Chapter 11
Structure and Function of Nopp140 and Treacle

Fang He and Patrick DiMario

11.1 Introduction

Nopp140 and treacle are believed to function as molecular chaperones, delivering small nucleolar ribonucleoprotein complexes (snoRNPs) to the nucleolus where preribosomal RNA is synthesized, cleaved, chemically modified, and assembled into large and small ribosomal subunits. Orthologs of Nopp140 have been identified in an evolutionarily wide range of eukaryotes from yeast to human, but treacle appears to be restricted to the vertebrates. Both proteins share an amino terminal Lis1 homology (LisH) motif in their amino termini, and a large central repeat domain consisting of alternating serine-rich acidic motifs and lysine/proline-rich basic motifs. The carboxy termini of Nopp140 and treacle are dissimilar denoting unique associations. Nopp140 associates preferentially with both box H/ACA snoRNPs that guide pseudouridylation of pre-rRNA, and weakly with box C/D snoRNPs that guide $2'$-O-methylation, while treacle interacts with box C/D snoRNPs with no detected interaction with box H/ACA snoRNPs. Nopp140 and treacle may be multifunctional: the large central domains of Nopp140 and treacle serve as scaffolds for delivering and positioning snoRNPs within nucleoli. Nopp140 also participates in Pol I and in one case, Pol II transcription; treacle also interacts with Pol I transcription machinery. Nopp140 is required for normal development in *Drosophila*, while treacle is critical for mammalian neural crest cell development. Loss of treacle function causes a nucleolar stress response that initiates p53-mediated apoptosis in embryonic neural epithelial and neural crest cells leading to the Treacher Collins–Franceschetti Syndrome in humans, a collection of craniofacial malformations.
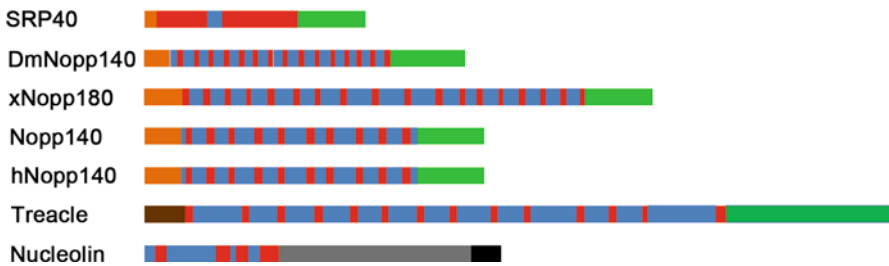
P. DiMario (✉)

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA
e-mail: pdimari@lsu.edu

11.2 Nopp140: Orthologs and Early Reports

Clearly, the majority of work on Nopp140 has been performed by U. Thomas Meier, first as a post doctoral fellow in Gunter Blobel's laboratory at Rockefeller University, and then in his own laboratory at Albert Einstein College of Medicine. While looking for proteins responsible for nuclear-cytoplasmic transport, Meier and Blobel (1990) identified p140 (as it was first called) in rat as a protein that could bind the nuclear localization signal (NLS) of the SV-40 large T antigen. Immuno-fluorescence microscopy showed that p140 localized to the nucleolus, but not to the nucleoplasm or cytoplasm. Colocalization with fibrillarin indicated that p140 localized to the fibrillar regions of the nucleolus. Later, Meier and Blobel (1992) deduced the amino acid sequence of Nopp140 as it is now called. They verified that antibodies against Nopp140 strongly labeled nucleoli, but with the better antibody they could detect weaker nucleoplasmic labeling. Injecting fluorescence labeled antibodies into the cytoplasm caused nucleolar accumulation of the antibodies, indicating that Nopp140 shuttles between the nucleus and the cytoplasm where it binds the antibody and then delivers the antibody to the nucleolus upon nuclear import. Nucleo-cytoplasmic shuttling of Nopp140 was later confirmed by Bellini and Gall (1999).

Nopp140 is conserved among eukaryotes, from yeast to humans (Fig. 11.1). It was actually observed prior to 1990 (Pfeifle and Anderer 1984; Schmidt-Zachmann et al. 1984; Pfeifle et al. 1986). Schmidt-Zachmann et al. (1984) initially characterized the *Xenopus* ortholog of Nopp140, which is now called xNopp180 (Cairns and McStay 1995). The protein has an apparent molecular weight of 180 kDa and a pI of ~4.2; it is enriched in nucleoli of oocytes and somatic cells. Immuno-gold

Fig. 11.1 Nopp140 protein compared to structurally related treacle and nucleolin proteins. Bar diagrams of yeast Srp40 (Meier 1996), *Drosophila* DmNopp140-True (Waggener and DiMario 2002), *Xenopus* xNopp140 (Cairns and McStay 1995), rat Nopp140 (Meier and Blobel 1992), human hNopp140 (Pai et al. 1995), human treacle (So et al. 2004), and human nucleolin (Srivastava et al. 1989) were drawn to show their different domains and motifs. Orange represents the conserved N-terminal domain of Nopp140 proteins. *Deep blue* within the amino termini of Nopp140 and treacle represents the LisH motif used as a possible homo-dimerization domain. *Red* represents acidic stretches, and *blue* represents basic stretches within the large central domains of Nopp140 and treacle and within the amino terminal region of nucleolin. *Green* represents the conserved C-terminal domain of Nopp140 proteins. *Gray* and *black* in nucleolin represent the four RNA binding domains and the RGG domain, respectively

labeling showed that the protein localizes to the dense fibrillar component (DFC) of interphase nucleoli, and immuno-fluorescence labeling showed that xNopp180 disperses to the cytoplasm during mitosis (metaphase and anaphase), with no apparent accumulation in the chromosomal nucleolar organizer regions (NORs). In telophase, xNopp180 rapidly reassembles into reforming nucleoli. Its cDNA was isolated from a *Xenopus* oocyte expression library using mAb G1C7 (Cairns and McStay 1995), and the deduced amino acid sequence shows up to 18 alternating acidic and basic stretches in the central domain (Fig. 11.1), while the N- and C-termini show 50 and 59% identities to the respective regions of rat Nopp140.

Pfeifle and Anderer (1984) described a nucleolar protein of 135 kDa in various mouse fibroblast, leukemia, and embryonic cell lines by immuno-fluorescence labeling and western blot analyses. Cross reacting proteins were observed in human cells (128 kDa), chicken cells (130 kDa), and *Drosophila* culture cells (118 kDa). Pulse labeling of enriched nucleoli from cultured mouse cells with [^{32}P] demonstrated preferential labeling of the mouse 135 kDa protein with a phosphoserine/phosphothreonine ratio of 47/1. An interesting point made in this early report is the apparent dependence of pp135 abundance on the cell cycle, with greater abundance of pp135 in rapidly dividing cells versus stationary cells. A subsequent paper (Pfeifle et al. 1986) reported localization of pp135 to NORs during mitosis.

Human Nopp140 was first predicted from cDNA clones that encoded proteins with sequence similarity to rat Nopp140 (Nomura et al. 1994). Pai et al. (1995) discovered human p130 when searching for proteins that fluctuated in abundance between interphase and M-phase. P130 is present in nucleoli of interphase cells, and it is heavily phosphorylated by casein kinase II (CKII) with hyper-phosphorylation occurring during mitosis, presumably by Cdk1. While p130 was undetectable by immuno-fluorescence during metaphase, it localized to prenucleolar bodies in telophase, and eventually to the nucleoli in interphase. Human p130 contains ten alternating acidic and basic repeat stretches (Fig. 11.1), and it shows 74% identity to rat Nopp140 (Pai et al. 1995). Interestingly, an isoform of p130 contains an additional 10 amino acid insertion in the fourth basic region (Pai and Yeh 1996). Both human isoforms (referred to as p130α and p130β) are coexpressed in various cell types, but the transcript encoding the β form (with the extra amino acids) is expressed to a lesser extent than the transcript encoding the α form. Both transcripts show a significant decrease in abundance upon cell cycle arrest.

