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**Title:** Functional characterization of protein-tyrosine phosphatases in zebrafish development using image analysis  
**Date:** 2012-09-12
Summarizing Discussion
During gastrulation the zebrafish morphology undergoes a drastic change from an ‘inverted cup’-shape to a narrow and long body frame. This impressive remodeling is due in great part to the evolutionary conserved CE movements, which are crucial for proper organ anlage positioning and as a consequence are essential for normal development. The cells collectively polarize, i.e. elongate and orient their longest axis in mediolateral direction, and migrate towards the dorsal midline, where cell intercalation occurs. Many genes have been identified regulating CE movements, although the exact mechanism remains unclear. However, mutants and morphant phenotypes have shown that a loss of the cell polarization is a key factor underlying the resulting, characteristic, shorter and broader embryo phenotypes.

Previously, we have identified all members of the family of classical PTPs in the zebrafish genome and provided a detailed analysis of the spatio-temporal expression patterns of these 48 PTPs during early embryonic development. In this thesis, we focus on the role of PTPs in gastrulation convergence and extension (CE) cell movements.

Functional screening of the family of classical PTPs using MOs in the zebrafish system

Using the remarkably efficient morpholino micro-injection knockdown techniques in zebrafish, we investigated the function of four zebrafish PTP genes (ptpra, ptprea/b, ptpn13, and ptpn20) in zebrafish CE cell movements. In Chapter 2 it is shown that ptpra, ptprea and ptpreb not only play a role in the polarization which underlies the gastrulation morphogenetic movements, we also speculate that they operate in a pathway which regulates cell polarity through Src family kinases Fyn and Yes, and RhoA in parallel to the non-canonical Wnt pathway. Both ptpra and ptprea/b morphants and ptpra -/- mutants show the characteristic shorter phenotypes apparent at 48 hpf (quantified by tailength) and loss of cell polarity associated with CE defects. It was shown that the knockdown could be alleviated by coinjection with constitutively active Fyn and Yes and RhoA, a well-known downstream effector of non-canonical Wnt signaling. Co-injection of mRNA of ptpra or ptpre with Wnt11 MO or vice versa did not result in alleviation of the phenotype, indicating a parallel pathway to the non-canonical Wnt signaling leading to RhoA activation.

An automated cell tracing algorithm was developed to determine the migration speeds of individual cells and was used to demonstrate a significant difference between the average convergence and extension rate of the gastrulation cell movements of ptpra -/- homozygous mutant embryos and wildtype (wt) siblings. For the determination of cell polarization of the presomitic mesoderm, a method was devised to more efficiently quantify the shapes of cells within a tissue
based on confocal images. In contrast to the conventional method, which relies on manual annotation of the cell outline, this method applies user determined image processing steps to filter out the outline of the cells in the images (an automated algorithm for this has been developed and presented in Chapter 5).

In chapter 3, we identified PTP-BL and Ptpn20 as structural paralogues and determined both to be critical to CE cell movements as components of a system regulating cell polarity in concert with RPTPα and RPTPε. We demonstrate that full knockdown of PTP-BL and Ptpn20 results in CE defects reminiscent of the phenotypes resulting from RPTPα and RPTPε knockdown. While knockdowns of RPTPα and RPTPε were rescued by co-injection of activated RhoA, knockdowns of PTP-BL and Ptpn20 were rescued by co-injection of dominant negative RhoA. Next, using co-injection of suboptimal amounts of MOs, genetic interactions were established between ngef (identified as a Rho-GEF) and ptpra/ptpre and between ahrgap29 (identified as a Rho-GAP) and ptpn13 and ptpn20. Our data shows that PTP-BL, Ptpn20, RPTPα and RPTPε function in pairs, where low dose co-knockdown of ptpra and ptpn13 resulted in a severe phenotype and co-knockdown of ptpra and ptpn20 did not. Similarly, ptpre and ptpn20, but not ptpn13 resulted in synergistic effects. We hypothesize that asymmetric distribution of RhoA-GTP and RhoA-GDP over the leading and trailing edge of the cell is required for proper polarity and migration. We propose that RPTPα and RPTPε act on one side of the cell as activators of RhoA, while PTP-BL and Ptpn20 exert their function on the opposite side as inhibitors. Full knockdown of either one of the components will reduce RhoA-GTP/RhoA-GDP levels to such an extent that cell polarity will be lost. Low-dose knockdown of the single components only mildly reduces RhoA-GTP/RhoA-GDP levels, but does not result in loss of polarity because of normal signaling on the opposing side. Combining low-dose knockdown on the other hand, leads to reduced activation of RhoA on one side and reduced inhibition of RhoA on the other side, resulting in loss of polarity, and as a consequence CE defects. It remains to be determined which mechanism of differential upstream activation of PTPs, subcellular localization, substrate specificity or cell type specific expression may underly the specificity of the combined knockdowns.

