKOs) of *Polr1a*, *Polr1c*, and *Tcof1* (*SI Appendix*, Fig. S7*A*). The levels of *Polr1a*, *Polr1c*, and *Tcof1* transcripts in NCCs were reduced in E9.5 $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ mutant embryos, respectively, relative to littermate $Polr1a^{NKO/+}$, $Polr1c^{NKO/+}$, and $Tcof1^{NKO/+}$ controls, confirming Cre-mediated excision of exons flanked by loxP sites (*SI Appendix*, Fig. S7*B*).

E9.5 $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ mutants presented with visibly hypoplastic frontonasal prominences and pharyngeal arches when compared with littermate controls, a phenotype that worsened considerably by E10.5 to E11.5 (Fig. 4 *A–D* and *SI Appendix*, Fig. S8 *A–H*). To determine whether this tissue hypoplasia was a consequence of perturbed NCC development, we crossed *ROSAeYFP* into the background of $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ mice to indelibly label the NCC lineage with YFP (43). This revealed that NCCs migrate into the facial prominences and pharyngeal arches in *NKO* mutants by E9.5 (*SI Appendix*, Fig. S8 *I–L*). However, the smaller facial outgrowths in these mutants appeared to correlate with reduced populations of NCCs, a phenotype that was even more pronounced at E10.5 in the *NKO* mutants compared with littermate controls (Fig. 4 *E–H* and *Q–T*). $Polr1a^{NKO/NKO}$ embryos had the most severe reduction in the NCC population, consistent with its essential role as part of the catalytic core of Pol I. The NCC population was also severely hypoplastic in $Polr1c^{NKO/NKO}$ and $Tcof1^{NKO/NKO}$ embryos, although to a slightly lesser degree than $Polr1a^{NKO/NKO}$ embryos. Reflecting this difference in severity, $Polr1a^{NKO/NKO}$ embryos died around E11.5, whereas $Polr1c^{NKO/NKO}$ and $Tcof1^{NKO/NKO}$ embryos survived until E12.5 and E13.5, respectively (*SI Appendix*, Fig. S7 *C* and *D*), conveying the relative importance of *Polr1a*, *Polr1c*, and *Tcof1* in NCCs for embryo survival.

NCCs differentiate into a wide variety of cell and tissue derivatives, including neurons in the peripheral nervous system and osteochondroprogenitors of craniofacial cartilage and bone. To examine NCC differentiation into neurons, we stained for neuron-specific class III β-tubulin (TuJ1) at E10.5. This revealed that $Polr1a^{NKO/NKO}$ and $Polr1c^{NKO/NKO}$ mutants exhibit hypoplastic cranial ganglia, especially the trigeminal (V), together with diminished nerve projections compared with littermate controls (Fig. 4 *I–K*). The trigeminal in $Tcof1^{NKO/NKO}$ mutants displayed altered morphology and smaller nerve projections, consistent with a reduced population of NCCs (Fig. 4*L*). The early lethality of *NKO* mutant embryos prevented analysis of NCC differentiation into mature cartilage and bone. Therefore, we investigated the specification of NCCs into osteochondroprogenitors. The expression of Sox9, a master regulator of chondrogenesis, and its downstream target, *Col2a1* (44), were both diminished in the facial prominences in E9.5 and E10.5 *NKO* mutants compared with controls (Fig. 4 *M–P* and *SI Appendix*, Fig. S8 *M–P′* and *Q–T*). The reduced domains of chondrogenic gene expression, especially the first and second pharyngeal arches, and hypoplastic cranial ganglia likely reflect the reduced number of NCCs within the arches in *NKO* mutants (Fig. 4 *E–H* and *SI Appendix*, Fig. S8 *I–L*). Furthermore, smaller craniofacial prominences and pharyngeal arches (Fig. 4 *A–D* and *Q–T*) suggest that *Polr1a*, *Polr1c*, and *Tcof1* play critical roles in NCC proliferation and/or survival.

Polr1a, _Polr1c_, and _Tcof1_ Loss-of-Function in NCCs Leads to Increased NCC Death. We hypothesized that decreased proliferation and/or increased apoptosis accounts for the reduced NCC population in *NKO* mutants. Transverse sections of E9.5 $Polr1a^{NKO/NKO}$;*ROSAeYFP*, $Polr1c^{NKO/NKO}$;*ROSAeYFP*, and $Tcof1^{NKO/NKO}$;*ROSAeYFP* embryos were stained for the mitotic proliferation marker pHH3. Quantification revealed that while mutant embryos displayed slightly fewer pHH3+ NCCs compared with littermate controls, the differences were not statistically significant at this stage (Fig. 5 *A–D* and *SI Appendix*, Fig. S9*A*). In contrast, $Polr1a^{NKO/NKO}$;*ROSAeYFP*, $Polr1c^{NKO/NKO}$;*ROSAeYFP*, and $Tcof1^{NKO/NKO}$;*ROSAeYFP* mutant embryos exhibit increased terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of NCCs (Fig. 5 *E–H* and *SI Appendix*, Fig. S9*B*), especially within the pharyngeal arches (Fig. 5 *E–H*).

