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# Identifying the targets and understanding the role of Hmx1

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During embryonic development, the neuroectoderm forms the neural retina, the pigmented epithelium and the optic stalk. H6 family homeobox 1 (HMX1) belonging to the omeobox (HMX) family of transcription factors, is widely expressed in the eye, peripheral ganglia and branchial arches. In Zebrafish eye formation, Hmx1 expression is gradually restricted to the nasal part of the ganglion cell layer. A mutation in HMX1, is linked to a subset of ocular defected termed Oculo-auricular syndrome of Schorderet-Munier-Franceschetti (OAS) (MIM#612109). HMX1 belonging to a larger homeobox (HMX) family of transcription factors containing HMX2, HMX3 and sensory organ homeobox 1 (SOHo1) genes presents a characteristic 60-amino acid homeobox domain, philogenetically conserved. Two other conserved domains are called SD1 and SD2.

Despite the advancement of our knowledge on HMX1, its contribution to the eye formation and generally to craniofacial development remains unclear. We therefore aimed at identifying the potential target genes and the biological processes in which they are involved. Our predictive promoter model (PPM) able to recognize a framework of two or more canonical CAAGTG transcription factor binding sites (TFBs) identified Ubiquitin-like Containing PHD and RING Finger Domains 1 (UHRF1) as candidate gene common to mouse and human genomes. UHRF1 acts as an epigenetic coordinator between DNA methylation and histone and together with partner proteins, they actively regulate chromatin modifications and cellular proliferation. We questioned whether in vitro HMX1 would affect the human UHRF1 promoter activity: a luciferase reporter was placed under the control of the identified UHRF1 promoter region and the interaction between HMX1 and UHRF1 was analyzed. Wildtype (WT) HMX1 significantly inhibits UHRF1 promoter activity, while deleted HD, SD1 and SD2 regions, exhibit no effects on UHRF1 promoter.

Then we investigated the potential activity exerted in vivo by Hmx1 on Uhrf1 in a Hsp70-hmx1 transgenic line which was previously generated by cloning the human Hmx1 coding sequence downstream of the hsp70 promoter. In response to the overexpression of Hmx1 we observed reduced expression of Uhrf1 at 1 dpf and increased expression at 5 dpf.

Uhrf1 with the partner Dnmt1 are implicated in regulating lens development as well as regulating DNA methylation process. Investigating the link between HMX1 and the partner genes UHRF1 may allow us to better characterize the OA-SMF phenotype.

# Expression of wilm's tumor genes in zebrafish cardiomyocytes during development causes atrial hyperplasia and fibrosis

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Wilm's tumor 1 is an oncogene often linked with pediatric kidney cancers. In the zebrafish two orthologues for this gene have been described: wt1a and wt1b. Both genes are associated with kidney and heart development. In the heart both orthologues are transiently expressed during embryogenesis in the epicardium. wt1b has also been shown to be expressed in few cardiomyocytes during early developmental stages. Here we demonstrate that overexpression of wt1a and wt1b in cardiomyocytes during embryogenesis results in atrial dilation and hyperplasia. We postulate that wilm's tumor 1 genes are necessary for heart development and that the expression levels of these genes in cardiomyocytes need to be tightly controlled for proper heart formation.

# Wilms' tumor 1b (wt1b) expression defines a pro-regenerative macrophage subtype and is required for organ regeneration in the zebrafish

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Organ regeneration is preceded by an inflammatory phase characterized by the recruitment of innate immune cells, which play an active role during repair and regrowth. Here we studied macrophage subtypes during organ regeneration in the zebrafish, an animal model with high regenerative capacity. We identified a novel macrophage subpopulation expressing wilms tumor 1b (wt1b), which preferentially accumulates within regenerating tissues. This wt1b-positive macrophage population exhibited an overall pro-regenerative gene expression profile and different migratory behavior compared to the remainder of macrophages labeled by mpeg1 expression. Functional inhibition of Wt1b in mpeg1+ cells increased their motility, showing that wt1b regulates migration to the site of injury. Further, homozygous wt1b mutant zebrafish presented a reduced number of myeloid lineage cells in the hematopoietic niche, suggesting impaired macrophage differentiation. In the context of regeneration, wt1b mutants showed reduced fin growth upon caudal fin amputation and cardiomyocyte proliferation following cardiac injury. This is the first description of a pro-regenerative macrophage subtype in the zebrafish and supports a new role for wt1b in organ regeneration.

# Can successive cryoinjuries exhaust the regenerative capacity of the zebrafish heart ?

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Mammals fail to regenerate their hearts after myocardial infarction. On the contrary, adult zebrafish can regenerate their damaged myocardium within 30-60 days post-cryoinjury. It remains unknown whether this regenerative capacity is efficient after multiple successive injuries. In this study, we assessed cardiac regeneration of adult zebrafish after a series of cryoinjuries interspaced by 30 days of recovery. First, we used transgenic cell-lineage tracing analysis to demonstrate that the second cryoinjury targets the regenerated tissue after the first cryoinjury. Then, we performed series of 2, 3 and 6 successive cryoinjures and analyzed the proportion of regenerating myocardium and connective tissue in the ventricles using histological and immunofluorescence staining. In all cases, the cortical layer of the myocardium was thickened as compared to the intact ventricular wall. We showed that after 2 and 3 successive cryoinjuries, 50% of zebrafish were capable of scar-free regeneration, whereas the remaining fish displayed remnants of fibrotic tissue even after 60 days. These results were similar to those obtained after a single cryoinjury. Consistently, by assessing cardiomyocyte proliferation and embryonic myosin expression during the regenerative process, we found that successive injuries did not markedly impair the myocardial activation. By contrast, after 6 successive cryoinjuries, no fish were able to perfectly regenerate their heart and displayed persisting collagenous tissue. In conclusion, our results show that the ability to resolve the scar and regenerate a new myocardium is fairly robust, but could decrease with many multiple cryoinjuries, suggesting a limit to cardiac regeneration.