Sequence similarity in the carboxy terminus of Nopp140 has been the criterion for identifying various orthologs. Srp40 is the ortholog of mammalian Nopp140 in *S. cerevisiae* (Meier 1996); it consists of two acidic clusters separated by one basic stretch. Its carboxy terminal domain is 59% identical to that in mammalian Nopp140. Like rat Nopp140, the acidic domains in Srp40 are rich in Ser residues that are likewise phosphorylated; a GST-Srp40 fusion has a calculated mass at 69 kDa, but it migrates in SDS gels at 110 kDa. Antibodies directed against rat Nopp140 cross react with Srp40, which colocalizes with the yeast fibrillarin ortholog, Nop1. Either the N-terminus or the central domain of Srp40 is sufficient to establish nuclear localization, while the carboxy-terminal domain alone is not (Ikonomova et al. 1997). Deletion of Srp40 retarded yeast growth mildly (it is not an essential protein),

and the nucleoli remained morphologically unaffected. The slight growth defects could be rescued by introducing full length rat Nopp140, and the large central repeat domain of rat Nopp140 alone seemed to fully restore the growth defects (Yang and Meier 2003).

Over-expression of Srp40 resulted in growth arrest. Although growth defects were observed when Srp40 was under-expressed or over-expressed, no abnormalities in rRNA transcription/maturation or translocation were detected. These early results suggested that Srp40 is not critical for preribosome assembly or transport. On the other hand, Srp40 is necessary for snoRNA localization to the yeast nuclear body, a structure comparable to the mammalian Cajal body (Verheggen et al. 2001). See below for a more detailed discussion of Nopp140's (Srp40's) role in snoRNP biogenesis.

A cDNA encoding a *Drosophila* Nopp140-like protein was originally isolated by screening a cDNA library prepared from stage 10 egg chambers with a subclone encoding the amino terminal region of *Xenopus* nucleolin (Waggener and DiMario 2002). We originally intended to recover *Drosophila* nucleolin (this was prior to the availability of the *Drosophila* genome). The recovered full length *Drosophila* cDNA, however, encoded a Nopp140-like protein that contained a large central domain of alternating acidic and basic motifs that are quite similar to those in prototypical rat Nopp140, but it has a distinctive Arg-Gly–Gly rich (RGG) carboxyl domain. RGG domains are often found in RNA binding proteins such as fibrillarin, vertebrate nucleolin, and many SR and hnRNP-type proteins (Ochs et al. 1985; Lischwe et al. 1985; Nichols et al. 2002). Just as we finished sequencing the *Nopp140-like* cDNA, the *Drosophila* genome became available, and a BLAST search identified conceptual gene *CG7421*, now called *Nopp140*. Two translation products were predicted by alternative splicing: the RGG-containing version that we had in hand and a true Nopp140 ortholog with 64 and 65% sequence identity in its carboxy terminus when compared to the carboxy termini of rat and human Nopp140, respectively. The two *Drosophila* isoforms are now referred to as Nopp140-RGG and Nopp140-True; they share the same amino-terminus and large alternating acidic and basic central domain, but they differ in their carboxy-terminal domains (Waggener and DiMario 2002). Both proteins contain CKII and Cdk1 phosphorylation sites, and they show slower than expected mobility on SDS-gels, migrating at approximately 125–127 kDa. Both proteins localize to nucleoli when expressed in *Drosophila* Schneider II culture cells or in transgenic embryos, larvae, and adults (McCain et al. 2006). Interestingly, with the *Drosophila* genome now well annotated, it appears that *Drosophila* does not encode a close homolog of vertebrate nucleolin, the original target of our cDNA library screen.

Two Nopp140 isoforms were also identified in *Trypanosome brucei* (Kelly et al. 2006). *Tb*Nopp140 and the *Tb*Nopp140-like protein (*Tb*NoLP) share the same central alternating acidic and basic repeat domain. While the C-terminus of *Tb*Nopp140 is similar to the C-terminus of yeast Srp40, the carboxy terminus of *Tb*NoLP also contains a RGG domain, similar to the Nopp140-RGG isoform in *Drosophila*. Both Nopp140 isoforms in *T. brucei* localize to nucleoli, both are phosphorylated, and interestingly, both can be coprecipitated with antibody directed against RNA Pol I (Kelly et al. 2006).

The existence of Nopp140-RGG isoforms in *Drosophila* and *Trypanosome* suggests that these isoforms may have similar interactions and perform similar functions as that of vertebrate nucleolin, at least with respect to its carboxy RGG domain. Similar RGG domains exist in many other RNA-associated proteins, usually near their carboxy termini. These RGG domains are known to bind RNA either directly or indirectly (Kiledjian and Dreyfuss 1992; Godin and Varani 2007). The arginines within the tripeptide repeats are asymmetrically dimethylated (reviewed by McBride and Silver 2001), the domain forms a series of β turns (collectively, a β-spiral) (Ghisolfi et al. 1992), and it likely binds G-quartet RNA structures (e.g., Darnell et al. 2001; Ramos et al. 2003). Precisely what the RGG domain in the Nopp140 *Drosophila* and *Trypanosome* splice variants is doing remains unknown. The analogous carboxy RGG domain in nucleolin has been reported to bind to both RNA and protein (reviewed by Ginisty et al. 1999; see Chap. 9).

11.3 Detailed Molecular Structures of Nopp140 Proteins

Figure 11.1 summarizes the linear domain organization of the various Nopp140 orthologs. The amino terminal portion of Nopp140 contains a LisH (Lis1-homology) motif that is generally accepted to be a dimerization domain (Kim et al. 2004). The LisH motif was originally described in Lis1 from *Mus musculus* (residues 6–39). Lis1 is required for normal neuronal migration during cerebral cortex development; mutations in the human *Lis1* gene lead to Miller-Dieker lissencephaly ("smooth brain"), a defect leading to severe retardation, epilepsy, and eventually death (Reiner et al. 1993; Emes and Ponting 2001). The LisH motif in human Nopp140 consists of amino acid residues 9–42 in which residues K17, K22, and A31 are well conserved in other LisH motifs (Kim et al. 2004). The LisH motif contains two alpha helices necessary for dimerization; two LisH motifs form a homodimer by assembling their alpha helixes into a four-helix anti-parallel bundle (Kim et al. 2004; Mateja et al. 2006). Presence of the LisH motif in the amino terminus of Nopp140 suggests homo-dimerization or perhaps hetero-dimerization with another LisH-containing protein.

Mammalian Nopp140 contains a large central domain consisting of 10 repetitive alternating acidic and basic motifs. Nopp140 is heavily phosphorylated (Pfeifle and Anderer 1984), and its pI is quite acidic; for instance, the pI for *Xenopus* xNopp180 is ~4.2 (Schmidt-Zachmann et al. 1984). The deduced rat protein is computationally determined to be 73.4 kDa, but shifts to the apparent weight of 140 kDa in SDS-gels. This shift is likely due to extensive phosphorylation and the resulting high charge density. Dephosphorylation by alkaline phosphatase dramatically shifts Nopp140 downward on SDS-gels compared to the phosphorylated form (Meier and Blobel 1992; Cairns and McStay 1995). Nopp140 expressed in bacteria can be phosphorylated extensively by CKII in vitro (Meier 1996). In fact, rat Nopp140 contains 82 serine residues within the 10 acidic motifs of the central domain; 45 of these serines are recognizable CKII consensus phosphorylation sites

(S/T-X-X-D/E), but once a particular serine is phosphorylated, it serves as the critical acidic residue at the C-terminal side of what becomes another CKII phosphorylation site (Meier and Blobel 1992). Interestingly, Nopp140 forms a stable complex with the β regulatory subunit of CKII in vitro and likely in vivo (Li et al. 1997). Thus the majority of the serine residues within the central domain are probably phosphorylated in vivo. This phosphorylation is required for Nopp140's interaction with snoRNPs (see below).