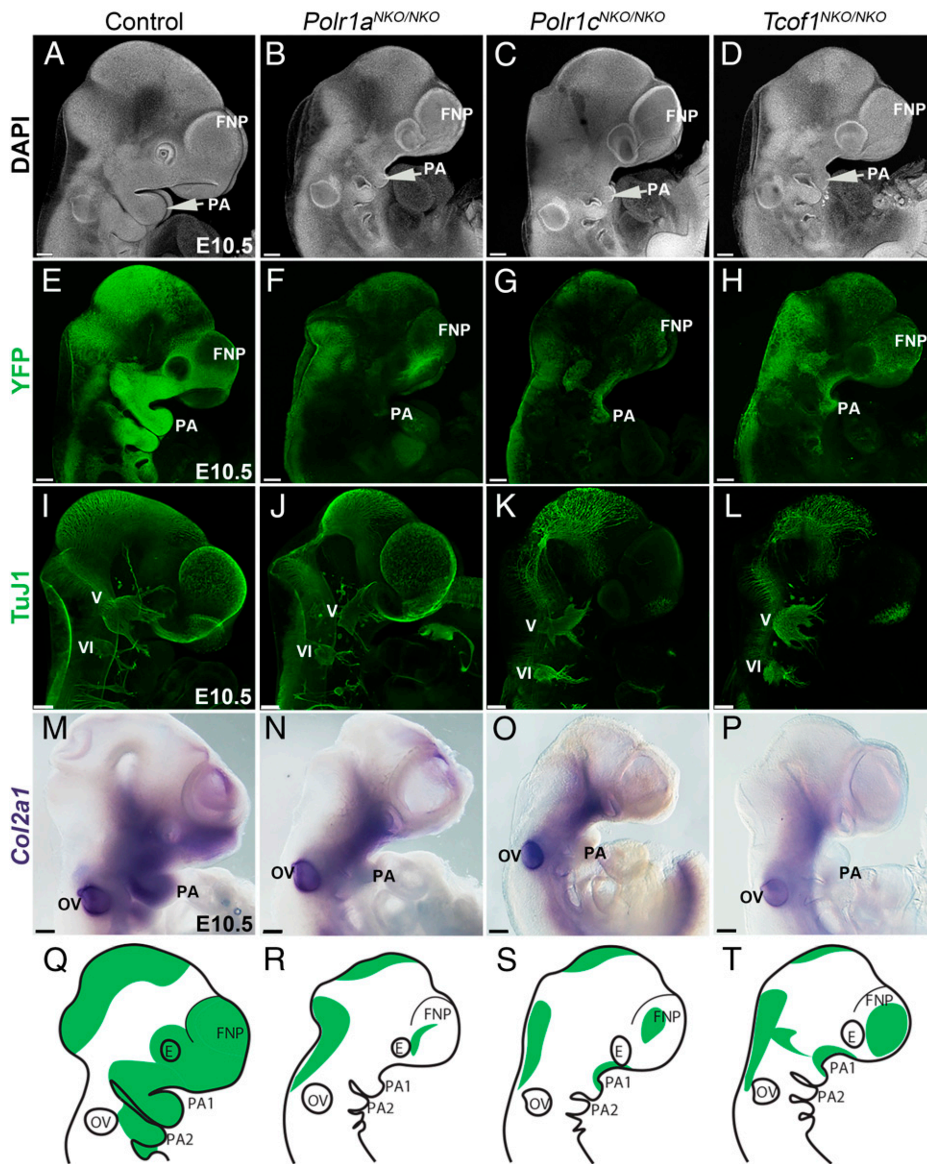


Fig. 4. *Polr1a*, *Polr1c*, and *Tcof1* are required for NCCs and craniofacial development in mice. (*A–D*) DAPI staining at E10.5 shows hypoplastic pharyngeal arches (white arrow) and frontonasal prominences in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos compared with control embryos. (*E–H*) $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ were bred into the background of *ROSAeYFP* mice to label the NCC lineage with YFP. YFP staining indicates fewer NCCs in the pharyngeal arches and frontonasal prominences in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos. (*I–L*) Neuron-specific class III β-tubulin (TuJ1) staining indicates that NCC differentiation to neurons and glia is disrupted in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos. The trigeminal (V) nerve ganglia are hypoplastic in all mutants. (*M–P*) In situ hybridization for chondrogenesis marker *Col2a1* shows reduced expression, especially within the pharyngeal arches in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos. (*Q–T*) Schematic figures depicting hypoplastic pharyngeal arches and frontonasal prominences as well as decreased NCCs (green) in mutants versus controls. FNP, frontonasal prominence; OV, otic vesicle; PA, pharyngeal arches. (Scale bars, 200 μm).

p53 is a well-known mediator of apoptosis (45), and its mRNA level (46) or protein activity (27, 47) have each been proposed to underlie tissue-specific defects in the neuroepithelium. We therefore quantified *p53* expression by qPCR and found no significant changes in *p53* transcription between NCCs and non-NCCs in wild-type embryos (*SI Appendix*, Fig. S10*A*), but *p53* was slightly reduced in the NCCs of $Polr1a^{NKO/NKO}$ mutants compared with $Polr1a^{NKO/+}$ controls (*SI Appendix*, Fig. S10*A*). This demonstrates that differences in *p53* mRNA levels do not underlie differences in cell death, consistent with our previous studies (26, 27). Interestingly, p53 protein is uniformly expressed across different tissues at very low levels in wild-type E8.5 embryos (27), and although *p53* was not affected at the transcript level, p53 protein was tissue-specifically increased in the neuroepithelium and pharyngeal arches in *NKO* mutants compared with their respective littermate controls (Fig. 5 *I–L* and *SI Appendix*, Fig. S9*C*). While p53 protein levels were not significantly increased in $Polr1a^{NKO/NKO}$ mice at this stage, examination of cell-cycle inhibitor and p53 target gene *p21* by qPCR demonstrated a significant increase in the NCCs of $Polr1a^{NKO/NKO}$ mutants (*SI Appendix*, Fig. S10*A*). This suggests that there may be an effect on proliferation downstream of p53 activation and that the difference in pHH3 observed (Fig. 5 *A* and *B*), while not statistically significant, may be biologically significant to the mutant phenotype. To confirm that the up-regulation of p53 is biologically relevant in the $Polr1a^{NKO/NKO}$ mutant mice, we treated these embryos and littermate controls with a p53 inhibitor, pifithrin-α (48). $Polr1a^{NKO/NKO}$ mice treated with pifithrin-α showed a considerable increase in the volume of the pharyngeal arches in concert with increased YFP+ cells in the arches and frontonasal prominences (n = 3/4) compared with $Polr1a^{NKO/NKO}$ mutants treated with dimethyl sulfoxide (DMSO) (*SI Appendix*, Fig. S10*B*). This indicates that increased p53-dependent cell death reduces the NCC population in $Polr1a^{NKO/NKO}$ mutants. However, these pifithrin-α treated $Polr1a^{NKO/NKO}$ embryos do not survive beyond E12.5, probably because inhibiting p53 does not rescue rRNA synthesis and ribosomal stress. Altogether, our results signify that the NCC population in *NKO* mutants is diminished primarily due to a cell-autonomous increase in p53 protein-dependent cell death (Fig. 5*M*).