# Induction of heart tumor in juvenile zebrafish

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The vertebrate heart is rarely associated with cancer. Here, we assessed whether a heart tumor model can be established in the zebrafish. We took advantage of this genetic model to investigate the effects caused by overexpression of an oncogenic HRasG12V mutation using the Gal4-UAS system. A previous study has revealed that a haematopoietic stem cell-specific promoter driver line, runx1:Gal4 combined with UAS:GFP-HRasG12V, resulted not only in blood phenotype, but also in heart overgrowth after 30 post-fertilization (dp= f). We identified that this phenotype can be due to the leaky expression of the runx1 promoter in a subset of cardiomyocytes located at the atrio-ventricular valve region. Consistently, we detected ectopic Ras-immunoreactivity in the overgrown heart of transgenic fish. To elucidate the mechanism und erlying this phenotype, we conducted histological staining and immunofluorescence analysis. At 28 dpf, the enlarged heart of larvae carrying the mutated HRasG12V comprised a misshaped ventricle with densely packed disorganized myofibrils and a decreased lumen, compared to control. Furthermore, visualization of cell cycle markers indicated a higher rate of cardiomyocyte proliferation. The embryonic cardiac myosin heavy chain protein, which is normally expressed only during the first two weeks of development, was still present in the heart at 28 dpf, suggesting a non-differentiated state of cardiac cells. These findings suggest a tumorigenic transformation of zebrafish cardiomyocytes caused by overexpression of HRasG12V in a subset of cardiomyocytes. To further examine the ability of zebrafish heart to develop cancer, we generated a new inducible Gal4-ERT transgenic line under the cardiac specific promoter cmlc2. Overexpression of UAS:GFP-HRasG12V using cmlc2:Gal4-ERT resulted in a formation of gigantic malformed heart, with highly proliferative undifferentiated cardiomyocytes. In conclusion, our study shows that the zebrafish heart can form cancer in response to HRasG12V overexpression, providing a unique model of cardiac tumorogenesis.

# Genetic diversity of zebrafish (Danio rerio, Hamilton, 1822) in Bangladesh using DNA barcoding and RAPD techniques

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Zebrafish is one of the most important model fish species used in the field of Biological Sciences. It is also broadly used as model species in aquaculture research. The study of genetic diversity of wild zebrafish (Danio rerio) by DNA barcoding and RAPD markers are important tools which will help in developing commercial fish stock for the sustainable aquaculture management. The present investigation was undertaken to study the morphometric variation and genetic diversity of the zebrafish collected from 4 distinct locations in Bangladesh (Mymensingh, Faridpur, Khulna and Munhigonj) and 12 of the samples were analyzed by RAPD marker and DNA barcoding techniques. Three random decamer primers were used for RAPD marker and two were used for DNA barcoding to amplify DNA fragments. 112 bands were scored by the primers, 22 were polymorphic. The percent polymorphic loci and gene diversity values varied within a range of 10.60–33.33 and 0.0645–1.2685 respectively. Nei's genetic similarity between populations across all the primers ranged from 0.2812 to 1.0000. The dendrogram based on Nei's genetic distance showed 11 clusters; the population of Faridpur was found to have maximum genetic diversity with 33.33% polymorphic loci and the Khulna population had a minimum with 10.60% polymorphic loci. Genetic diversity also found among the populations of Mymensingh and Munshigonj with 15.15% and 19.69% polymorphic loci. The clusters also revealed strong correlation with the species. For DNA barcoding, we amplified the cytochrome oxidase subunit 1(COI) barcode sequence (680 bp long) for 4 specimens, and we compared them with reference sequences from different databases (GenBank and BOLD). Though the database is undergoing continual development, it was able to provide species matches of > 99% sequence similarity for 4 samples tested. The overall sequence alignment similarity among our sampled specimens was 99.35%.This result provides baseline data for future work on population structure and genetic variation analysis of zebrafish.

# Phenotype severity is influenced by mutation type in SRP54 deficiency

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Whole exome sequencing analyses are increasingly performed on patients presenting with suspected inherited disease but lacking classical mutations linked to presented phenotypes. Using whole-exome sequencing in sbds-negative Shwachman-Diamond Syndrome (SDS) families, we recently identified three independent patients, each of whom carried a de novo missense variant in srp54 (encoding signal recognition particle 54 kDa). The SRP54 protein is a key component of the ribonucleoprotein complex that mediates the co-translation al targeting of secretory and membrane proteins to the endoplasmic reticulum (ER). Here, we use a novel Sanger zebrafish mutant to further confirm our previous findings, as well as injection approaches in zebrafish embryos to demonstrate that the mutation type influences phenotype severity. We show that homozygous srp54 mutant zebrafish die early during development. Heterozygous siblings, however, are viable and display neutropenia but no pancreas defect. In vivo injections of patient-derived mutant mRNAs of human SRP54 induce differential phenotypes resembling those seen in patients, and impair ATRA-driven morphologic differentiation and CD11b surface induction when expressed in HL-60 cells, an established in vitro model system of neutrophil induction. Taken together, we here describe a novel disease model of SDS that founds on SRP54 as molecular driver and impaired neutrophil differentiation as functional output.