Besides the acidic motifs, the basic motifs within the large central domain of rat Nopp140 contain a total of 19 protein kinase C (PKC) consensus sites (Meier and Blobel 1992), suggesting regulation by calcium dependent signaling pathways. Most of these PKC sites also form Cdk1/cyclin B phosphorylation sites, and as expected, Cdk1/cyclin B phosphorylation of Nopp140 increases in mitosis, suggesting a link between its M-phase phosphorylation and initial redistribution to the cytoplasm (Pai et al. 1995). Finally, a highly conserved protein kinase A (PKA) site resides in the carboxy terminus of Nopp140 (Meier 1996; Chiu et al. 2002; Kim et al. 2006).

The carboxy terminus of Nopp140 is the most conserved region of the various Nopp140 proteins described (Meier 1996). The terminus actually consists of two identifiable subdomains (NoppCa and NoppCb) encoded by their own exons. This is also the case for the Nopp140-True isoform in *Drosophila* (Waggener and DiMario 2002), indicating exon conservation in *Nopp140* gene organization. The precise functions of these carboxy subdomains remain unknown, but their properties in rat Nopp140 have been well described (Isaac et al. 1998). For instance, the conserved PKA phosphorylation site (Ser_{685} in rat Nopp140 and Ser_{670} in *Drosophila* Nopp140-True) suggests that Nopp140 may be a substrate for signal transduction-mediated phosphorylation cascades that regulate molecular interactions within nucleoli or Cajal bodies (CBs) (Meier 1996).

11.4 Nopp140's Nucleolar Locations and Associations

The location of Nopp140 inside nucleoli has been somewhat controversial: early reports claimed that Nopp140 resides in the DFC of interphase cells (Schmidt-Zachmann et al. 1984; Pfeifle et al. 1986), while later reports (Vandelaer and Thiry 1998; Thiry et al. 2009) using different fixation techniques indicate that Nopp140 can be detected in the fibrillar centers, preferentially on the peripheral edge. There is no significant amount of Nopp140 in the granular regions or in the nucleoplasm.

The location of Nopp140 during mitosis has been equally controversial. While three reports (Pfeifle et al. 1986; Weisenberger and Scheer 1995; Vandelaer and Thiry 1998) describe the association of Nopp140 with M-phase NORs, other reports claim that Nopp140 does not localize to the NORs (Schmidt-Zachmann et al. 1984; Pai et al. 1995; Dundr et al. 1997; Tsai et al. 2008). The most recent study shows that Nopp140 localizes initially to the nucleoplasm in between the

chromosomes during prophase, and that it redistributes to perichromosomal regions, to nucleolar derived foci (NDF), and to the cytoplasm, but not to NORs from prometaphase to telophase (Thiry et al. 2009). In telophase, Nopp140 enters reforming nucleoli without detectable association with prenucleolar bodies (Dundr et al. 1997; Thiry et al. 2009).

Similarly, McCain et al. (2006) used GFP-Nopp140 as a marker for nucleologenesis in *Drosophila* embryogenesis, and showed that initial nucleolar formation in stage 13 and 14 blastoderm nuclei occurred without Nopp140's apparent localization to prenucleolar bodies. Interestingly, the first cells to form in the *Drosophila* embryo are the primordial germ cells (pole cells), but these cells lack nucleoli during the blastoderm stages because of repressed DNA transcription (Deshpande et al. 2004). Again, GFP-Nopp140 appeared dispersed within the pole cell nuclei during the blastoderm stages. Pole cells form nucleoli within minutes just as they begin their migration at the start of gastrulation. The dispersed GFP-Nopp140 coalesced rapidly into the forming nucleoli. Contrary to these studies that showed a diffuse distribution of Nopp140 prior to nucleologenesis, Baran et al. (2001) showed that Nopp140 in one-, two-, and four-cell mouse embryos, localizes to peripheral patches of nucleolus precursor bodies (NPBs, the peripheral zones being analogous to prenucleolar bodies) and that Nopp140 shifts to the cortex of the NPB (analogous to the NOR) as rDNA transcription begins in the two-cell embryo.

11.5 Molecular Interactions Indicate Function

Neither deletion of yeast Srp40 (Meier 1996), nor the knock-down of *Drosophila* Nopp140 (Cui and DiMario 2007) seemed to alter nucleolar structure. When over-expressed, however, full length human Nopp140 caused the redistribution of RNA Pol I and largely disrupted nucleolar integrity (Chen et al. 1999). Similar findings were reported for both *Drosophila* Nopp140 isoforms: when the *Drosophila* GFP-Nopp140-True was over-expressed in transgenic larvae, nucleoli within the polyploid cells appeared swollen and disorganized. Over-expression of the GFP-Nopp140-RGG isoform completely disrupted the nucleoli (Cui and DiMario 2007). Taken together, these observations suggest that Nopp140 is not required for nucleolar formation, but its over-expression disrupts nucleolar integrity and function.

Isaac et al. (1998) carefully examined what roles the individual amino terminal, the large central repeat domain, and the carboxy terminal domains have in nucleolar and Cajal body localization and retention. The amino terminal domain (NoppN) was expressed as a fusion (GFP-NLS-HA-NoppN); it localized to the nucleoplasm and the cytoplasm, but it failed to localize to nucleoli or CBs. NoppN contains a putative NES, perhaps explaining its cytoplasmic enrichment. Endogenous Nopp140 was not affected by the over-expression of NoppN.

When over-expressed as a fusion to HA, the conserved C-terminus of Nopp140 (referred to as HA-NoppC) localized to nuclei, but it acted as a dominant negative

in that it caused the redistribution of full length Nopp140, the Nopp140-associated protein of 57 kDa (NAP57), and fibrillarin from nucleoli to nucleoplasmic granules. Nucleolin and UBF remained within the nucleoli, which maintained their normal structural integrity. Over-expression of NoppC also caused the disassembly of CBs as judged by the dispersion of p80 coilin to the nucleoplasm. As described above, the carboxy terminal domains of mammalian Nopp140 and *Drosophila* Nopp140-True are encoded by two separate exons. Isaac et al. (1998) showed over-expression of the individual peptides encoded by the individual exons (NoppCa and NoppCb) caused the same dominant-negative phenotypes on nucleoli (redistribution of nucleolar components) and CBs (disruption) as did NoppC itself.

Interestingly, Isaac et al. (1998, 2001) showed that over-expression of the large central domain of Nopp140 (referred to as NoppR) in COS-1 cells caused the formation of phase-dark nuclear rings of 0.5–5 µm diameter. They called these structures R-rings. Over-expression of full-length human Nopp140 can also form R-rings (Kittur et al. 2007). Like NoppC, NoppR caused a dominant-negative effect by redistributing endogenous Nopp140, fibrillarin, NAP57, UBF, and Pol I from nucleoli to the R-rings; however, neither nucleolin nor B23 were affected. Newly synthesized p80 coilin also localized to R-rings, but other Cajal body components (e.g., Sm antigens) failed to redistribute to the R-rings. Subsequent examination of R-rings (Isaac et al. 2001) revealed multilamellar membrane stacks that appear identical to the previously described nucleolar channel system (NCS) found only in postovulation human endometrial cells that are receptive to blastoderm implantation (see references in Isaac et al. 2001).