Excision of *Polr1a*, *Polr1c*, and *Tcof1* Results in Decreased rRNA and Protein Synthesis. Multiple stressors can activate p53 and lead to increased apoptosis or cell-cycle arrest, and the degree of

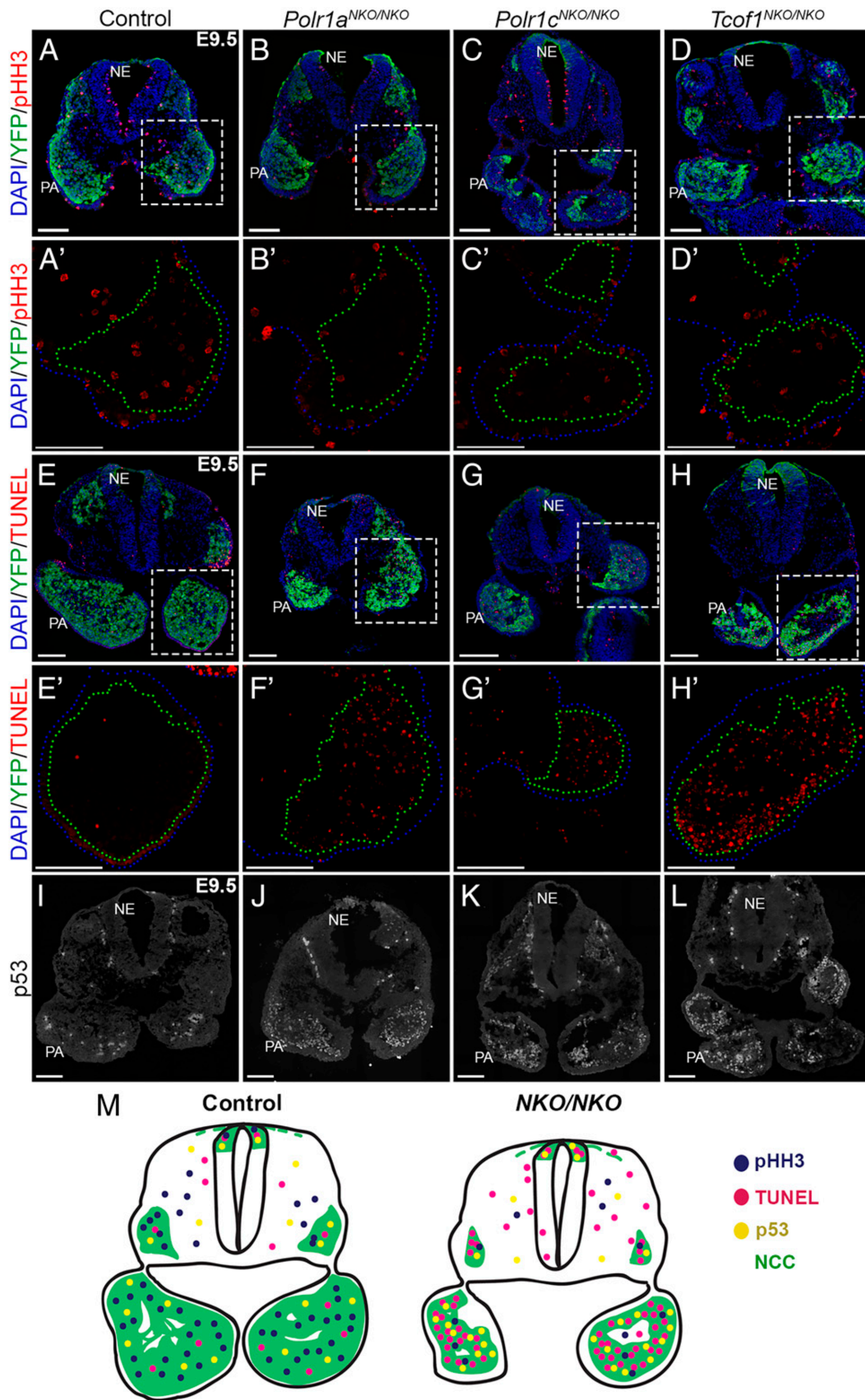


Fig. 5. Reduced proliferation and increased p53-dependent cell death underlies the reduced NCC population in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ mice. (*A*–*D*) Proliferation (pHH3; red) is reduced in NCC (YFP+) of $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos. (*A′*–*D′*) Higher-magnification view of boxed region in *A*–*D*. Pharyngeal arches are outlined in green, indicative of the YFP-expressing cell boundary, and blue, indicative of DAPI-labeled cell boundary. (*E*–*H*) TUNEL staining shows increased cell death in YFP+ NCC in the pharyngeal arches of $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos at E10.5. (*E′*–*H′*) Higher magnification of the pharyngeal arches, with the YFP-expressing region outlined in green and DAPI with blue. (*I*–*L*) Increased p53 staining (white) in the pharyngeal arches in $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$ embryos suggests p53-dependent cell death. (*M*) Summary schematic of control and NCC-specific mutant (representative of $Polr1a^{NKO/NKO}$, $Polr1c^{NKO/NKO}$, and $Tcof1^{NKO/NKO}$) sections depicting decreased levels of pHH3 (blue), increased cell death (pink), and p53 (yellow) levels within NCC (green). NE, neuroepithelium; PA, pharyngeal arches. (Scale bars, 100 μm).