# Alternative mechanism of angiogenesis during the zebrafish fin regeneration

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Angiogenesis, the formation of new blood vessels from pre-existing ones, is an essential process involved in many physiological and pathological processes. Beside two main types of angiogenesis, namely sprouting and intussusception (splitting), additional way called “vascular mimicry (VM)” has been described. By the latter, tumor cells transform and become endothelial cell contributing to the de novo formation of the microvasculature and tumor vascular supply. The process has been demonstrated in different cancer types such as melanoma, breast, kidney and ovary but has never been reported as a normal physiological process.

The aim of this project is to investigate whether VM appears as well in normal tissue employing zebrafish fin regenerative assay. After amputation, fin regeneration was monitored from day 1 to day 14 using in vivo light, confocal microscopy, electron and 3view microscopy, and different staining.

During the fin regeneration, different steps indicating vascular mimicry have been partially documented: (i) islands of extravasated erythrocytes are formed in front of the expanding vascular front; (ii) precursor cells surrounding the islands attenuate and adopted endothelial phenotype; (iii) so formed blood cysts are integrated in the microvasculature.

In conclusion, the first evidence supporting a “vascular mimicry” hypothesis in normal tissue have been collected. Additional work including zebrafish lines will be included to document the process of erythrocytes extravasation and integration of the vascular cysts to the circulation. In addition, the transition and differentiation of blastemal precursor cell to endothelial cells has to be exactly elucidated.

# Mitochondria in embryogenesis: an organellogenesis perspective

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Organogenesis is well characterized in vertebrates. However, the adaptation of intracellular compartments remains unknown. Last year, we presented the timing of mitochondria patterns of change in early stages of development but the dialogue between organogenesis and mitochondria still needs to be defined. Here, we will expand on the mechanistical point of view, particularly the identification of Shh signaling as a major conductor able to synchronize mitochondria modifications with tissue development. Further, we demonstrate the co-dependence between maturation of mitochondria and maturation of myofibers. Finally, we reveal that mitochondria quality represents a key factor to control embryonic progress. Our findings establish the subtle dialogue between organogenesis and mitochondria as an integral component of the proper management of early vertebrate development.

# Diet effects on growth, mortality, RNA:DNA ratio and gene expression of zebrafish

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A experiment was conducted to study the effects of some commercial and natural diets on growth, mortality, RNA:DNA ratio and gene expression of zebrafish. Total eight combinations of diets (T1: egg yolk, T2: egg yolk+ Artemia, T3: Horlicks, T4: Horlicks+ Artemia, T5: Tetra-Bits, T6: TetraBits+ Artemia, T7: NovoTom, T8: NovoTom+ + Artemia ) were fed to zebrafish larvae for a period of 45 days. Total 360 first feeding larvae were equally stocked in triplicate and fed them twice per day to a satiation point. Growth parameters data were analyzed using SPSS software and gene expressing data were analyzed using Relative Expression Software tools 2009 (REST). Significantly highest length (2.53±0.04 cm) was obtained in T2 followed by T6 (2.38±0.9 cm). Lowest length (1.85±0.08 cm) was obtained in T3 group fish. Similarly, significantly highest weight gain (0.25±0.02 g) was observed in T2 followed by T6 (0.20±0.03 g). Significantly lowest weight (0.14±.01g) was observed in T3 when compared with T2 and T6. But there was no significant weight difference between T1, T3,T4,T5, T7 and T8. Significantly lowest condition factor (1.40±0.07) was found in T8 group fish and highest (2.41±0.43) was recorded in T3 group fish. Highest RNA:DNA ratio was found (1.01) in T5 followed by T4 (0.95) and lowest was found in T1 (0.77). Similar result was observed for Growth hormone (GH) gene expression. Highest gene expression was found in T5 followed by T4 and lowest in T2. The result of the present work could provide significant contribution in the field of global zebrafish research.

# Using zebrafish to identify causative genes for sexual development in the 16p11.2 locus

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The 16p11.2 BP4-BP5 deletion and duplication syndromes are associated with a complex spectrum of neurodevelopmental phenotypes that include developmental delay and autism spectrum disorder, with a reciprocal effect on head circumference, brain volume, and body mass index. In this study, we observe that dosage of the 16p11.2 BP4-BP5 interval was negatively correlated with age at menarche in the UK and Estonian biobanks and 16p11.2 clinical cohorts, with a directionally consistent trend for pubertal onset in males. We also report a negative correlation between the 16p11.2 dosage and hypothalamic volume both in humans and mice, intimating a perturbation in the gonadotropin-releasing hormone (GnRH) axis. To investigate the contribution of transcripts located within the 16p11.2 BP4-BP5 region to the puberty phenotypes observed in CNV carriers, we used two independent approaches: Mendelian randomization and in vivo modeling in zebrafish to mimic altered gene dosage. The processes by which the GnRH axis are established in zebrafish are mostly conserved with mammals; zebrafish are missing a GnRH1 ortholog but express GnRH2 in the midbrain tegmentum, and GnRH3 in the forebrain areas of olfactory bulb-terminal nerve, preoptic area, and hypothalamus. Importantly, GnRH3 neurons perform hypophysiotropic roles in control of neurogenesis, neuronal migration, and reproduction in zebrafish. We monitored GnRH3 neurons live using a Tg(GnRH3:EGFP) transgenic zebrafish model with an automated in vivo imaging technology. Firstly, to model the 16p11.2 BP4-BP5 duplication condition, we first