Kittur et al. (2007) examined the R-rings in even greater detail. The rings apparently form by invagination of the inner nuclear membrane into the nucleoplasm. They used immuno-fluorescence microscopy to show that R-rings form by the accumulation of the highly charged NoppR in patches on the underside of the nuclear envelope. They showed that Nopp140 complexes with calcium in a phosphorylation-dependent manner, and then used electron spectroscopic imaging to show that R-ring formation likely occurs via a calcium-mediated interaction between the multiple phosphates on NoppR and the inner nuclear membrane. The stacked membranes of the R-rings lie within an electron dense matrix that contains Nopp140, its bound calcium, and associated nucleolar components. R-rings are often found in close proximity to the nuclear envelope and nucleoli. Because of their derivation from the nuclear envelope and thus the ER, R-rings contain a mix of rough and smooth ER-associated membrane and luminal proteins. These include calnexin, Sec61, the IP_3 calcium channel, the receptor for the signal recognition particle, BiP, PDI, HMG-CoA reductase, and glucose-6-phosphatase. R-rings, however, are distinct from the nuclear envelope in that they lack the lamin-associated protein, LAP2, nucleoporin p62, and lamin B. R-rings are morphologically indistinguishable from NCSs, and like R-rings, NCSs contain calnexin, BiP, and glucose-6-phosphatase. NCSs, however, contain less Nopp140 and calcium than do the R-rings. What induces NSC formation, what role NSCs play in the receptive phase of the human endometrium, and what molecular relationships they may have with nucleoli remain exciting avenues of exploration.

11.6   Nopp140, a Chaperone for Small Nucleolar Ribonucleoproteins (snoRNPs)

The first indication that Nopp140 interacts with snoRNPs was the discovery that rat NAP57 (dyskerin in humans, Cbf5p in yeast) could coimmunoprecipitate with Nopp140 (Meier and Blobel 1994). Immuno-fluorescence microscopy showed that NAP57, like Nopp140, localized to both nucleoli and CBs, with NAP57 localized primarily in the nucleolar DFCs (Meier and Blobel 1994). NAP57 is a pseudouridy-lase, a component of box H/ACA RNPs, which consist of four proteins (NAP57, GAR1, NHP2, and NOP10) and one of several box H/ACA guide RNAs (Ganot et al. 1997; Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998). Immunoprecipitation of Nopp140 also identified intact snoRNP complexes that contained H/ACA guide RNAs (Yang et al. 2000). Box H/ACA snoRNPs function in site-specific pseudouridylation of pre-rRNA processing, pre-mRNA splicing, and telomere maintenance (reviewed by Meier 2005). In vitro pseudouridylation by box H/ACA snoRNP complexes occurred in an energy and helicase independent reaction *without* the association of Nopp140 (Wang et al. 2002), suggesting that Nopp140 itself is not required for the snoRNP enzymatic reaction. The functional model as proposed by Wang et al. (2002) states that within the nucleolus, Nopp140 acts as a scaffold for multiple snoRNPs as they modify the pre-rRNA. Further, Nopp140's association with at least the box H/ACA snoRNPs is dependent on its extensive CKII phosphorylation, indicating the association between Nopp140 and snoRNPs is electrostatic and reversible.

Besides box H/ACA RNPs, Nopp140 weakly associates with C/D box snoRNPs (Yang et al. 2000) that perform site-specific methylation of the pre-rRNA (Tollervey et al. 1993; Kiss-László et al. 1996; Nicoloso et al. 1996). Components of box C/D RNPs include the four core proteins, NHP2L1/15.5, NAP65, Nop56, and fibrillarin, which is the RNA methyl-transferase. Fibrillarin and NAP65 were found in Nopp140 immunoprecipitates, but under less stringent conditions, suggesting that Nopp140's interactions with C/D box snoRNPs are not as strong as its interactions with box H/ACA snoRNPs (Yang et al. 2000). In a study to determine association between Nopp140 and the specific box U3 C/D box snoRNP complex, Watkins et al. (2004) found Nopp140 associated with both precursor and mature U3-containing snoRNPs in nuclear extracts, but antibodies against U3 snoRNP core proteins (e.g., Nop56, Nop58, fibrillarin) failed to coprecipitate Nopp140 from *nucleolar* extracts. Watkins et al. (2004) concluded that Nopp140, along with two other putative assembly factors, TIP48 and TIP49, participates as a snoRNP biogenesis factor in the nucleo-plasmic phase of U3 RNP assembly (perhaps in CBs, see below), but that Nopp140 dissociates from the mature form of the U3 snoRNP once inside the nucleolus.

Determining the role of yeast Srp40 in snoRNP biosynthesis/maintenance has been difficult because of its dependence on the *Shm2* (previously called *LES2*) gene product (Yang et al. 2000; Yang and Meier 2003). *Shm2* encodes a cytosolic serine hydroxymethyltransferase involved in one-carbon metabolism, converting tetrahy-drofolate (THF) to 5, 10-methylene THF. Yang et al. (2000) first showed that the

single mutations, *srp∆* or *shm2*, have slight growth defects, and that the double mutant can be rescued by *SRP40* expression from a plasmid either from its own endogenous promoter or from the *GAL10* (conditional) promoter. Depleting Srp40 in the conditional double mutant led to reductions of several box H/ACA RNAs (snR3, snR10, snR11, snR42, and the required snR30), but not the box C/D RNAs U3, U14, and U24 as determined by Northern analyses (Yang et al. 2000; Yang and Meier 2003). The observation indicates that Srp40, like Nopp140, is likely to have a greater role in box H/ACA snoRNP interaction/biosynthesis than it has in that of box C/D snoRNPs. In fact, loss of box H/ACA snoRNAs by depletion of Srp40 is similar to phenotypes observed for the loss of individual H/ACA box proteins, Cbf5, Nhp2p, and Nop10p (Henras et al. 1998; Lafontaine et al. 1998; Watkins et al. 1998).

To further define how *Shm2* might interact with *Srp40*, Yang and Meier (2003) first showed that the triple mutant strain, *srp40∆ shm3 ade3,* is synthetic lethal. Like Shm2, ADE3 is a cytosolic enzyme involved in one-carbon metabolism, producing 5, 10-methylene THF from formate and THF in three steps. Yang and Meier (2003) showed that *SHM2*, *SRP40*, or *ADE3* could rescue the triple-synthetic lethality when expressed separately from *LEU2* plasmids using their own endogenous promoters. The mechanistic link between Srp40 and the two cytosolic enzymes involved in one-carbon metabolism is perplexing, but Yang and Meier (2003) showed that *catalytic* mutants of Shm2, expressed in the synthetic lethal strain, actually complemented growth. This indicates that loss of one-carbon metabolites is not the reason for lethality, and that Shm2 (and perhaps Ade3) may have secondary, non-catalytic functions related to Srp40. This possibility was strengthened by over-expressing Lsm5 which partially restored growth of the triple mutant. Lsm5 normally resides in yeast Sm-like complexes, and Lsm5 likely has several roles in nuclear RNA (e.g., tRNA) processing. Yang and Meier (2003) showed that ectopic Lsm5 expression provided a partial growth rescue which correlated with partial restorations in box H/ACA snoRNPs snR3 and snR10 abundance. Apparently, Lsm5 interacts with Shm2 as determined by a genome-wide two-hybrid assay, and Yang and Meier (2003) concluded that Lsm5 links the cytosolic enzyme, Shm2, with box H/ACA snoRNPs, and therefore Srp40. The mechanistic details of these interactions, however, remain unknown.

11.7 Nopp140 and Cajal Bodies

Isaac et al. (1998) showed that newly synthesized Nopp140 in transfected culture cells localizes first to nucleoli and then to CBs, suggesting that Nopp140 shuttles snoRNPs from nucleoli and CBs. Using the *Xenopus* oocyte system, Bellini and Gall (1999) showed that shuttling Nopp140 appears simultaneously within nucleoli and CBs as it reenters the nuclei from the cytoplasm, and they reasoned there may be a difference between newly synthesized Nopp140 just arriving to the nucleus for the first time (i.e., Isaac et al. 1998) and mature Nopp140 that shuttles between the nucleolus, CBs, and cytoplasm. What these differences may be remains unknown,

but one could easily imagine extensive CKII phosphorylation on the mature Nopp140 versus nascent Nopp140 as a possible determinant in CB localization. Regardless of the differences, it is now well accepted that Nopp140 is the likely chaperone for snoRNPs between CBs and nucleoli.

