Chapter 6 Discussion

In this thesis, I have characterised the phenotype of the loss-of-function *nol9*^{sa1022} zebrafish mutant and demonstrated that it exhibited defects in the development of the pancreas, liver and intestine. I further predicted that a loss-of-function mutation in *las1l*, a gene encoding a Nol9-interacting protein, would result in a similar phenotype to the *nol9*^{sa1022} mutant, which I confirmed upon morphological characterisation of the *las1l*^{sa674} zebrafish mutant. The mRNA expression profiles of *nol9*^{sa1022} and *las1l*^{sa674} mutant were then studied and compared to those of two other zebrafish rRNA processing mutants, *tti*^{s450} and *set*^{s453}, revealing shared genes and pathways that were differentially expressed in all four mutants. This chapter summarises the overall findings of the work and discusses the potential future directions for the project.

The purpose of my study was to determine the function of Nol9 in zebrafish pancreas development and would contribute to our expanding knowledge of ribosomal biogenesis mutants. In Chapter 3, I described the conserved function of Nol9 between human and zebrafish, observing that zebrafish Nol9 is also involved in the processing of the rRNA precursor molecules and formation of the large ribosomal subunit. The characterisation of the *nol9*^{sa1022} mutant showed that development of the exocrine pancreas, liver and intestine in these fish is impaired after 3 days post fertilisation (d.p.f.) whereas there were no other apparent morphological defects and the formation of the endocrine pancreas namely the pancreatic islets and the secondary islets was unaffected. In Chapter 4, I described morphological defects in the digestive organs and impaired exocrine pancreas development after 3 d.p.f. resulting from a disruptive mutation in the Nol9-interacting protein Las1l. Although a more detailed characterisation of the digestive organs of *las1l*^{sa674} mutant is required, the results strongly suggest a critical role for members of the Nol9-Las1l complex in the development of digestive organs in zebrafish. Previous studies have shown that Nol9 and Las1l are involved in processing of rRNAs of the large ribosomal subunit and are crucial for ribosome biogenesis (Castle et al., 2012; Castle et al., 2010; Heindl and Martinez, 2010). Our findings provide an additional link between functions in ribosomal biogenesis and digestive organ development (Boglev et al., 2013; Chen et al., 2005; Mayer and Fishman, 2003; Provost et al., 2012; Provost et al., 2013; Qin et al., 2014). Interestingly, we also found that the phenotype in *nol9*^{sa1022} mutants is milder, occurs later than *npo* and *def* mutants and does

not include jaw defects, in contrast to the *npo*, *def*, *tti* and *nom1* mutants. The possible reasons for an overall attenuated *nol9*^{sa1022} phenotype include a less critical role of *nol9* in ribosome biogenesis or a higher level of maternal mRNAs available. Further investigation into the protein activity and levels of the respective genes in each mutant and their effects on rRNA processing and ribosome biogenesis may provide greater insight into this area.

The tissue specificity of mutants of ribosomal biogenesis proteins and ribosomopathies is a puzzling phenomenon as these proteins are ubiquitously expressed and all cells and tissues require ribosomes for protein synthesis (Freed et al., 2010; McCann and Baserga, 2013; Narla and Ebert, 2010). The current hypothesis to explain the tissue-specific phenotype in zebrafish involves the exhaustion of maternally derived ribosomal biogenesis mRNA in tissues that are rapidly dividing and thus requiring large numbers of ribosomes. In Chapter 3, it was described that *nol9* is initially ubiquitously expressed and subsequently high level expression becomes restricted to highly proliferating organs including the pancreas, liver, intestine and head. This result agrees with the developmental expression pattern of previously published ribosomal biogenesis genes (Boglev et al., 2013; Chen et al., 2005; Mayer and Fishman, 2003; Provost et al., 2013; Qin et al., 2014). More research is needed to better understand the heightened sensitivity of digestive organs to mutations in ribosomal biogenesis genes. In particular, there are several hypotheses that can be explored; firstly, it is possible that a different spectrum of mRNAs is translated if the numbers of fully functional cytoplasmic ribosomes do not meet the cell's demand for protein synthesis. To test this possibility, the zebrafish ribosome biogenesis mutant lines could be crossed to a transgenic line e.g. (*Tg(XlEef1a1:GFP)*^{s854}) expressing GFP specifically in the digestive organs, sorting GFP+ and GFP- cells and subsequently studying their mRNA expression profiles (Ober et al., 2006; Stuckenholz et al., 2009). Secondly, Nol9 and other ribosomal biogenesis proteins can have tissue-specific interacting proteins and may have additional functions in digestive organs.