evaluated the phenotypic consequences of overexpressing each of the 30 human mRN. As of the genes mapping to the BP4-BP5 interval. We found that overexpression of a single transcript, ASPHD1, was sufficient to induce a 19% reduction of GFP signal in comparison with controls at 5 days post fertilization. To test epistasis, a phenomenon that is well-documented for genes within the 16p11.2 BP4-BP5 region, we co-injected ASPHD1 with four candidates suggested by Mendelian randomization; KCTD13 induced an exacerbated phenotype suggesting genetic interaction between both genes. Secondly, to model the 16p11.2 BP4-BP5 deletion condition, we used CRISPR/Cas9 genome editing to deplete endogenous asphd1 transcript and observed a 13% decrease in GFP signal in asphd1 F0 mutant larvae in comparison with controls. ASPHD1 is predominantly transcribed in brain and pituitary gland and is implicated in peptidyl-aspartic acid hydroxylation. Together, our data identify ASPHD1 as a major reproductive phenotype driver in 16p11.2 CNV carriers, and highlight the power of an interdisciplinary approach to elucidate disease etiologies underlying complex traits.

# Distribution and restoration of serotonin-immunoreactive paraneuronal cells during caudal fin regeneration in zebrafish

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Aquatic vertebrates possess diverse types of sensory paraneuronal cells in their skin for the detection of stimuli in water. However, the presence of such cells has only rarely been studied in the locomotory appendages in adult zebrafish, a common model organism. Here, we identified serotonin (5-HT)-immunopositive cells scattered in the epidermis of the caudal fin. These cells were distinct from keratinocytes as revealed by their low immunoreactivity for keratin and desmosome markers. Instead, they were detected by Calretinin (Calbindin-2) and Synaptic vesicle glycoprotein 2 (SV2) antibodies, indicating their calcium-regulated neurosecretory activity. Consistently, electron microscopy revealed secretory organelles in small round cells in the fin epidermis. Based on the three immunomarkers, 5-HT, Calretinin and SV2, we referred to these cells as HCS cells. We found that HCS cells were spread throughout the entire caudal fin at an average density of 300 cells per mm2 on the fin surfaces. These cells were strongly enriched at ray bifurcations in wild type and elongated fins of another longfin mutant fish. To determine whether hydrodynamics play a role in the distribution pattern of HCS cells, we applied an interdisciplinary approach with a kinematic analysis. Measurements of particle velocity with a fin model revealed differential fluid velocities between bifurcated rods as compared to adjacent non-bifurcated regions. The obtained values suggest that the accumulation of HCS cells at the sites of bone bifurcation may represent a biological adaptation for sensing water parameters. The significance of this distribution pattern is reinforced by the fact, that after fin amputation, the spatial pattern of HCS cells was reproduced in the newly regenerated fin. Chemical inhibition of serotonin synthesis did not impair regeneration of HCS cells, suggesting that this neurotransmitter is not essential for the restorative process. In conclusion, our study provides the first report of a specific population of solitary paraneuronal cells in the fin, whose distribution correlates with fluid velocities.

# Molecular characterization of heterogeneous cardiomyocyte populations in the adult zebrafish ventricle

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Despite the simple architecture of the adult zebrafish heart, the ventricle comprises heterogeneous populations of cardiomyocytes. Furthermore, in response to heart injury, cardiac cells acquire new properties associated with regeneration. The molecular mechanisms regulating cardiomyocyte heterogeneity and plasticity are poorly understood. We have previously identified a transgenic reporter, called careg, which is expressed in subcortical cardiomyocytes forming the primordial layer, and which is transiently induced in the peri-injured myocardium during heart regeneration. Interestingly, the primordial layer displays lower regenerative capacity than underlying trabecular and overlying cortical cardiomyocytes. To gain further insights into the different regenerative competence of cardiac cells, we performed a transcriptome profiling of careg-expressing cardiomyocytes of the primordial layer and the peri-injured trabecular myocardium. Using a combination of immuno fluorescence, transgenic reporters, pharmaceutical approach and dominant negative overexpression, we are currently investigating the function of potential candidates involved in the regulation of the intrinsic plasticity of cardiomyocytes in the zebrafish heart.

# Actomyosin dynamics and the Bmp pathway drive apical extrusion of proepicardial cells

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The epicardium, the outer mesothelial layer enclosing the myocardium, plays key roles in heart development and regeneration. During embryogenesis it arises from the proepicardium (PE), a cell cluster that appears in the dorsal pericardium close to the venous pole of the heart. Little is known about how the PE emerges from the pericardial mesothelium. Using the zebrafish model and a combination of genetic tools and pharmacological agents we investigate the molecular mechanisms of PE formation. Moreover, we developed new powerful tools for the quantitative analysis of in vivo imaging. Using these tools we reveal that a coordinated collective movement of the dorsal pericardium drives PE formation, which goes along with drastic changes of the cell shape. The dynamics of the emerging PE are orchestrated by actomyosin activity. Even though actomyosin dynamics proved critical, our results reveal that overexpression of Bmp can rescue a Myosin II inhibition and thereby apical extrusion of PE cells. More generally, by comparison to cell extrusion for the elimination of unfit cells from epithelia, our results describe a mechanism where extruded cells play an important role in development to form the epicardium.