p53 activation may contribute to the tissue specificity of developmental syndromes (47). Given the essential role of Pol I subunits and associated factor Treacle in rRNA transcription, we hypothesized that p53 is activated in the *NKO* mutants through a ribosomal stress or nucleolar surveillance response (15, 27, 28). When rRNA transcription is disrupted, this could lead to an imbalance in ribosomal protein to rRNA production, triggering p53 activation. To determine if disruptions in rRNA transcription underlie the increased p53-dependent cell death observed in *NKO* mutants, we analyzed rRNA transcription in fluorescence-activated cell (FAC)-sorted NCCs by qPCR. At E9.5, ∼24 h after Cre excision, 5′ETS expression was significantly down-regulated in $Polr1a^{NKO/NKO}$;*ROSAeYFP* and $Tcof1^{NKO/NKO}$;*ROSAeYFP* NCC when compared with respective control NCCs (Fig. 6*A*). While 5′ETS was not significantly changed in $Polr1c^{NKO/NKO}$;*ROSAeYFP* NCC compared with controls, 28S rRNA, which reflects the level of the precursor 47S transcript and the mature 28S rRNA, was significantly reduced (Fig. 6*A*). Overall, our data demonstrate that rRNA transcription begins to decrease in *Polr1a*, *Polr1c*, and *Tcof1 NKO* mutants as early as E9.5.

Previous studies have shown that reductions in rRNA transcription result in reduced ribosome biogenesis and protein

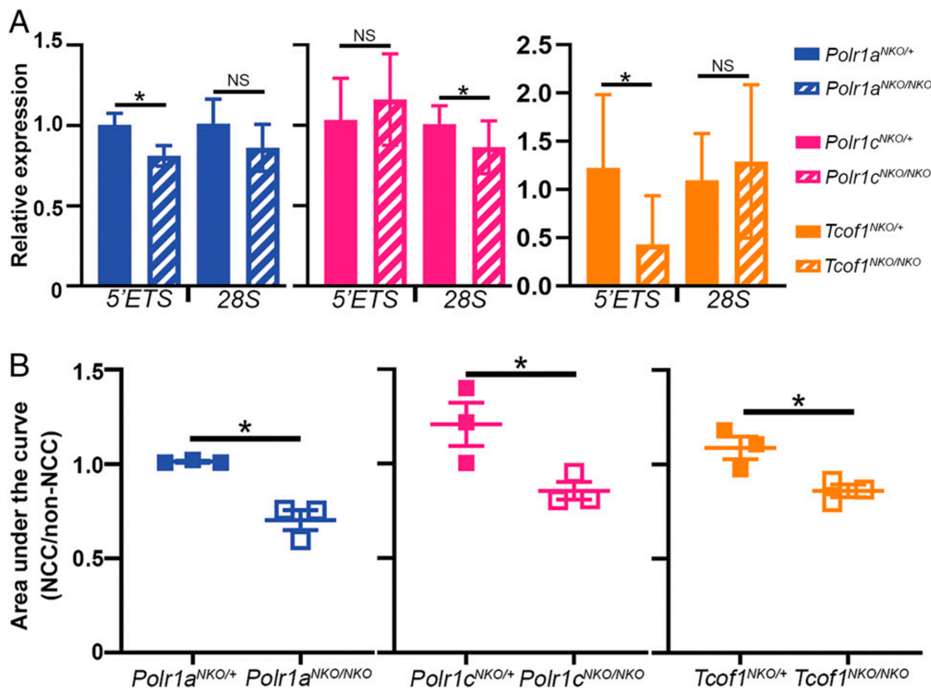


Fig. 6. rRNA transcription and total protein are reduced in NCCs in *Polr1a*$^{NKO/NKO}$, *Polr1c*$^{NKO/NKO}$, and *Tcof1*$^{NKO/NKO}$ mice. (*A*) qPCR for the 5′ETS region of rRNA is significantly reduced in the sorted NCCs of *Polr1a*$^{NKO/NKO}$ and *Tcof1*$^{NKO/NKO}$ embryos, while the mature 28S rRNA transcript is not significantly changed. In *Polr1c*$^{NKO/NKO}$ embryos, 28S rRNA is reduced, while 5′ETS is not significantly affected. (*B*) Quantification of silver staining demonstrates that the total protein in NCCs is significantly reduced compared with non-NCCs in *Polr1a*$^{NKO/NKO}$, *Polr1c*$^{NKO/NKO}$, and *Tcof1*$^{NKO/NKO}$ embryos at E10.5. A total of 2,000 cells of the YFP+ and YFP− populations of each genotype were used to perform silver staining. NS, not significant. *$P < 0.05$.

synthesis (29), demonstrating that rRNA transcription is a rate-limiting step in ribosome biogenesis. We therefore hypothesized that protein synthesis would be reduced in *Polr1a*, *Polr1c*, and *Tcof1 NKO* NCC as a consequence of reduced rRNA transcription. Protein was extracted from equal numbers of FAC-sorted NCC (YFP+) and non-NCC (YFP−) cells from E10.5 *Polr1a*$^{NKO/NKO}$;*ROSAeYFP*, *Polr1c*$^{NKO/NKO}$;*ROSAeYFP*, and *Tcof1*$^{NKO/NKO}$;*ROSAeYFP* mutant embryos and their respective controls. Silver staining (49) revealed a significant decrease in total protein in *Polr1a*, *Polr1c*, and *Tcof1* NKO NCCs relative to control NCCs (Fig. 6*B* and *SI Appendix*, Fig. S11). This demonstrates that *Polr1a*, *Polr1c*, and *Tcof1* loss-of-function in NCC leads to a cell-autonomous reduction in rRNA transcription and total protein, which results in increased p53-dependent NCC apoptosis and, consequently, craniofacial anomalies.

