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# Appendix

English Summary

Nederlandse Samenvatting

Riassunto Italiano

Curricula Vitae  
&  
List of Publications

Abbreviations

## Summary

This thesis describes the use of zebrafish to study Noonan- (NS) and LEOPARD syndromes (LS). In the first three chapters, we provide a background for the following six chapters. In **Chapter 1**, we briefly discuss zebrafish gastrulation and the associated signaling mechanisms. The use of zebrafish to study protein tyrosine phosphatases (PTP) is discussed in **Chapter 2**. A brief overview of previous studies on PTPs using zebrafish is given and genetic tools available in zebrafish are discussed. As our lab is interested in gastrulation, we further outline the tools we use to study zebrafish gastrulation. Moreover, phosphoproteomics is discussed as a method to study PTPs *in vivo*. **Chapter 3** describes the role of *PTPN11* (protein-tyrosine phosphatase, non-receptor type 11), the gene encoding SHP2, a cytoplasmic PTP that is essential for vertebrate development. Mutations in *PTPN11* are associated with NS and LS, two autosomal dominant disorders with overlapping symptoms. Intriguingly, while NS mutations result in a more 'active' state of Shp2, LS mutations give rise to a PTP defective protein. NS and LS patients display various symptoms, including short stature, craniofacial defects and heart abnormalities. Interestingly, the cardiac phenotype of NS and LS patients is quite distinct; in this sense NS patients present pulmonary stenosis (PS) while hypertrophic cardiomyopathy (HCM) is the most common cardiac defect present in LS.

In **Chapter 4** we have used the zebrafish as a model to investigate the role of Shp2 in embryonic development. We characterized in details the role of the two *ptpn11* zebrafish isoforms (*ptpn11a* and *ptpn11b*). We show that *ptpn11a* is expressed constitutively and *ptpn11b* expression is strongly upregulated during development. In addition, the products of both *ptpn11* genes, Shp2a and Shp2b, are functional. Target-selected inactivation of *ptpn11a* and *ptpn11b* revealed that double homozygous mutants are embryonic lethal at 5-6 days post fertilization (dpf). *Ptpn11a*<sup>-/-</sup>*ptpn11b*<sup>-/-</sup> embryos showed pleiotropic defects from 4 dpf onwards, including reduced body axis extension and craniofacial defects, which was accompanied by low levels of phosphorylated Erk at 5 dpf. Interestingly, defects in homozygous *ptpn11a*<sup>-/-</sup> mutants overlapped with defects in the double mutants albeit they were milder, whereas *ptpn11b*<sup>-/-</sup> single mutants did not show detectable developmental defects and were viable and fertile. *Ptpn11a*<sup>-/-</sup>*ptpn11b*<sup>-/-</sup> mutants were rescued by expression of exogenous *ptpn11a* and *ptpn11b* alike, indicating functional redundancy of Shp2a and Shp2b.

Using phosphoproteomics, in **Chapter 5** we describe the identification of Fer kinase as a potential downstream protein in the etiology of both NS and LS. Phosphotyrosine immunoprecipitation of lysates from zebrafish injected with wild type, NS or LS Shp2 followed by mass spectrometry showed a decrease in a phosphopeptide corresponding to the tyrosine kinase Fer in both NS and LS. We showed that *fer* is expressed in early embryos and that loss of Fer results in developmental defects, including convergence and extension defects. Partial loss of Fer cooperated with low expression of NS and LS variants of Shp2 but not with wild type Shp2 expression. Thus, the loss of Fer contributed to NS and LS phenotypes in zebrafish.

In **Chapter 6** a similar approach is used in NS mouse hearts to identify Protein zero related (Pzr) as the most hyper phosphorylated protein in NS. Loss of Pzr leads to gastrulation defects in zebrafish and phenotypes reminiscent of loss of Shp2, NS and LS. Pzr is hyper phosphorylated at its immunoreceptor tyrosine inhibitory motifs (ITIMs) in multiple tissues and is shown to be dependent on Src activation. Co-immunoprecipitation studies show that NS and LS Shp2 exhibit enhanced binding to Src and PZR, consisting of a complex. Moreover, the ITIMs, binding sites for

Shp2 are essential for induction of the zebrafish convergence and extension phenotype. Thus, NS and LS Shp2 form a complex with Src and Pzr to induce developmental defects in zebrafish.

In **Chapter 7** we investigated the role of the most common NS and LS Shp2 mutations in zebrafish in cardiac development. Defective heart development is a prominent symptom of both NS and LS, but how the Shp2 variants affect cardiac development is unclear. We showed that the heart function was impaired in embryos expressing NS and LS variants of Shp2. The cardiac anomalies consisted of reduced cardiomyocyte migration, coupled with impaired leftward heart displacement. Expression of specific laterality markers was randomized in embryos expressing NS and LS variants of Shp2. Ciliogenesis and cilia function in Kupffer's vesicle was impaired, likely accounting for the left/right asymmetry defects. Mitogen activated protein kinase (Mapk) signaling was activated in embryos expressing NS and LS Shp2-variants. Interestingly, inhibition of Mapk signaling prior to gastrulation rescued cilia length and heart laterality defects suggesting that NS and LS Shp2-variant mediated hyperactivation of Mapk signaling leads to impaired cilia function in Kupffer's vesicle, causing the heart impairment at later stages.

Finally we investigated the role of Alpha-2-Macroglobulin-Like-1 (A2ML1) in NS in **Chapter 8**. To date, all mutations known to cause NS are dominant and they enhance the RAS/MAPK signaling pathway. However in 25% of cases, the genetic cause of NS remains unknown, suggesting that factors other than those involved in the canonical RAS/MAPK pathway may also play a role. In our study, we used family-based whole exome sequencing of a case-parent trio and identified a *de novo* mutation, p.(Arg802His), in *A2ML1* which encodes the secreted protease inhibitor Alpha-2-Macroglobulin-Like-1. Subsequent resequencing of *A2ML1* in 155 cases with a clinical diagnosis of NS led to the identification of additional mutations in two families, p.(Arg802Leu) and p.(Arg592Leu). Functional characterization of these human *A2ML1* mutations in zebrafish showed NS-like developmental defects, including a craniofacial defects and cardiac malformations. The crystal structure of A2M, which is highly homologous to A2ML1, led us to the identification of the intramolecular interaction partner of p.Arg802. Mutation of this residue, p.Glu906, induced similar developmental defects in zebrafish, strengthening our conclusion that mutations in *A2ML1* cause a disorder clinically related to NS. We showed for the first time, the involvement of an extracellular factor in a disorder clinically related to RASopathies, providing potential new leads for better understanding of the molecular basis of this family of developmental diseases.

In **Chapter 9**, we discuss each previous chapter separately and provide an integrated view on the findings of this thesis.