# Adult sox10+ cells contribute to myocardial regeneration in the zebrafish

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During heart regeneration in the zebrafish, fibrotic tissue is replaced by newly formed cardiomyocytes derived from pre-existing ones. It is unclear whether all cardiomyocytes have an equal capacity to replace lost myocardium. In opposition to mammals, sox10+ neural crest cells have been proposed to contribute to the embryonic zebrafish myocardium. Here we examined the contribution of embryonic sox10-derived cells to the adult zebrafish myocardium and found that while they persisted in adult hearts they did not participate in regeneration. However, sox10 genetic fate mapping identified a population of cardiomyocytes in the adult heart with a distinct gene expression profile that expanded massively after cryoinjury. Genetic ablation of sox10+ cardiomyocytes severely impaired cardiac regeneration revealing that sox10 marks a subset of cardiomyocytes with high regenerative potential. RNA-Seq analysis indicated that sox10-derived CMs not only preferentially contribute to heart regeneration, but that they also represent a myocardial cell population in the adult zebrafish heart with a unique gene signature. Collectively, these data strongly suggest that a ventricular sox10-derived CM population present in the adult zebrafish heart is required for cardiac regeneration.

KEYNOTE LECTURE 1

# Towards European zebrafish guidelines

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Zebrafish is today the second most used in vivo model in biomedical research. The increased use of this small fish species accentuates the value with a framework for common husbandry standard operating procedures. It will secure animal welfare and at the same time support a development towards more standardised protocols and reproducible results. Work from a FELASA-EUFishBioMed joint working group with a mandate from FELASA to propose recommendations for zebrafish housing and husbandry will be discussed.

# Zebrafish model of the auculoauricular syndrome, recent advance in the role of hmx1 and hmx4

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## Background

In human, homozygous HMX1 mutations lead to the oculoauricular syndrome (OAS) (OMIM: 612109). The OAS phenotype is characterized by an unusual ear lobule, microphthalmia and by ophthalmic anomalies affecting the retina resulting in decreased visual acuity in patients. In mouse, homozygous Hmx1 mutations lead to similar phenotype including unusual ear shape, microphthalmia and visual impairment. In this project we are interested in building a zebrafish model of the corresponding homeobox gene which is either hmx1 or hmx4. In contrast to vertebrates such as human, mouse or chicken; the zebrafish possess four distinct hmx genes. In order to further study the OAS and open up the opportunity of high throughput screening, we developed several models of hmx1 and hmx4 mutants.

## Methods

We developed several zebrafish models using CRISP-Cas9 and zinc finger nuclease mediated deletion of either or both hmx1 and hmx4. On different mutant strains we obtained visual testing of the optokinetic reflex. We analysed the retina structure at 5dpf with chemical staining and immunohistochemistry labelling of the retina's cell populations and we also analysed the cartilaginous structure of the larvae. Furthermore, we began to study the effect of either the deletion or promoter based expression of hmx gene on potential target known from other vertebrate model.

## Results

Here we present a new zebrafish model for ocular anomalies. Hmx4 mutant eyes are microphthalmic, and the 5dpf larvae have decreased visual acuity. Photoreceptor structure is altered but the ratio of the retina to the lens and the eye is preserved. As previously reported by Gongal et al., abnormal closure of the neural tube is confirmed in our model. We also present some preliminary data on potential target that could help understand the regulatory and developmental role of hmx gene in the zebrafish.

# Investigation of the coupling mechanisms driving the synchronization of the segmentation clock

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The elongating body axis of vertebrate embryos is patterned via the sequential and rhythmic production of segments from a posterior unsegmented tissue called the presomitic mesoderm (PSM). The rhythmicity of this process is controlled by a population of genetic oscillators termed the segmentation clock. In each cell of the zebrafish PSM, the expression of a set of genes oscillates in a cell-autonomous manner. Cellular oscillators are locally coupled and they generate spatiotemporal waves of gene expression that sweep the tissue from posterior to anterior and reiterate during the formation of each segment. While it is now established that Notch signaling is involved in synchronization of cellular oscillators, a quantitative and molecular understanding of synchronization is still lacking. Here, we investigate the molecular mechanisms underlying the coupling between neighboring cells by developing a microfluidic platform to pair single cells isolated from the zebrafish PSM. We aim at estimating the strength of the coupling at the cellular level by monitoring how phases of cell-autonomous oscillators change when cells interact with each other. This microfluidic platform offers great versatility and allows to pair cells from two different populations. By estimating the coupling strength of cells from different genetic backgrounds, we want to determine the relative contributions of Notch signaling components to the coupling. Simultaneously, we intend to investigate how different Notch pathway components impact the amplitude of oscillations at the cellular-level. Our work should also yield further molecular insights into the Delta-Notch signaling system and the mechanisms of synchronization in biological systems.