***Polr1a*, *Polr1c*, and *Tcof1* Deletion Results in Rpl5 and Rpl11 Binding to Mdm2 and p53 Stabilization.** To investigate the molecular mechanism by which Pol I disruption activates p53-dependent apoptosis, we generated mouse embryonic fibroblasts (MEFs) from *Polr1a*$^{flx/flx}$, *Polr1c*$^{flx/flx}$, and *Tcof1*$^{flx/flx}$ mice crossed to tamoxifen-inducible *Cre-ER*^{T2} mice, hereafter referred to as tamoxifen-inducible knockouts (*tKO*s). We observed recombination in nearly 70% of the *Polr1a*$^{tKO/tKO}$, *Polr1c*$^{tKO/tKO}$, and *Tcof1*$^{tKO/tKO}$ cells 24 h after tamoxifen treatment (*SI Appendix*, Fig. S12 *A–C*). As expected, *Polr1a*, *Polr1c*, and *Tcof1* transcripts were decreased in *Polr1a*$^{tKO/tKO}$, *Polr1c*$^{tKO/tKO}$, and *Tcof1*$^{tKO/tKO}$ MEFs compared with *Polr1a*$^{flx/flx}$, *Polr1c*$^{flx/flx}$, and *Tcof1*$^{flx/flx}$ control MEFs 48 h after tamoxifen-induced Cre activation (*SI Appendix*, Fig. S12 *D–F*). Consequently, rRNA transcripts, labeled with the Y10b antibody, were significantly down-regulated in the nucleolus and cytoplasm of *tKO* mutant MEFs (*SI Appendix*, Fig. S12*G*), as was nascent protein synthesis (*SI Appendix*, Fig. S12*H*). At the same time, the levels of p53 were, conversely, increased in *Polr1a*$^{tKO/tKO}$, *Polr1c*$^{tKO/tKO}$, and *Tcof1*$^{tKO/tKO}$ MEFs (*SI Appendix*, Fig. S13), demonstrating the mechanistic equivalency between *tKO* MEFs and *NKO* embryos.

During normal cell growth and proliferation, p53 typically exhibits a short half-life, due in large part to MDM2, which binds to and ubiquitinates p53, targeting it for degradation (50). Mdm2 prevents the accumulation of excess p53, even under conditions of cell stress. However, it has been proposed from in vitro studies that when there is an imbalance in the normal stoichiometric ratio of rRNA and ribosomal proteins, free or excess ribosomal proteins, particularly Rpl5 (uL18) and Rpl11 (uL5), bind to Mdm2, inhibiting its function (24, 51–54). rRNA transcription is decreased in *NKO* embryos and *tKO* MEFs; however, Western blots showed that the levels of Mdm2, and ribosomal proteins Rpl5 and Rpl11, remain unchanged in *tKO* MEFs compared with controls (*SI Appendix*, Fig. S13). Interestingly, immunoprecipitation followed by immunoblotting revealed increased binding of Rpl5 and Rpl11 to Mdm2, in concert with decreased binding between Mdm2 and p53 in *tKO* MEFs compared with controls (Fig. 7). These results suggest that disruptions in Pol I–mediated rRNA transcription alter the stoichiometric balance between rRNA and ribosomal proteins, resulting in increased Rpl5 and Rpl11 binding to Mdm2. This diminishes Mdm2 binding and ubiquitinating p53, leading to tissue-specific p53 accumulation, which can account for the tissue-specific neuroepithelial cell and NCC apoptosis, reduction in NCCs, and craniofacial anomalies characteristic of many ribosomopathies (*SI Appendix*, Fig. S14).

Discussion

rRNA transcription is essential for normal embryo development, and our mouse knockouts of *Polr1a*, *Polr1c*, *Polr1d*, and *Tcof1* demonstrate that Pol I function is critical for preimplantation whole-embryo survival, as well as tissue-specific NCC survival. However, why craniofacial development is highly sensitive to perturbations in global rRNA transcription and Pol I function in humans and animal models (15, 17, 26, 28) remains a critical gap in our knowledge.

Our data demonstrate that rRNA synthesis is tissue-specifically regulated in vivo during mouse embryogenesis and