The mRNA expression profiles of *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants were analysed and described in Chapter 5. The findings from this study are subject to two major limitations: firstly, whole zebrafish larvae were used for gene expression profiling potentially making it more difficult to detect tissue-specific changes and secondly, mRNA expression analysis was carried out on 5 d.p.f. larvae, at least one day after the impaired development of digestive organs first occurred. Despite these potential shortcomings, the study has found that

genes involved in cell cycle regulation were significantly upregulated in *nol9*^{sa1022} mutants, which is corroborated by the results from the cell cycle analysis that showed an increased number of cells at the G1 phase in these mutants and impaired cell proliferation of *ptf1a*-expressing cells in the exocrine pancreas. Altogether, these findings suggest that impaired cell proliferation contributes to the developmental defects of organs observed in *nol9*^{sa1022} mutants. An increase in cell death was not observed in *nol9*^{sa1022} mutants but functional analysis of the mRNA expression profiles of *nol9*^{sa1022} mutants revealed an upregulation of cell death genes. Since autophagy has been found to upregulated in the intestinal epithelium in *titania* and in red blood cells from *rps7*-deficient zebrafish embryos and DBA and SBDS patients, we surmise that autophagy also operates in *nol9*^{sa1022} mutants (Boglev et al., 2013; Heijnen et al., 2014). The study of the relationship between *nol9* function and autophagy will lead to a better understanding of autophagy as a cell survival mechanism that leads to defects in the development of digestive organs in *nol9*^{sa1022} mutants.

Another finding from the analysis of mRNA expression profiles in Chapter 5 was that genes belonging to the Tp53 signalling pathway are upregulated in *nol9*^{sa1022} mutants. This indicated that a Tp53-dependent mechanism could contribute to the developmental defects in the digestive system of *nol9*^{sa1022} mutants. This is consistent with the involvement of Tp53 in mediating clinical features of ribosomopathies in human, including the craniofacial dysmorphology in Treacher Collins syndrome (Jones et al., 2008), some of the red cell abnormalities in Diamond Blackfan anaemia (Boultwood et al., 2012), the macrocytic anaemia in 5q^{-} syndrome (Barlow et al., 2010) and the hepatobiliary dysfunction in North American Indian Childhood Cirrhosis (Wilkins et al., 2013). However, our study had shown that genetic loss of Tp53 did not rescue the small pancreas phenotype of *nol9*^{sa1022} mutants, confirming previous findings in some ribosome biogenesis zebrafish models and providing additional evidence for the involvement of Tp53-independent mechanisms in failed expansion of zebrafish exocrine pancreas following impairments in ribosome biogenesis (Boglev et al., 2013; Provost et al., 2012; Provost et al., 2013; Qin et al., 2014). Tp53-independent mechanisms have yet to be described in ribosomopathies. It is probable that the same Tp53-independent mechanism that is seen in the zebrafish rRNA processing mutants, also contributes to the exocrine pancreas defects in Shwachman-Bodian Diamond syndrome (SBDS) (Provost et al., 2012) and some of haematological defects in Diamond Blackfan anemia (Danilova et al., 2011; Jia et al., 2013; Song et al., 2014; Taylor et al., 2012). Future studies should concentrate on elucidating these Tp53-independent nucleolar stress pathways

as they will enhance our understanding of the pathogenic mechanisms of ribosomopathies. In addition such insights may influence the development of chemotherapeutic therapies since it is estimated that TP53 function is disrupted/lost in more than 50% of all human tumours (Soussi et al., 2000). The commonly used anti-cancer treatments induce cellular stress that activates TP53-mediated cell cycle arrest and/or death, and tumours with defective TP53 are less sensitive to those drug treatments than tumours with functioning TP53 (Harris, 1996). Studies on TP53-independent mechanisms could potentially identify new targets for chemotherapeutic applications.