# Studying the role of CACNA2D4 in a rare type of retinal dysfunction

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CACNA2D4 encodes the auxiliary α2δ-4 subunit of a voltage-gated calcium channel expressed in the retina. Patients carrying mutations in this gene suffer from a rare form of retinal dysfunction, which mainly affects cone vision. In this study, we exploit the advantages of the cone-dominant zebrafish to assess the role of this protein in calcium channel trafficking and signal transmission. To this end, we created CRISPR/Cas9-KO lines of the paralogous zebrafish genes cacna2d4a and cacna2d4b, respectively, and studied retinal morphology and function using electroretinography (ERG), immunohistochemistry and light- and electron microscopy. Our findings suggest, that cacna2d4b is involved in calcium channel trafficking and show that a loss of both subunits impairs cone-driven retinal function without causing retinal degeneration. Thus, the cacna2d4-KO zebrafish is a suitable model to study the human condition and its underlying pathophysiological mechanism.

# Comparative retinal transcriptomics of zebrafish ciliopathy mutants cc2d2a and talpid3

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The primary cilium is a microtubule-based structure, protruding from most differentiated cell types. It serves as sensory organelle that processes extracellular signals and transmits them into the cell. Types of transmitted signals depend on the cell type and include developmental signaling pathways such as Hedgehog or Wnt. Light sensation in photoreceptors (PRs) also occurs in cilia which have evolved into highly specialized “outer segments” (OSs). Cilia are anchored inside the cell through a modified centriole becoming the basal body (BB). Defects in the formation and/or function of cilia result in a variety of human diseases called ciliopathies, which frequently display retinal degeneration given the role of cilia in PRs. Ciliopathies are genetically very heterogeneous, since mutations in >30 different genes can cause the same specific disorder. To address how dysfunction of so many different genes results in the same phenotype, our research uses zebrafish mutants in 2 genes associated with the same disorder, cc2d2a and KIAA0586/talpid3, to understand the shared and unique consequences resulting from their dysfunction. Retinal phenotypes of cc2d2aw38 and talpid3i264 mutants were previously described: While Cc2d2a controls ciliary protein content by facilitating fusion of incoming vesicles carrying ciliary proteins, Talpid3 is required for BB docking in the first steps of ciliogenesis. Consequently, cc2d2a-/- PRs display extended but dysmorphic OSs, while talpid3-/- PRs mostly form no OSs at all. Transcriptomic analysis by RNA-sequencing of whole eyes from each mutant and its respective control was performed and pair-wise comparisons identified differentially expressed genes. Preliminary results show a strong overlap of PR-specific genes downregulated in both mutants, including phototransduction-cascade components, while several genes involved in retinal protection and degeneration were upregulated in both mutants. The majority of genes differentially regulated between the two mutants are metabolic genes, reflecting differences in the genetic background of the zebrafish strains harboring the mutations. We are currently following up those hits that are up- or downregulated only in one of the mutants. This includes components of the so-called BBSome, known to control movement into and out of the cilium, which are downregulated in talpid3 mutants only, or new candidates with limited information with respect to their ciliary function such as hap1, which is upregulated in cc2d2a mutants only.

# Knockout of the glutamate transporter eaat2a elicits spontaneous epileptic seizures in the larval zebrafish

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Excessive excitatory signaling of glutamate may cause epilepsy, one of the most prevalent neurological brain disorders worldwide. In physiological conditions, glutamate is removed from the synaptic cleft mainly by the excitatory amino acid transporter 2 (EAAT2).
Malfunctioning of this transporter has been associated with glutamate accumulation, prolonged neuronal excitation and epileptic seizures in mice. However, the exact mechanisms of seizure propagation are not yet clear. As such, we developed a new model in the zebrafish, a simple vertebrate, in order to unravel the underlying epileptic networks.

We show that CRISPR/Cas9-mediated knockout (KO) of the paralogue eaat2a in zebrafsh leads to a distinct epileptic phenotype in larvae. First, homozygous mutant fish display specific epileptic behavior, represented by episodes of rapid swirling and bursts of activity. Furthermore, neuronal and glial calcium imaging revealed sudden bursts of excessive activity spreading across the whole brain of KO larvae. These results suggest that eaat2a mutants suffer from spontaneous generalized seizures. Therefore, eaat2a KO zebrafish can serve as a chronic model of epilepsy and has the potential to lead to valuable insights into cellular networks and molecular mechanisms during spontaneous seizures.

# Lack of OXPHOS cIII-cIV superassembly decreases metabolic efficiency

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Mitochondrial respiratory chain complexes can super-assemble into quaternary structures called Supercomplexes, process in which the Supercomplex Assembly Factor 1 (SCAF1, also known as Cox7a2l) is the responsible of the interaction between complexes III (cIII) and IV (cIV). Although a function of optimization of cellular metabolism was attributed to Supercomplexes, the phenotypical consequences of the lack of SCAF1 have never been studied in animal models. To study the role of cIII-cIV superassembly, we have generated a null mutant model of the SCAF1 orthologous gene in zebrafish (known as cox7a3) using CRISPR/Cas9 technology. As a first step, we have corroborated that zebrafish respiratory chain complexes can also superassemble into Supercomplexes by Blue Native-PAGE. Although they show differences in the assembly pattern compared to mice, the zebrafish SCAF1 orthologous is also required for cIII-cIV assembly and the lack of the protein leads to the lack of Supercomplexes containing cIIIcIV. Next, we characterized the phenotypic consequences of SCAF1 null mutant. Zebrafish SCAF1 -/- are smaller than their wild type siblings, present reduced fertility due to delayed ovary maturation and differences in fat deposition. Surprisingly, these phenotypic alterations were restored upon additional feeding, suggesting that SCAF1 -/- zebrafish are metabolically less efficient than wild types. In conclusion, cIII-cIV superassembly driven by SCAF1 leads to a better optimization of metabolic resources and therefore an advantage in situations of food limitation in zebrafish. Our results provide for the first time a direct evidence for a physiological role of respiratory complexes superassembly.