Most snoRNAs are encoded as introns, and they assemble into snoRNPs without ever leaving the nucleus. Conversely, snoRNAs U3, U8, and U13 are transcribed from their own genes by RNA Pol II and thus have an initial m_7G cap, but they too remain in the nucleus where their 5′ caps are trimethylated, and where they too assemble into snoRNPs prior to their delivery to the nucleolus (Narayanan et al. 1999; Verheggen et al. 2002; Boulon et al. 2004). The CB is the nuclear compartment associated with spliceosomal assembly, preassembly of transcription complexes, and the processing of snoRNAs (reviewed by Nizami et al. 2010). Meier and Blobel (1994) first found Nopp140 in CBs by immuno-fluorescence microscopy. This was later confirmed by immuno-electron microscopy with the colocalization of Nopp140 and p80 coilin (Vandelaer and Thiry 1998), a generally accepted marker protein for CBs (see Nizami et al. 2010). Nopp140 can interact with amino terminus of p80 coilin (Isaac et al. 1998); however, the retention time of Nopp140 in CBs is shorter than that of p80 coilin or the survival of motor neuron (SMN) protein; in fact, the transit time for Nopp140 in CBs is similar to the transit times for snoRNP proteins GAR1 (box H/ACA snRNPs) and fibrillarin (box C/D snoRNPs), suggesting an interaction between Nopp140 and these snoRNPs, perhaps for their biogenesis or remodeling while in the CBs (Dundr et al. 2004).

Several studies have examined either the appropriate levels of Nopp140 required for CB integrity, or the localization of Nopp140 to CBs that are depleted of other known constituents. For instance, when over-expressed, the dominant negative NoppC described above disrupts CBs (Isaac et al. 1998). Conversely, depletion of yeast Srp40 disrupts the nucleolar body which may be the yeast complement of the metazoan CB (Isaac et al. 1998; Verheggen et al. 2001).

Lemm et al. (2006) described the RNAi knockdown of SMN or hTGS1 (the methyl-transferase that further methylates m_7G caps to yield 2,2,7-trimethyl G caps on U snRNAs and snoRNAs), and showed that residual coilin-containing nuclear foci maintained snoRNP proteins fibrillarin and Nop58. They reported (but did not show) that Nop56 and Nopp140 were also found in similar residual coilin-containing foci. Lemm et al. (2006) concluded that factors necessary for snoRNP assembly localize to a subclass of coilin-containing nuclear foci that still form in the absence of hTGS1 or SMN.

In a pivotal paper, Renvoisé et al. (2009) showed an inverse correlation between Nopp140 levels in CBs within spinal muscular atrophy (SMA) fibroblasts and the severity of the disease, suggesting the SMN protein is required for Nopp140 localization within CBs. SMA is a neuronal degenerative disease marked by low levels of SMN (for review, see Lorson et al. 2010); SMN is required for snRNP biogenesis both in the cytoplasm and in CBs (Carvalho et al. 1999). As SMN interacts with fibrillarin and GAR1 (Jones et al. 2001; Pellizzoni et al. 2001), it may also function in snoRNP assembly or maturation. Localization of Nopp140 to CBs is significantly reduced in SMA cells, and this reduction is correlated with reduced levels of box

H/ACA snoRNP proteins, GAR1, and NAP57/dyskerin, within CBs. Renvoisé et al. (2009) showed that Nopp140 localizes to CBs in nearly all (96%) COS cells that had been transiently transfected to over-express wild type SMN, while a reduced number (56%) of nontransfected control cells contained Nopp140 within their CBs. Three SMN mutants (SMN472Δ5, SMNexΔ7, and SMNE134K) display progressively severe phenotypes, and they reduce the accumulation of Nopp140 in CBs to correspondingly greater extents (Renvoisé et al. 2009). RNAi knockdown of SMN in control fibroblasts also reduced Nopp140 levels in the CBs, while over-expression of wild-type SMN in primary SMA cells restored Nopp140 levels in the CBs. Although Nopp140 has been shown to interact directly with p80 coilin (Isaac et al. 1998), it is now apparent that wild-type SMN is required for the accumulation of Nopp140 within CBs. The precise function of Nopp140 in CBs remains unknown, but it is becoming increasingly clear that Nopp140 acts with SMN in vital aspects of snoRNP biogenesis or remodeling within the CBs. In the least, Nopp140 can now be used as a CB marker to gauge the severity of SMA (Renvoisé et al. 2009).

11.8   Nopp140 as a Transcription Factor

Intriguing studies indicate that Nopp140 acts as a transcription factor for at least one Pol II gene (Lee et al. 1996; Miau et al. 1997). A C/EBP family member, AGP/EBP, was previously known to induce the acute phase response α_1-acid glycoprotein (*AGP*) gene. In searching for other factors that coactivate the *AGP* gene, the authors identified Nopp140 by coimmunoprecipitation with AGP/EBP followed by LC/MS/MS. Control experiments verified that Nopp140 bound to AGP/EBP in a defined complex rather than to the AGP/EBP antibody. Cotransfection of BHK cells with a reporter construct, *AGP-CAT*, and either *CMV-Nopp140* or *CMV-Nopp140-Reverse* showed enhanced CAT activity only with Nopp140 expression. As there are no known nucleic acid binding domains in vertebrate Nopp140, its coactivation of *AGT-CAT* must be via interaction with identifiable DNA-binding transcription factors.

To verify this possibility, Miau et al. (1997) cotransfected *CMV-Nopp140* and *CMV-AGP/EBP* expression plasmids along with the reporter plasmid and showed that both Nopp140 and AGP/EBP interact synergistically to activate expression of the *AGP-CAT* reporter gene. Functional (CAT) assays using deletions for both Nopp140 and AGP/EBP initially suggested that the carboxy terminal portion of Nopp140 (residues 347–704) is required to interact with the amino-terminal portion of AGP/EBP (residues 21–151). The authors initially concluded that Nopp140 bound to AGP/EBP by way of these identified regions as AGP/EBP bound to its three cognate DNA elements within the *AGP* promoter region. Further work revealed that Nopp140's role in coactivation of *AGP-CAT* is mediated by an additional interaction between Nopp140 and TFIIB. Specifically, the carboxy terminal portion of Nopp140 is critical for its in vitro interaction with TFIIB. The main conclusion of

the 1997 paper is that synergistic activation of *AGT-CAT* reporter gene is via a Nopp140-AGP/EBP-TFIIB ternary complex. The one caveat in these experiments is the possible over-expression of Nopp140 and AGP/EBP from strong CMV promoters. A follow-up report (Chiu et al. 2002) found that PKA phosphorylates rat Nopp140 at Ser^{113}, Ser^{627}, and Ser^{628}. Nopp140 phosphorylated by PKA activates *AGP* gene expression in a synergistic manner with CREB and C/EBPβ, while a mutant version of Nopp140 devoid of the site Ser^{627} could not achieve this synergistic activation.