The mammalian target of rapamycin (mTOR) complex can regulate ribosome biogenesis by promoting rDNA transcription and playing a role in rRNA processing (Iadevaia et al., 2012). Additionally it increases the preference of ribosome in translating terminal oligopyrimidine (5'-TOP) mRNAs including ribosomal biogenesis genes through phosphorylation of S6 kinase (S6K) 1, which in turns phosphorylates RPS6 (Jefferies et al., 1997; Mayer and Grummt, 2006; Reiter et al., 2004). Recently, Heijnen *et al*. reported a TOR-dependent increase in phosphorylation of S6K in human cells with RP loss and in RP-deficient zebrafish, a response that is triggered by reactive oxygen species (ROS) (Heijnen et al., 2014). Conversely, Boglev *et al.* found that autophagy induction in *tti*^{s450} is independent of mTOR (Boglev et al., 2013). Future directions for the project will include studying whether ROS and the mTOR pathway contribute to the exocrine pancreas defects in *nol9*^{sa1022} mutants.

The mRNA expression profiling revealed an enrichment of ribosome- and translation-related functions amongst genes that were significantly upregulated in *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants, a result previously observed following knockdown of *sbds* and in *nom1* mutants (Provost et al., 2012; Qin et al., 2014). These findings further support the idea that a compensatory mechanism operates in cells following disruptions in ribosome biogenesis whereby cells attempt to increase the expression of genes involved in transcription and translation in order to fulfil the demands of rapidly proliferating tissues for protein synthesis (Provost et al., 2012). Further research is needed to determine the nature of this compensatory mechanism, more specifically how it is triggered, its effects on tissues and whether it is restricted to affected organs. Interestingly, this study revealed that genes functioning in haematopoiesis were differentially expressed in *nol9*^{sa1022} and *las1l*^{sa674} mutants. To our knowledge a role for an rRNA processing gene in haematopoiesis has not yet been described in zebrafish. A detailed examination of haematopoiesis in *nol9*^{sa1022} and

las1l^{sa674} mutants is currently being undertaken and can potentially provide insight into the process of haematopoiesis and the mechanisms operating in red blood cell defects in Diamond Blackfan anemia and 5q^{-} syndrome and neutropenia in Shwachman-Bodian-Diamond syndrome. In addition, it was found that the expression of the eye-specific gene *galectin related inter-fiber protein* is significantly downregulated in *nol9*^{sa1022}, *las1l*^{sa674}, *tti*^{s450} and *set*^{s453} mutants, although the gross morphology of the eye of *nol9*^{sa1022} and *las1l*^{sa674} mutants appeared normal. Further work needs to be done to establish whether the eye development of *nol9*^{sa1022} and *las1l*^{sa674} mutants is impaired.

Understanding the effects of aberrant ribosome biogenesis in an *in vivo* model is important to deciphering the underlying mechanisms of ribosomopathies and developing therapeutic treatments for these disorders. These studies may also benefit cancer research where there is a great deal of interest in developing therapeutics that target ribosome biogenesis in malignant cells (Drygin et al., 2010). An extensive knowledge of the molecular mechanisms following impaired ribosome biogenesis is required in order to safely modify them since targeting ribosome biogenesis could have devastating consequences and potentially result in changes in DNA regulation and/or mRNA translation patterns, eventually leading to an increased cancer risk (Holmberg Olausson, 2012).

This thesis has demonstrated that *nol9* and *las1l* are essential for normal exocrine pancreas development in zebrafish. Through this discovery, this work has provided a novel link between ribosome biogenesis and pancreas development, and helped to place the importance of rRNA processing proteins in a tissue-specific context. Further study of other Nol9 interacting proteins may help strengthen this link. The continued research on *nol9*^{sa1022} and *las1l*^{sa674} mutants will not only contribute to our knowledge of pancreas development and ribosomopathies but also contribute to the field of cancer and regeneration.