# Membrane Sphingolipid composition modulates palmitoylation of the Anthrax Toxin Receptor to regulate oriented cell division

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The orientation of cell divisions is determined by the position of the mitotic spindle and plays crucial roles in early embryo axis determination, cell fate diversity and tissue morphogenesis. In zebrafish, dorsal epiblast cells are an excellent system to study oriented cell division in a developing tissue. Throughout gastrulation, these cells display divisions that are robustly oriented along the embryonic axis and are under the control of the Wnt/Planar Cell Polarity (PCP) pathway, which induces the formation of an asymmetrically polarized actin cap aligned with respect to the embryonic axis. This cortical actin cap triggers the spatial redistribution of the transmembrane protein Anthrax Toxin Receptor 2a (Antxr2a), which becomes enriched at the actin cap. Once at the cap, Antxr2a interacts with the small GTPase RhoA to activate the actin nucleat or Diaphanous (Dia). Dia is required to exert torque on the spindle to position it perpendicular to the actin/Antxr2a cap and therefore along the embryonic axis. Many of these molecules that we have uncovered to play a role in oriented divisions of epiblast cells are lipid modified, prompting us to investigate the role of membrane lipids in this process. Here, we first found that Antxr2a is palmitoylated, a reversible lipid modification, that determines the role of the receptor in oriented division: expression of a palmitoylation defective Antxr2a results in division randomization of epiblast cells. Secondly, we undertook a system level approach to study the lipidome of zebrafish embryos and found that the Wnt/PCP pathway regulates the levels of Sphingolipids. Genetically altering the metabolic production of these membrane lipids results in randomization of divisions of epiblast cells. Sphingolipids mediate the position of the mitotic spindle by regulating the palmitoylation state of Antxr2a. Biochemical and quantitative image analysis showed that Antxr2a needs to be fully palmitoylated to be able to interact with RhoA and therefore to activate Dia responsible to align the mitotic spindle with the embryonic axis. We have therefore uncovered a novel level of regulation by the Wnt signaling that involves Sphingolipids, which are required to regulate the palmitoylation state of the Anthrax Toxin receptor. Palmitoylation of the receptor is in turn regulating the intracellular signal that is required to orient the mitotic spindle during oriented divisions of epiblast cells.

# Time-course effect of exercise on zebrafish muscle

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Regular exercise is known to provide many health benefits, particularly in skeletal muscle. Although the impact of exercise on muscle metabolism is well described in humans and animal models, little is known about the time-course of such changes. The aim of this study was to identify the amount of exercise training necessary to produce effects on muscle architecture and mitochondria adaptation. Following a time course design, zebrafish were submitted to an endurance training protocol for 2, 5, 8, 12, 15 and 18 days. Fish were euthanized after 24 hours of starvation since the last training session. Protein content and gene expression levels at the different time points were compared to non-exercised controls. Interestingly, changes of the different electron transport chain proteins were deferentially distributed, plateauing either after 5 days or after 12 days of training. Key players of mitochondrial fusion and fission were synchronized and peaking at the same time. This work may be of importance for those interested in eliciting exercise stimuli in zebrafish skeletal muscle. Importantly, it shows that specific dose-response adaptations need to be taken into account depending of the specific outcome of interest. We are currently evaluating whether the timing of these modifications is similar in older and younger fish to appraise the impact of aging on muscle plasticity.

# Leveraging auto-generated ethograms to produce realistic fish behaviour in groups of zebrafish

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Many behavioural studies on animals rely on the use of behavioural “catalogs”, called ethograms, that are manually compiled by experts of the field. Ethograms often contain a detailed description of behavioural patterns to allow an expert to discern between them and, thus, they require numerous work hours of meticulous visual inspection to classify each behaviour over the course of time. In the case of robot animal interaction studies, the ethograms could potentially provide insight into behaviour specific locomotive patterns which the robot could learn to reproduce. Here, we present an unsupervised methodology to: (1) infer the number of distinct behaviours and generate an ethogram of the interactions in groups of zebrafish Danio rerio, and (2) leverage the per behaviour information as a reward for a reinforcement learning procedure that «learns» to behave like a fish. We compare this approach to a supervised learning procedure and show that our reward driven approach performs in a more realistic manner. In future work we will compare artificial trajectories generated by our methodology to ones from state-of-the art particle models. Are computers «learning» the same model or not? We also aim to use the artificially generated model in our robotic system to interact with real individuals and validate the extent to which the robot can adapt to the interaction dynamics.

# Attractive and aversive responses of zebrafish larvae to frequently detected classes of insecticides

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**Background:** Insecticides are extensively used in Switzerland and all over the world to control pests and pathogens in agriculture. Via spray drift, leaching or run-off they find their way into the aquatic environment where they pose a risk to non-target organisms, such as fish. Toxic effects from insecticides can occur at different organisational levels and may range from easily observable lethal to very subtle behavioural effects. Selecting appropriate behavioural responses is crucial for the survival of the single organisms as well as the whole population. Such behavioural responses are based on sensing and processing stimuli from the environment. Organisms possess an innate ability to escape from threatening situations they are naturally exposed to. Such situations can be posed by unfavourable environmental conditions, predators or alarm substances. Similarly, they are attracted to favourable cues such as food sources, social cues or mating partners. As most insecticides are designed to interfere with neuronal signalling, they are able to adversely affect this ability to process sensory input and react appropriately to environmental stimuli with extensive ecological consequences.