Nopp140 also has a putative role in Pol I transcription. Chen et al. (1999) immunoprecipitated endogenous human Nopp140 from CEM and HeLa cells and showed by SDS-PAGE and mass spectroscopy that the 190 kDa subunit of RNA Pol I coprecipitated. The other protein to coprecipitate was the alpha subunit of CKII, suggesting that Nopp140, CKII, and Pol I form a complex. They reported the same coprecipitation using anti-FLAG to pull down exogenously expressed FLAG-Nopp140. As other reports documented (Schmidt-Zachmann et al. 1984; Pfeifle et al. 1986), Chen et al. (1999) showed Nopp140 colocalizes with Pol I in dot-like structures within the nucleolar DFCs, suggesting a potential interaction. Actinomycin D-mediated segregation of nucleoli maintained similar colocalizations between Nopp140 and Pol I. With low level expression, FLAG-Nopp140 localized to similar dot-like structures within the DFCs, but over-expression of FLAG-Nopp140 clearly disrupted nucleolar morphology, producing large hypertrophied nucleoli. With this over-expression, nucleolin redistributed to the nucleoplasm while Pol I and fibrillarin remained associated with the FLAG-Nopp140 in the enlarged nucleoli, again suggesting possible interactions. Chen et al. (1999) went on to use a Nopp140 deletion series and coimmunoprecipitation to show that the region spanning residues 204–382 (middle portion of the large central domain) interacts with RPA194, the large subunit of Pol I. Exogenous expression of this Nopp140 region (residues 204–383) now tagged with FLAG and an NLS appeared to displace endogenous Pol I in a dominant negative manner. Over-expression of full length Nopp140 or Nopp140 depleted for its carboxy half (Nopp140N382, still containing residues 204–382) resulted in segregation of nucleoli and a block in Pol I transcription as measured by Br-UTP incorporation, similar to the effects of actinomycin D. Chen et al. (1999) concluded that Nopp140N382 competed in a dominant-negative manner with endogenous Nopp140 for Pol I. This was the first description of Nopp140 affecting rRNA transcription.

Yang et al. (2000) then showed that expression of just the conserved carboxy tail of Nopp140 (NoppC) displaced endogenous Nopp140 from nucleoli in a dominant-negative manner, and blocked Pol I transcription as monitored by BrUTP incorporation. Interestingly, Pol I remained in position within these nucleoli. Kelly et al. (2006) also coprecipitated both isoforms of Nopp140 in *Trypanosome* using an antibody against Pol I, adding more validity to the possibility that Nopp140 directly interacts with Pol I as a transcription factor in rDNA transcription. One of the most intriguing hypotheses put forth regarding Nopp140 is that its association with Pol I could provide a molecular link between pre-rRNA transcription and processing, perhaps providing a feedback mechanism to regulate Pol I transcription when ribosome production is perturbed (Chen et al. 1999; Yang et al. 2000).

11.9   Organismal Depletion of Nopp140

We finish our discussion of Nopp140 by describing perturbations in development
when it is depleted (Cui and DiMario 2007). RNAi-mediated depletion of *Drosophila*
Nopp140 mRNAs was measured by RT-PCR, and the loss of Nopp140 was deter-
mined by immunofluorescence microscopy. Depletions of *Nopp140* transcripts by
50% or greater caused late larval and pupal lethality; however, a partial depletion of
30% permitted adults to survive, but these adults displayed deformed legs, wings,
and cuticle. The defects were reminiscent of craniofacial malformations associated
with the Treacher Collins syndrome due to the loss of the related nucleolar protein,
treacle (see below). Our initial results suggested that larval diploid precursor cells
(imaginal disc cells that generate legs and wings, and histoblasts that generate the
adult cuticle) have higher demands for ribosome biogenesis, and are thus more sen-
sitive to ribosome loss. Preliminary results clearly show abundant anti-caspase 3
labeling in wing discs isolated from larvae that express RNAi that depletes Nopp140.
Loss of imaginal wing disc cells by apoptosis is thus the most likely explanation for
the morphological defects due to loss of Nopp140. Terminally differentiated larval
polyploidy cells (i.e., larval midgut cells) appear to respond differently to the loss of
Nopp140 by inducing autophagy rather than apoptosis. How different cells respond
to the loss of ribosomes may prove to be much more complicated than originally
anticipated.

11.10   Treacle and the Treacher Collins–Franceschetti
Syndrome 1

Treacle is a nucleolar phosphoprotein structurally related to Nopp140 (Wise et al.
1997; Marsh et al. 1998; Winokur and Shiang 1998; Isaac et al. 2000; Fig. 11.1).
Treacle has been studied primarily in mammals (human, mouse, dog) (Dixon et al.
1997; Paznekas et al. 1997, Haworth et al. 2001), but an ortholog exists in *Xenopus*
(Gonzales et al. 2005a, b) indicating that treacle is a vertebrate protein. Human
treacle is encoded by the *TCOF1* gene at 5q32-q33.1 (Jabs et al. 1991; Dixon et al.
1993; Dixon 1996). *TCOF1* is greater than 20 kbp in length, and it contains 27
exons (see So et al. 2004). Three isoforms of human treacle exist because of alter-
native splicing; the original human isoform described is 1,411 amino acids in
length, but the predominant isoform in terms of abundance is 1,488 residues in
length (So et al. 2004). Human treacle has a highly conserved amino terminus of
213 amino acid residues. Like Nopp140, treacle contains a LisH motif (amino
acids 5–38) (Emes and Ponting 2001; Kim et al. 2004). The amino terminus is fol-
lowed by 11 repeating units (10 in the originally described isoform). Each repeat
consists of an acidic and a basic motif. Similar to Nopp140, the acidic motifs in
treacle are serine-rich with many putative CKII and PKC phosphorylation sites,
while the basic motifs are rich in lysine, alanine, and proline. Human treacle

expressed in *E. coli* has a predicted size of 144 kDa (Marsh et al. 1998), but the native, highly phosphorylated protein from human cells migrates anomalously at ~220 kDa on SDS-gels presumably because of the extensive phosphorylation and charge density (Isaac et al. 2000).

The carboxy tail of human treacle contains several functional NLSs, and the last 41 residues are necessary for nucleolar retention (Marsh et al. 1998; Winokur and Shiang 1998; Isaac et al. 2000); nonsense mutations yield treacle truncations that fail to translocate into the nucleus or localize within nucleoli (Marsh et al. 1998; Winokur and Shiang 1998). Similar truncations are frequently associated with the Treacher Collins syndrome in humans (Wise et al. 1997).

In mouse and humans, treacle is expressed in a wide variety of embryonic and adult tissues (Dixon et al. 1997; Paznekas et al. 1997), but most significantly, treacle expression is elevated in the embryonic neural folds just prior to neural tube fusion and in the first pharyngeal arch, coincident with primordial tissues known to give rise to craniofacial structures. Mutations in *TCOF1* give rise to the autosomal dominant Treacher Collins–Franceschetti syndrome (TCS; Fazen et al. 1967; Dixon 1996, Trainor et al. 2009). TCS is the most common of congenital craniofacial disorders in humans, afflicting 1 in 50,000 live births (Wise et al. 1997). Defects include hypoplasia of the facial mandible and zygomatic complex, coloboma (lesion) of the lower eyelids, a lack of eye lashes medial to the eye lid defect, downward slanting palpebral fissures, a high incidence of cleft palate, and conductive hearing loss due to malformation of the outer ear and the middle ear ossicles (Dixon 1996). Higher than expected polymorphisms exist within *TCOF1* (Teber et al. 2004), and they may account for the variable expressivity of the TCS.