In chapter 6, we performed a screen of the family of classical PTPs to identify genes with a role in gastrulation cell movements in zebrafish. To our knowledge, this is the first time a MO-screen has been performed on an entire family of proteins. We designed 2 independent splice site morpholinos for each of the 48 genes, encoding PTPs. In the first round all embryos were analyzed morphologically, by quantifying the taillength at 72hpf, as a measurement for body axis extension, a hallmark of CE defects. Positive hits were identified based on both MOs causing similar phenotypes. This resulted in the identification of four candidate genes with a potential role in CE. These positive hits were analyzed in detail for CE defects in the second round by in situ hybridization using well-established
markers for CE cell movements. The tools described in Chapter 5 were used to assess defects in cell polarization of the presomitotic mesoderm. Based on the comparative analysis of the results with four positive controls, i.e. PTPs with a known role in CE cell movements (ptpra, ptprea/b, ptpn11, ptprua), one additional PTP, ptprda, was identified as essential for normal gastrulation cell movements.

The relative ease of combining MOs by co-injection allows for an efficient method for simultaneous knockdown of multiple genes and has proven to be a valuable technique in identifying genes operating in the same signaling pathway and identifying redundancy between ohnologs (chapter 2 and 3). One obvious setback in their use for screening for CE phenotypes is the fact that the phenotype, which is considered positive in the morphological analysis, i.e. a shorter body axis, is also a known non-specific morpholino induced artefact, making the use of controls for morpholino specificity warranted [1].

Identification and characterization of NRas-I24N, a novel Noonan-associated mutation

Noonan syndrome is a relatively common dominantly inherited genetic developmental disorder that is characterized by reduced growth, orbital hypertelorism, and congenital heart defects. It is classified as a RASopathy, a group of genetic disorders in which the underlying cause is associated with dysregulation of the Ras-mitogen-activated-protein-kinase (RAS-MAPK) signaling pathway. ~50% of Noonan patients present with activating mutations in PTPN11, a gene encoding SHP2, a PTP which regulates signaling upstream in the RAS-MAPK pathway [2]. Other mutations associated with Noonan syndrome are also found in this pathway, namely SOS1 [3, 4], KRAS [5, 6], SHOC2[7], RAF1 and BRAF [3, 5, 8]. Earlier work has also shown that Noonan associated mutations in Shp2, the zebrafish ortholog of SHP2 induce gastrulation defects in zebrafish [9].

Recently two mutations in NRAS were reported to be associated with Noonan syndrome, T50I and G60E [10]. In chapter 4, we report a mutation in NRAS, resulting in an I24N amino acid substitution, that we identified in individual bearing typical Noonan syndrome features. The I24N mutation activates N-Ras, resulting in enhanced downstream signaling. This is the first report of an I24 mutation in any RAS isoform. The surrounding region of the mutation, which is localized between the P-loop, involved in nucleotide binding, and Switch I, involved in effector binding, is highly conserved between N-RAS, K-RAS, and H-RAS. This together with the fact that mutations in Q22 have been found in patients diagnosed with Noonan (Q22R) and other Rasopathies (Q22E and Q22K) [11, 12], suggests a regulatory role for this region of which the mechanism remains to be elucidated.

Expression of N-Ras-I24N, N-Ras G60E or the strongly activating mutant
N-Ras G12V, which we included as a positive control, resulted in the developmental defects in zebrafish embryos, demonstrating for the first time that these activating N-Ras mutants are sufficient to induce developmental disorders that resemble the defects induced by active mutants of downstream factors, B-RAF, RAF-1 and MEK, from Noonan and related syndromes [13]. These defects in zebrafish embryos are reminiscent of symptoms in individuals with Noonan syndrome. We were able to show that these symptoms appear to be a result of defective cell polarization and resulting aberrant gastrulation CE cell movements. MEK inhibition, using the small molecular inhibitor CI-1040, completely rescued the activated N-Ras-induced phenotypes, demonstrating that these defects are mediated exclusively by Ras-MAPK signaling. This finding also raises the possibility of using pharmacological inhibitors in the treatment of RASopathies or other developmental disorders caused by overactivation of the RAS-MAPK pathway.