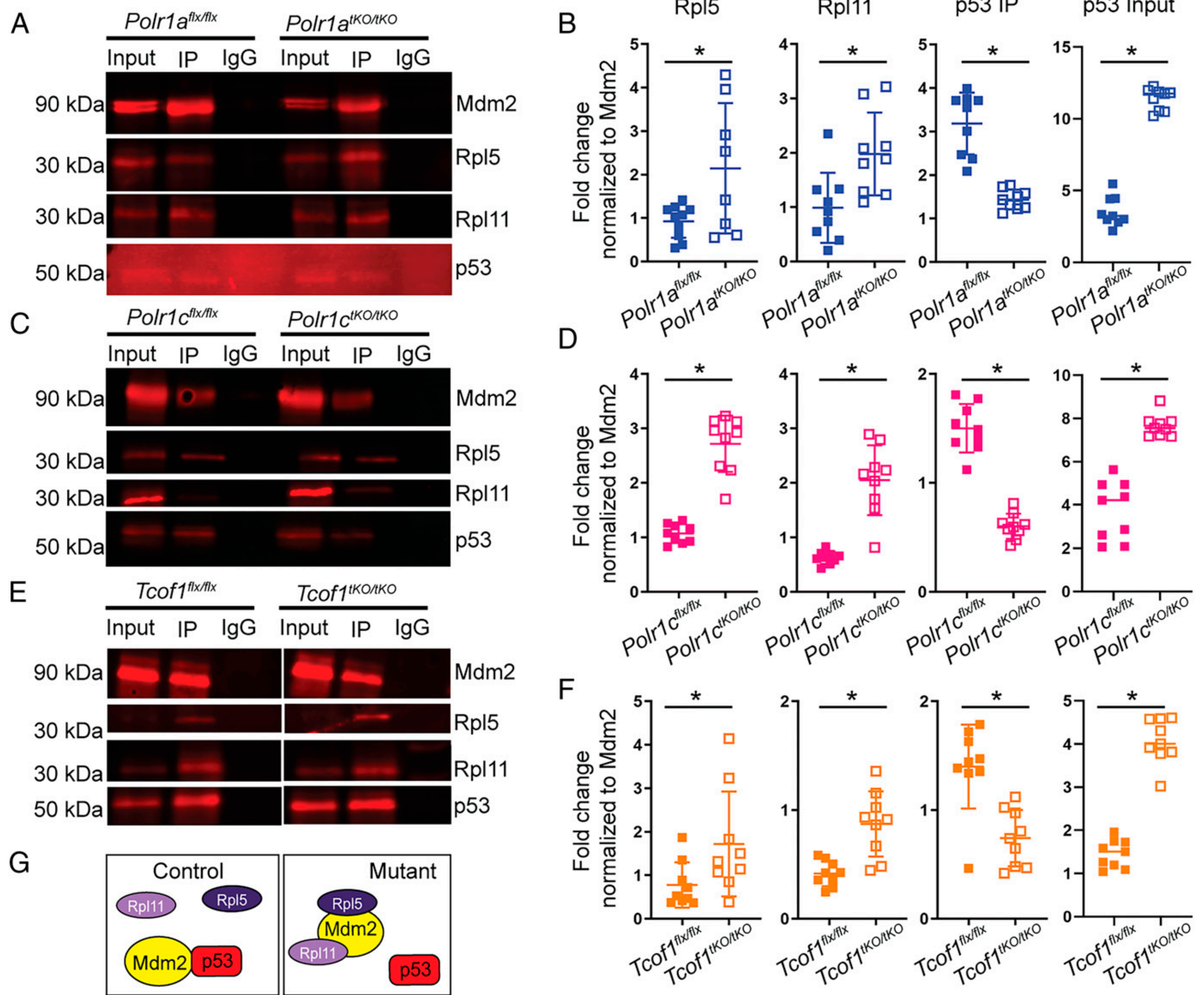


Fig. 7. p53 is activated as a result of higher ribosomal protein binding to Mdm2 in mutant MEFs. MEFs derived from *Polr1a* $^{flx/flx}$, *Polr1a*$^{tKO/tKO}$ (*A* and *B*), *Polr1c* $^{flx/flx}$, *Polr1c*$^{tKO/tKO}$. (*C* and *D*), *Tcof1*$^{flx/flx}$, and *Tcof1*$^{tKO/tKO}$ (*E* and *F*) embryos were treated with tamoxifen and used for immunoprecipitation (IP) assays. Pull-down with Mdm2 and immunoblotting for Rpl5 and Rpl11 revealed increased binding of Mdm2–Rpl5 and Mdm2–Rpl11 in *Polr1a*$^{tKO/tKO}$, *Polr1c*$^{tKO/tKO}$, and *Tcof1*$^{tKO/tKO}$ MEFs compared with their respective control MEFs. Conversely, p53 binding to Mdm2 is reduced in *Polr1a*$^{tKO/tKO}$, *Polr1c*$^{tKO/tKO}$, and *Tcof1*$^{tKO/tKO}$ MEFs compared with controls, consistent with the increased levels of p53 observed in the *Polr1a*$^{NKO/NKO}$, *Polr1c*$^{NKO/NKO}$, and *Tcof1*$^{NKO/NKO}$ embryos. Band intensities were measured as the ratio between Mdm2 and Rpl5, Rpl11, or p53. (*G*) Summary schematic showing Mdm2–p53 binding in control and Rpl5, Rpl11–Mdm2 binding in mutant resulting in free p53. *$P < 0.05$, Student's *t* test.

that this correlates with tissue-specific threshold sensitivities to disruptions in rRNA transcription. Quantification of 47S pre-rRNA transcription showed that the neuroepithelium and NCCs exhibit endogenously high levels of rRNA transcription compared with surrounding non-NCCs (Fig. 1 and *SI Appendix*, Fig. S14*A* versus *SI Appendix*, Fig. S14*C*), which is mechanistically underpinned by dynamically enriched expression of *Tcof1*, *Polr1a*, *Polr1c*, *Polr1d*, and other Pol I subunit transcripts and protein in the neuroepithelium and NCCs in mice (Fig. 2 and *SI Appendix*, Fig. S4). This correlates with elevated translation in the neuroepithelium and NCC progenitors, which is necessary to meet high proliferation needs and possibly other demands, such as the requirement to translate new proteins for cytoskeletal rearrangement during epithelial-to-mesenchymal transitions (EMTs) (55) (Fig. 1 and *SI Appendix*, Figs. S1 and S14*A*).

Global disruption of Pol I transcription using BMH-21 results in apoptosis specifically in neuroepithelial cells and NCC progenitors in E8.5 mouse embryos (Fig. 1 *J*–*L*). Further, craniofacial anomalies are more severe and 100% penetrant in *Tcof1*$^{+/-}$; *Polr1a*$^{+/-}$, *Tcof1*$^{+/-}$;*Polr1c*$^{+/-}$, and *Tcof1*$^{+/-}$;*Polr1d*$^{+/-}$ double heterozygous mutants compared with craniofacial anomalies observed in *Tcof1*$^{+/-}$ mutant embryos (Fig. 3). Therefore, taken together with our previous observations in zebrafish (15, 28), this indicates that high rRNA transcription in NCC progenitors leads to their high sensitivity to disruptions in rRNA synthesis, while non-NCC-derived tissues are affected to a lesser degree (Fig. 3 and *SI Appendix*, Fig. S14*B* versus *SI Appendix*, Fig. S14*D*).