Disruption of the murine *Tcof1* gene caused severe craniofacial anomalies and perinatal death in *Tcof1*^{+/−} mice (Dixon et al. 2000; Dixon et al. 2006). Deletions of 1–40 nucleotides are the most common genetic defects, but insertion-type, splicing, and nonsense mutations also exist (Trainor et al. 2009). The craniofacial defects were traced back to apoptosis in embryonic neural crest cells within the cranial neural folds, specifically a subset of cephalic neural crest cells that display relatively high *Tcof1* expression. To better establish the link between *Tcof1*/treacle and the TCS, Dixon et al. (2000) replaced the first exon in the mouse *Tcof1* gene with the neomycin resistance cassette in embryonic stem cells. Germ line chimeric males were prepared and crossed to wild-type females to produce heterozygous *Tcof1*^{+/−} embryos. These heterozygous embryos displayed several major craniofacial deformities beginning at day 8 of development (E8). Whole mount TUNEL assays of E9 *Tcof1*^{+/−} embryos showed excessive amounts of apoptosis in the neuro-epithelium of the cranial neural folds and in the neural tube compared to wild-type litter mates. Anti-neurofilament labeling of E10.5 *Tcof1*^{+/−} embryos showed a loss of neural crest cell-derived structures such as cranial ganglia, the ophthalmic branch of the trigeminal nerve, the glossopharyngeal ganglia, and an underdevelopment of the dorsal root ganglia. These heterozygous *Tcof1*^{+/−} mice died shortly after birth. Interestingly, the particular genetic background of the heterozygous *Tcof1*^{+/−} embryos has a significant effect on the penetrance and severity of the cranial defects (Dixon and Dixon 2004). For example, *Tcof1*^{+/−} mice with inbred CBA, C57BL6, or C3H genetic

backgrounds were lethal displaying severe morphological abnormalities, while the majority of *Tcof1*^{+/−} mice with DBA/1 and BALB/c backgrounds were normal and viable. This variation is likely due to factors in the different genetic backgrounds, but the identity of these factors remains unknown.

Dixon et al. (2006) showed that treacle is required in a cell-autonomous, spaciotemporal manner for rapidly proliferating cephalic neural crest cells with apparent high demands in protein synthesis. Cells of the neuro-epithelium and neural crest-derived craniofacial mesenchyme in *Tcof1*^{+/−} E8.75–E9 embryos showed relatively few ribosomes by antibody labeling (mouse monoclonal anti-rRNA antibody, Y10B) compared to wild-type littermates. Induction and migration of cephalic neural crest cells were not affected by the loss of treacle. Rather, treacle was required for ribosome biogenesis, and spatiotemporal haplo-insufficiency of treacle led to apoptosis and loss of these neural epithelial cells finally resulting in TCS. But why these particular neural crest cells are sensitive to the loss of treacle (ribosomes) at this point in development remains uncertain. Malformations associated with TCS are restricted to the head and neck regions, suggesting that other embryonic progenitor cells must have either sufficient amounts of treacle (ribosomes) or lower demands for protein synthesis.

In a seminal study, Jones et al. (2008; see also McKeown and Bronner-Fraser 2008; Sakai and Trainor 2009) showed that the partial loss of treacle in *Tcof1*^{+/−} mouse embryos (E8.5–E10.5) led to p53 stabilization in the neuroepithelium, and in turn to p53-induced G_1-cell cycle arrest, apoptosis, and ultimately hypoplasia of cranioskeletal structures. Remarkably, however, they were able to rescue this hypoplasia by deleting the *p53* gene. Rescue occurred in a *p53* dose-dependent manner; that is they observed complete rescue with *p53*^{−/−} versus partial rescue with *p53*^{+/−}. Immuno-staining with the Y10 mAb showed that deleting the *p53* gene had no effect (neither decline, nor restoration) in ribosome biogenesis in the neural crest cells. This indicates that loss of ribosomes was not the direct inducer of apoptosis in these cells, but rather a nucleolar stress response caused by treacle insufficiency somehow triggered p53 stabilization, cell cycle arrest, and finally apoptosis.

The nucleolar stress response is just now coming into focus. While Isaac et al. (2000) showed that the abundance of full length treacle does not vary by more than twofold in fibroblasts derived from both normal individuals and TCS patients, Jones et al. (2008) suggested that the embryonic neural crest cells have a higher threshold requirement for a specific level of treacle due to its high rate of proliferation (Trainor, pers. comm.). A resulting deficiency in ribosome biogenesis thus leads to stress in these neural crest cells. All forms of cell stress disrupt nucleoli to some extent (Rubbi and Milner 2003), and strong evidence now indicates that nucleoli act as stress sensors (Rubbi and Milner 2003; Olson 2004; Ma and Pederson 2008). For example, one hypothesis holds that stress-induced nucleolar disruption in mammalian cells releases p19^{ARF} to the nucleoplasm where it blocks the p53-specific ubiquitin ligase, MDM2. Activated p53 acts as a negative regulator of ribosome biogenesis by disrupting normal interactions between RNA Pol I and the upstream binding factor (UBF) and the selectivity factor (SLI) (Zhai and Comai 2000),

thereby compounding the loss of nucleolar function. Once stabilized, p53 induces proapoptotic *Bcl* family member genes *Bax* and *Bak* whose protein products facilitate release of cytochrome c from the mitochondria thus inducing a cascade of caspase activity and the initiation of apoptosis.

11.11 Treacle Function

Within mammalian cells, treacle localizes to nucleolar DFCs, but unlike Nopp140, it fails to localize to CBs (Isaac et al. 2000). As far as we know, treacle's only role is in nucleolar ribosome biogenesis (Trainor, pers. comm.). Immunoprecipitations showed a potential association between treacle and the alpha catalytic subunit of CKII, and according to Isaac et al. (2000), there is no apparent interaction between treacle and Nopp140, or between treacle and box H/ACA snoRNP components NAP57 and GAR1. Lin and Yeh (2009), however, did detect an interaction between treacle and Nopp140 by coimmunoprecipitation, specifically between Nopp140 and treacles's carboxy terminus (a robust interaction with residues 962–1488, but a weakened interaction with residues 1294–1488).

Hayano et al. (2003) performed a proteomic analysis of Nop56, a component of nucleolar box C/D small nucleoprotein complexes that direct site specific 2′-*O*-methylation of pre-rRNA. Treacle coprecipitated with Nop56-associated pre-rRNP complexes, and its association with Nop56 was independent of RNA, suggesting a protein–protein interaction between treacle and the box C/D snoRNP complexes. Conversely, precipitation of FLAG-tagged treacle-associated complexes identified Nop56. Gonzales et al. (2005b) confirmed a direct interaction between Nop56 (its C-terminal residues 367–594) and treacle, and while no direct interaction was found between fibrillarin and treacle, fibrillarin could be coprecipitated with FLAG-tagged treacle, suggesting an indirect association. The two studies therefore, indicate an interaction between treacle and C/D box snoRNPs mediated by a direct interaction with Nop56.

Gonzales et al. (2005b) further demonstrated that RNAi-mediated depletion of treacle in *Xenopus* oocytes blocked 2′-*O*-methylation of nucleotide C_{427} in the 18S region of pre-rRNA, and showed that *Tcof1*$^{+/-}$ mouse embryos with either CBA or C57BL/6 genetic backgrounds that are lethal (Dixon and Dixon 2004) were also deficient in pre-rRNA methylation of the corresponding nucleotide, C_{463}. Conversely, *Tcof1*$^{+/-}$ mouse embryos with a BALB/c genetic background have no craniofacial malformations (Dixon and Dixon 2004), and they showed normal pre-rRNA methylation. While 2′-*O*-methylation was adversely affected in *Tcof1*$^{+/-}$ mice with a CBA background, pseudouridylation of U_{1642} in the 18S region was not impaired in these embryos. Hayano et al. (2003) suggested that treacle acts as a chaperone similar to Nopp140, but that treacle and Nopp140 interact with box C/D snoRNPs at different stages during ribosome biogenesis. An equally intriguing possibility is that treacle may preferentially chaperone box C/D snoRNPs while Nopp140 chaperones the box H/ACA snoRNPs preferentially, and box C/D snoRNPs only marginally.