In conclusion, mutations in NRAS from individuals with Noonan syndrome activated N-Ras signaling and induced developmental defects in zebrafish embryos, indicating that activating mutations in NRAS cause Noonan syndrome.

*Image processing and automated algorithms*

Quantitative analysis and clear representation of the data is essential to modern day biology. Studying collective cell migrations, for instance during gastrulation CE cell movements requires the analysis of the net effect of cell polarization and/or displacements of a large number of cells within a tissue rather than measurements of a few individuals [14].

For analyzing CE defects in zebrafish, quantifying the coordinated orientation, or randomization evidently requires the analysis of quite a number of cells in order to obtain reliable data. The annotation of the cell membranes required for analysis of cell polarization is usually carried out manually, making it time consuming, tedious, and subjective. In *Chapter 5* we describe the development of an automated algorithm Cell Outliner for the detection of cell membranes, to facilitate data collection for polarization analysis. The method was proven to be effective using different fluorescent markers and on data sets both from zebrafish and mice. Additionally, a novel method of graphical data representation is introduced called Cell Roses, which adds the advantage of showing the correlation between both defining quantitative parameters of cell polarization, cell elongation and medio-lateral orientation, by plotting them on one comprehensive graph rather than separate ones. As a proof-of-principle this method was used to analyze the presomitic mesoderm of *ptpra/-* mutant zebrafish, a well-described CE phenotype. The Cell Roses clearly showed the concomitant loss of elongation and mediolateral orientation of the cells in the mutant tissues in comparison to wt siblings.
Several automated image processing algorithms were also developed for the detection and tracking of nucleus center positions, the latest version named Sugar Rush (available upon request). In Chapter 2 automated cell tracing is used to determine and compare the average extension and convergence speeds of zebrafish \textit{ptpra/-} to wt sibling samples. While multiple programs are available for standard object tracing tasks, none of the available (online or in house) programs were able to trace efficiently or accurately, the large numbers of cells needed for estimating these average migrating speeds, when applied on a standard laptop computer (in this instance defined as a Apple® MacBook Pro 2.7 GHz Intel® core i7 early-2011). Sugar Rush proved to be a very efficient and accurate cell tracer.

The most critical aspect in developing automated algorithms for image analysis is efficient segmentation, the separation of foreground objects from the background, i.e. detection. Typically, confocal images contain fluctuations (biological noise) in signal intensity and occasionally contain intensity gradients on top of mechanical shot noise, inherently linked to the physical principle used by confocal microscopes. The efficiency of both Sugar Rush and Cell Outliner lies in series of simple non-computationally-intense image processing steps used for segmentation, designed to efficiently eliminate the noise (biological or mechanical) in the image. As a result the original image is converted to a binary version containing white objects on a black background. This format immensely facilitates computational analysis of the objects, whether they need to be traced in a time series or scored for shape parameters.

The necessity of determining and scoring cell parameters such as collective migration speed or cell polarity is not restricted to the study of gastrulation. Angiogenesis [17], the establishment of the highly organized structures of auditory and vestibular epitheilia [18], neurogenesis [19], metastatic processes [20], wound healing [15, 16] and numerous more examples can be found in which the determination of tissue migration and cell polarization is critical in a quantitative analysis. The advent more sophisticated imaging technology, and the continuous development of more sensitive and specific fluorescent probes, allows the visualization of biological processes in unprecedented detail. Data analysis can be greatly facilitated by the use of algorithms like Cell Outliner and Sugar Rush.

Conclusion

In this thesis 6 PTP genes (\textit{ptpra, ptprea, ptpreb, ptpn13, ptpn20}, and \textit{ptprda}) were identified as having a role in zebrafish CE cell movements. Five of these (\textit{ptpra, ptprea, ptpreb, ptpn13, ptpn20}) have been further characterized and shown to function within signaling pathways which converge on downstream effectors of the non-canonical Wnt pathway, a pathway well-known to regulate CE cell movements, like RhoA. Additionally, for 2 PTPs (\textit{ptpn11} and \textit{ptprua}), which
were already indicated to have a role in CE cell movements, direct evidence was
generated for their function in cell polarization. It will be interesting to investigate
whether and, if so, how ptprda interacts with the non-canonical Wnt pathway. Ad-
ditionally, it will also be interesting to investigate crosstalk among the PTPs that
have been identified to play a role in CE cell movements. Further, a novel Noonan-
associated mutation was reported and shown to hyperactivate FGFR-RAS-MAPK
signaling, causing developmental defects. Finally, new tools were generated to
quantify gastrulation cell movements and cell polarization which also could be
useful outside of the domain of zebrafish gastrulation.

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