Furthermore, Pol I-mediated transcription functions in a cell-autonomous manner during mouse NCC development. NCC-specific deletion of *Polr1a*, *Polr1c*, and *Tcof1* genes results in NCC-autonomous down-regulation of rRNA transcription (Fig. 6), leading to increased p53-dependent cell death (Fig. 5) and, consequently, craniofacial anomalies (Fig. 4). While mechanistically similar, there are subtle differences between *Polr1a*$^{NKO/NKO}$, *Polr1c*$^{NKO/NKO}$, and *Tcof1*$^{NKO/NKO}$ embryos.

For example, the NCC population is more severely reduced in *Polr1a*$^{NKO/NKO}$ embryos compared with *Polr1c*$^{NKO/NKO}$ (Fig. 4), corresponding with previous work in zebrafish (15, 28). *Tcof1*$^{NKO/NKO}$ embryos exhibit the least severe phenotype in comparison with *Polr1a*$^{NKO/NKO}$ and *Polr1c*$^{NKO/NKO}$. This is consistent with Polr1a forming part of the catalytic site of Pol I, whereas Polr1c functions to hold Polr1a and Polr1b together, but does not form part of the catalytic site, while Tcof1/Treacle is an associated factor that interacts with Pol I (56). Interestingly, while Polr1a is a component of Pol I only, Polr1c is a subunit of both Pol I and Pol III and, therefore, may impact Pol III transcription in addition to Pol I, resulting in differences in how rRNA transcription is affected in NCCs (Fig. 6*A*). Modeling of pathogenic variants in *POLR1C* in HeLa cells suggests that the variants associated with TCS primarily affect Pol I function (57), although studies modeling similar pathogenic variants in yeast have indicated that some TCS variants can affect both Pol I and Pol III (25). The role of Pol III in NCCs and craniofacial development is an interesting area for future investigation, and it remains to be determined whether this also involves p53-dependent effects.

Tcof1/Treacle, however, does have additional roles to its function in rRNA transcription, namely, in reactive oxygen species-induced DNA damage repair (*SI Appendix*, Fig. S5 and Table S1), which, when perturbed, can also lead to p53-dependent apoptosis (21, 58, 59). Consistent with this known role of Tcof1/Treacle in DNA damage repair (21), we detected γ-H2AX expression in the pharyngeal arches of *Tcof1*$^{NKO/NKO}$ mutants and in *Tcof1*$^{tKO/tKO}$ MEFs (*SI Appendix*, Fig. S15 *D* and *H*). However, γ-H2AX only labeled a minority of NCCs in *Tcof1*$^{NKO/NKO}$ mutants, suggesting that Tcof1/Treacle's function in DNA damage repair is secondary to its primary role in rRNA transcription (21, 60). Moreover, antioxidant treatment to reduce reactive oxygen species-induced DNA damage ameliorates the craniofacial anomalies in about 30% of *Tcof1*$^{+/-}$ mice (21), but a much higher percentage (75%) are rescued via genetic p53 inhibition (27), again indicating that Tcof1/Treacle is mainly required for rRNA transcription (60). In contrast, we observed no evidence for γ-H2AX–labeled double-strand breaks in *Polr1a*$^{NKO/NKO}$ or *Polr1c*$^{NKO/NKO}$ mutant embryos (*SI Appendix*, Fig. S15 *B* and *C*) and *Polr1a*$^{tKO/tKO}$ or *Polr1c*$^{tKO/tKO}$ MEFs (*SI Appendix*, Fig. S15 *F* and *G*). Thus, the TUNEL staining observed in association with Polr1a and Polr1c loss-of-function is not due to DNA damage. Therefore, given that p53 was increased in all the *NKO* embryos (Fig. 5) and *tKO* mutant MEFs (*SI Appendix*, Fig. S13), this further substantiates the correlation between deficiencies in rRNA transcription and the Rpl5/11–Mmd2–p53-mediated tissue-specific apoptotic effects on NCCs in the pathogenesis of ribosomopathy-associated craniofacial anomalies. Altogether, while there is more to learn about the ribosomal and extraribosomal functions of Pol I subunits and associated factors, it remains clear that *Polr1a*, *Polr1c*, and *Tcof1* are required in NCCs and that the phenotypes of the *Polr1a*$^{NKO/NKO}$, *Polr1c*$^{NKO/NKO}$, and *Tcof1*$^{NKO/NKO}$ embryos are unified by perturbation of rRNA transcription and an increase in p53-dependent cell death.