Besides acting as a chaperone for box C/D snoRNPs, treacle may also function in rDNA transcription. Treacle colocalizes with the upstream binding factor (UBF) and Pol I on mitotic NORs, suggesting a role for treacle in the rDNA transcription machinery (Valdez et al. 2004; Lin and Yeh 2009). Treacle also maintains its localization with UBF within the nucleolar caps of actinomycin D-segregated nucleoli (Valdez et al. 2004), and with Pol I in nucleolar condensed spots when UBF is depleted by siRNA (Lin and Yeh 2009). Immunoprecipitation of the FLAG-tagged and nucleolar-localized carboxy-terminal half of treacle successfully pulled down UBF, and yeast two-hybrid confirmed the interaction. Small interfering RNA-mediated depletion of treacle caused a 47% drop in pre-rRNA, suggesting an inhibition in pre-rRNA transcription which was confirmed by RNase protection assays, ^{32}P-metabolic labeling, and BrUTP incorporation (Valdez et al. 2004). Later Gonzales et al. (2005b) used ChIP analysis to show that human treacle binds rDNA within nucleotides −240 to +370, a region that contains the proximal promoter and the 5′ end of the rDNA gene encoding the 5′ ETS of the pre-rRNA.

Lin and Yeh (2009) refined these treacle interactions to −321 to −22 in the HeLa cell rDNA promoter region, and then attributed this interaction to the carboxy-terminal region (residues 1294–1488) of treacle. They showed that siRNA-mediated depletion of treacle redistributed Pol I, UBF, and Nopp140 from nucleoli, even though their overall abundance did not change as assayed by immunoblots. Lin and Yeh (2009) further showed that the central repeat domain of treacle interacts with the Pol I complex in a robust manner but that the carboxy terminus of treacle binds UBF, Nopp140, and rDNA, the last one either directly or indirectly via UBF or Nopp140. Over-expression of this carboxy terminus behaved as a dominant negative in that it caused the redistribution of Pol I, UBF, and Nopp140, resulting in a decline in rDNA transcription as determined by BrU labeling. This dominant-negative behavior of treacle's carboxy terminal domain is reminiscent of the over-expression of NoppC as described above for Nopp140. Lin and Yeh (2009) concluded that central repeat domain of treacle interacts with the Pol I complex to maintain the transcription machinery in the nucleolus, and that the carboxy terminus of treacle is responsible for interacting with the rDNA promoter to help recruit UBF.

The model emerging from the combined observations (Valdez et al. 2004; Gonzales et al. 2005b; Lin and Yeh 2009) indicates that treacle, like Nopp140, links pre-rRNA processing with rDNA transcription. A haplo-insufficiency in vertebrate treacle would therefore disrupt ribosome production at the transcriptional and pre-rRNA processing levels. A resulting loss of functional ribosomes could then stress particularly sensitive embryonic neural crest cells leading to their apoptosis and the loss of critical progenitor cells that normally give rise to adult craniofacial structures. This model explains treacle function in vertebrate cells, but many questions remain unanswered. For example, are there functional overlaps between Nopp140 and treacle in vertebrate cells in rDNA transcription and pre-rRNA processing? Or does Nopp140 perform a preferential function (e.g., the delivery of box H/ACA snoRNPs to the nucleolus) while treacle performs a chaperone function preferentially for box C/D snoRNPs? What replaces treacle function in metazoans that apparently lack treacle (e.g., *Drosophila* and *C. elegans*)? With respect to TCS, why

is *Tcof1$^{+/-}$* haplo-insufficient in some genetic backgrounds but not in others? What are the genetic factors affecting *Tcof1* gene expression, and how do other genes and environmental factors affect TCS severity (i.e., the nucleolar stress that leads to p53 stabilization and apoptosis)? Questions like these will require diversified research approaches. For example, in a recent study, Dauwerse et al. (2011) examined patients who clearly displayed TCS phenotypes, but who did not have mutations in their *Tcof1* genes. Instead, these patients had mutations in their *POLR1D* or *POLR1C* genes that encode RPA40 and RPA16, respectively. RPA40 and RPA16 are shared subunits of RNA polymerases I and III, and are known to interact (Yao et al. 1996). Thus, TCS is a ribosomopathy (Dauwerse et al. 2011) caused by mutations in diverse genes whose protein products are required for ribosome biogenesis.

11.12 Other Related Nucleolar Proteins

The principal common feature between Nopp140 and treacle is the large central domain consisting of alternating acidic and basic domains. Other nucleolar proteins that share related domains include nucleolin (its yeast homolog is NSR1), B23, p100 in *Chironumus*, and NPI46 in yeast. Nucleolin and B23 are reviewed in Chaps. 9 and 10, respectively.

Sun et al. (2002) described a novel nucleolar phosphoprotein p100 in *Chironumus tentans*. It too has large central domain consisting of 12 alternating acidic and basic regions quite similar to those in mammalian Nopp140. Its predicted molecular weight is 63 kDa, but it too has an anomalous migration at 100 kDa on SDS gels. Immediately following the alternating acidic/basic domain is an RGG domain that is about half the size of that found in nucleolin. Interestingly, two C4-type zinc fingers follow the RGG domain in p100, and these domains are followed by a tryptophan-rich carboxy terminus. Because p100 maintains its nucleolar association after RNase treatment, Sun et al. (2002) suggest that p100 is a component of a putative proteinaceous nucleolar framework.

Finally, Shan et al. (1994) described NPI46 in *S. cerevisiae*. It is a non-essential nucleolar protein with a predicted mass of 46.5 kDa, but it migrates at about 70 kDa. Its central domain has three acidic domains reminiscent of those in Nopp140. A basic domain separates acidic domains 1 and 2, and a second basic domain follows the third acidic domain. The stretch of amino acids separating acidic domains 2 and 3 is not considered basic. The acidic domains contain a few CKII phosphorylation sites, but they are not as prevalent as in Nopp140 or treacle. Interestingly, the carboxy terminus (106 amino acids) of NPI46 is homologous to prolyl *cis-trans* isomerases that are classified by their ability to bind FK506, an immunosuppressant drug. Shan et al. (1994) showed that NPI46 has proline isomerase activity, and they suggest that NPI46 functions in ribosome biosynthesis perhaps to fold ribosomal proteins during the assembly process. We should keep in mind, however, that nonribosomal nucleolar proteins such as Nopp140, treacle, nucleolin, etc. have basic regions rich in proline, and prolyl isomerase activity could be invoked in the delivery and release of snoRNPs.

11.13 Future Explorations

Future work on Nopp140 will likely take us into regulatory pathways governing cell growth and cell stress responses (e.g., Ferreira-Cerca and Hurt 2009). For instance, a functional genomics assay identified the *Nopp140* gene in *Drosophila* as one of many genes necessary for ribosome biogenesis that are regulated by dTOR signaling (Guertin et al. 2006). Other *Drosophila* genes identified in the same assay were *Nop60b*, which encodes the H/ACA snoRNP pseudouridylase, and *CG3983* (now called *NS1*), which encodes nucleostemin. Nopp140 itself may be the target of multiple regulatory events as it shuttles from the cytoplasm back into the nucleus, on to CBs where it participates in snoRNP assembly/modification, then to nucleoli where it delivers these snoRNPs and provides a scaffold for their site-directed chemical modification of pre-rRNA, and finally to Pol I where it may regulate rDNA transcription. One can easily imagine differential phosphorylation of Nopp140 by CKII, PKC, PKA, and various phosphatases in response to cell proliferation or cell stress signals. The challenge before us is to determine what regulates post-translational modifications of Nopp140. Like Nopp140, differential phosphorylation of treacle could potentially regulate box C/D snoRNP function and Pol I transcription. The putative roles that Nopp140 and treacle may have in feedback regulation of Pol I transcription remain a fascinating avenue of future investigation. Can Nopp140 and treacle respond to perturbations in pre-rRNA processing or the loss of cytoplasmic ribosomes by regulating Pol I transcription? If so, would they act as transcription activators or repressors? Do Nopp140 and treacle participate in nucleolar stress response, which is only now coming into focus? Just as the nucleolus is considered "plurifunctional" (Pederson 1998), its constituent proteins are likely to be multifunctional as well.

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