While p53 signaling has been implicated in multiple ribosomopathies (45) and developmental syndromes (47), how disruptions in rRNA transcription and ribosome biogenesis result in cell-type-specific apoptosis and the molecular mechanism underlying elevated p53 levels in different pathologies remains unclear (24). Contrary to previous literature that implicates higher transcription of *p53* in the NCCs compared with surrounding cells as a potential reason for neurocristopathies (46), we observe that *p53* transcript quantity is similar in NCCs and other craniofacial cell types during early embryogenesis (*SI Appendix*, Fig. S10). In addition, previous data show that p53 protein levels are uniformly low in the neuroepithelium and surrounding tissues in wild-type embryos (27). However, in the absence of Pol I subunits or *Tcof1*, p53 protein is up-regulated in a tissue-specific manner (Fig. 5 and *SI Appendix*, Figs. S9 and S10). We demonstrate that posttranslational p53 activation in *Polr1a*, *Polr1c*, and *Tcof1* loss-of-function mutants results from an imbalance between rRNA transcription and ribosomal proteins, triggering a nucleolar surveillance response. Excess Rpl5 and Rpl11 bind to Mdm2, limiting Mdm2's ability to bind and ubiquitinate p53 (Fig. 7), which leads to p53 protein accumulation and, ultimately, NCC death (Fig. 5 and *SI Appendix*, Fig. S14). Further contributing to the tissue-specific impact of perturbed rRNA transcription and p53-dependent activation is that different tissues, including the neuroepithelium and cultured cranial NCCs, may be more sensitive to p53 activation (46) and, therefore, likely to undergo cell death in response to p53 activation (47), as opposed to other responses to p53 activation, such as cell-cycle arrest or senescence, the latter of which we did not observe. Our work therefore suggests that the initial trigger for p53 activation and accumulation in the neuroepithelium in TCS or AFDCIN may be through a nucleolar surveillance mechanism and that the sensitivity of the neuroepithelium and NCCs to p53 activation arises, at least in part, from their elevated requirement for rRNA transcription (*SI Appendix*, Fig. S14). Consistent with this model, the levels of rRNA transcription correlate with their susceptibility to p53-dependent cell death in cancer cell lines. Cancer cells with relatively high levels of rRNA transcription tend to undergo cell death after inhibition of rRNA synthesis, whereas cells with relatively low levels of rRNA transcription undergo cell-cycle arrest and are more likely to survive (24). Altogether, a nucleolar surveillance mechanism may also contribute to other ribosomopathies, in which deficiencies in specific ribosomal proteins or increased rRNA transcription (61) are associated with p53-dependent cell death (62, 63). Moreover, it emphasizes the importance of balanced rRNA and ribosomal protein production in the pathogenesis of these pathologies.

Our data suggest that the tissue-specific regulation of rRNA transcription has important implications across multiple diseases and tissue types. While tissue-specific expression and function of ribosomal proteins and preribosomal factors contribute to the pathogenesis of several developmental ribosomopathies (64, 65), the dynamic cell- and tissue-specific regulation of rRNA expression during development is not as well understood. Recent studies have observed tissue-specific expression of rRNA in the mouse eye and ovary (32, 33), during forebrain development (66), and during EMTs (55, 64). The level of rRNA in these tissues was hypothesized to correlate with levels of proliferation, similar to our data for the neuroepithelium and NCCs in E8.5 embryos. Interestingly, the differential levels of rRNA transcription in NCCs compared with surrounding cells begins to decrease by E9.5, suggesting that NCC progenitors are more sensitive during their formation, proliferation, and migration stages of development, than later during differentiation. Consistent with this idea, reductions in rRNA transcription have been observed in association with differentiation in other systems (66–68).

Other factors may also contribute to dynamic tissue-specific rRNA transcription beyond a proliferation and survival versus differentiation demand. This includes epigenetic changes in rDNA (69), rDNA copy-number variation (70), tissue-specific

expression of variant rRNA alleles (69), or regulation of rRNA synthesis by transcription factors such as Snail1 (55) and Runx2 (71), which are involved in EMTs or osteochondroprogenitor differentiation, respectively. Our data suggest that endogenous differential Pol I subunit and *Tcof1* gene expression contribute to the dynamic tissue-specific regulation of rRNA, which underlies the craniofacial defects in TCS and AFDCIN. However, further work is needed to determine the upstream mechanisms that modulate the expression of Pol I and Pol I–mediated transcription, especially in the context of development and disease.

In summary, our work has uncovered a dynamic tissue-specific regulation and requirement for rRNA transcription during mammalian embryonic development, which mechanistically accounts for the corresponding tissue-specific threshold sensitivities to disruptions in rRNA transcription, particularly in NCCs during craniofacial development. Loss-of-function of Pol I catalytic subunit (Polr1a), noncatalytic subunit (Polr1c), and associated factor (Tcof1) result in similar phenotypes, illustrating the conserved mechanisms underpinning the etiology and pathogenesis of Pol I-related craniofacial birth defects in ribosomopathies, such as TCS and AFDCIN. Furthermore, we found that the rRNA–Rpl5/Rpl11–Mdm2–p53 molecular pathway, which has been previously studied in the context of cancer in yeast and cell lines (24, 53), accounts for the post-translational activation of p53 protein in response to perturbed rRNA transcription. This explains why p53 inhibition is able to suppress apoptosis and rescue craniofacial anomalies in mouse (27) and zebrafish (28, 29) models of rRNA transcription deficiency and raises the possibility that re-establishing the stochiometric ratio between rRNAs and ribosomal proteins could provide a broadly applicable avenue for the therapeutic prevention of ribosomopathies.

Materials and Methods

Animal Husbandry. All mice were housed in a 16h-light:8h-dark light cycle. All animal experiments were conducted in accordance with Stowers Institute for Medical Research Institutional Animal Care and Use Committee (IACUC)-approved protocol (IACUC no. 2019-097). Transgenic mouse lines were generated at the Stowers Institute for Medical Research Laboratory Animal Facility or by the Virginia Commonwealth University Transgenic/Knockout Mouse Facility (Virginia Commonwealth University IACUC no. AM10025). Details of their generation and maintenance can be found in *SI Appendix*, *Materials and Methods*. MEFs were derived from transgenic mice as described (72).

Molecular and Phenotypic Analysis. Skeletal staining, in situ hybridization, and immunohistochemistry were performed according to published methods (73, 74). Descriptions of RNA and proteomic assays, along with quantification and statistical analyses, are provided in *SI Appendix*, *Materials and Methods*.

Data Availability. Original data underlying this manuscript can be accessed from the Stowers Original Data Repository (https://www.stowers.org/research/publications/LIBPB-1604) (75). PyImageJ scripts that interface with ImageJ from Jupyter Notebooks and which are noted in the methods and were used for quantitative analyses can also be found there. Single-cell RNA-sequencing data have been deposited in the Gene Expression Omnibus (accession no. 168351) (76). All study data are included in the article and/or *SI Appendix*.

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