**Aim:** We are investigating whether different classes of insecticides lead to attractive or aversive responses of zebrafish larvae, and are additionally interested in the neuronal mechanism underlying the observed behavioural response. We aim to better understand how insecticides change natural behavioural responses of fish in order to better predict their impact on the ecosystem.

**Methods**: Zebrafish larvae are exposed to a gradient of the test chemical while the behaviour is tracked with an automated video recording system. The larva's space use and locomotor activity are evaluated. After the behavioural experiment, neuronal regions that were activated during exposure are visualised by staining the larvae for an endogenous activity indicator (pERK).

**Results:** For nicotine we found an attractive response at 1 µM, expressed by an increased dwell time in the nicotine containing zone. Higher concentrations (10 µM), on the other hand, appear to be clearly aversive, and larvae try to escape the dish. Attractive and aversive responses have been reported to be attributed to activity levels in the Habenula with according activation or inhibition of the Dorsal Raphe nucleus, a part of the reward system in the teleost brain. In comparison to nicotine, two tested neonicotinoids, thiacloprid and imidacloprid, showed no significant effect. Larvae that were exposed to pirimicarb at 100 µM showed a significant attractive response, while insecticides of the organophosphate class did not elicit an effect. We are currently investigating whether other classes of insecticides (e.g. organophosphates and carbamates) that are frequently found in Swiss surface waters trigger similar or dissimilar behavioural patterns.

**Outlook**: We will dissect which higher brain areas and which chemosensory systems are involved in the behavioural reactions to environmental chemicals. This will advance our understanding of the impact of chemicals on fish behaviour and their underlying mechanisms.

# Analysis of inter-individual variability in locomotor activity of larval zebrafish as a basis to improve neurotoxicity testing of chemicals

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Many environmental contaminants, such as pharmaceuticals, pesticides and heavy metals, have been shown to interfere with the nervous system of different species including humans and fish, thereby creating deficiencies in sensation and behavior. Behavior is the integrated output of multisensory, neuroendocrine and neuromuscular signals and is an important, ecologically relevant and very sensitive endpoint of toxicity measurement. In aquatic toxicology, the analysis of locomotor behavior of chemically exposed zebrafish larvae has become a prominent method for the assessment of neurotoxicity, because of the easy-to-handle, high-throughput and automated manner of testing. However, the detection of group differences can be hampered by high inter-individual variability among larval zebrafish. Therefore, the aim of this study was to investigate behavioral inter- and intra-individual variability in zebrafish larvae as a basis to better estimate effects of chemicals on behavioral responses. We assessed the variability of locomotor behavior in larval zebrafish from 5 to 7 days post fertilization for some frequently used protocols (spontaneous swimming, light/dark phases, tapping stimuli) and collected physiological and morphological data of the individual larvae in order to explore whether they correlate with locomotor activity. Our results show that the variability in locomotor activity is lowest during dark phases, but for morphological parameters (length of the larva, size of the swim bladder, size of the yolk) and cardiophysiological measurements (heart rate), there was no correlation with individual locomotor activity. When looking into consistency of activity of the individuals, we found that during dark phases, activity levels correlated well between measurements taken in the morning and afternoon of each day and the activity of individual larvae was highly consistent between day 6 and 7. Overall, our study indicates that taking the variability of locomotor activity into account will improve the evaluation of chemical effects on behavioral responses of larval zebrafish and has the potential to reduce the number of animals to be used for neurotoxicity testing. These results might also offer new insights into mechanisms of toxicity considering that every individual's response to a chemical differs based on their genetic make-up.

# Systems toxicology approach for the assessment of imidacloprid neurotoxicity in zebrafish larvae

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Understanding the molecular basis of chemical toxicity would better inform subsequent risk assessment. To address this, we have developed a comprehensive systems toxicology approach to evaluate neurotoxicity in zebrafish larvae. We have chosen the insecticide imidacloprid for this assessment because of its ability to interfere with the function of the nervous system in fish.

Zebrafish larvae were exposed to two concentrations of imidacloprid according to the OECD fish embryo toxicity test for 120 hours. Treated larvae displayed shorter body length but were otherwise morpologically normal. Functional cardiac assays did not reveal significant differences between control and treated fish. Behavioral analysis indicated that the exposed larvae moved slower in response to light and vibrational stimuli. To gain mechanistic insight into imidacloprid toxicity, protein profiling using mass spectrometry was performed on chemicaly treated larvae. In total 3,533 proteins were quantified across nine samples. Out of these, 64 proteins showed significant differences between control and the low concentration of imidacloprid. 110 proteins were significantly changed between control and the high concentration group. In agreement with the behavioral assessment, gene ontology analysis revealed that proteins involved in vision and muscle contraction are differentially expressed in exposed individuals.

To complement the proteomic, behavioral, and morphological assessments, we have developed a network model describing pathways that lead to neurotoxicity in zebrafish embryos. To construct the model, we have curated 90 publications and coded the observations therein into a computable form. Key signaling nodes in the model, such as protein activities, are linked to information about downstream gene expression. Transcriptomic analysis of genes downstream of these nodes can be used to infer activity of the upstream protein – a process termed “network scoring.” Scoring of the network highlights the most affected nodes by a given treatment, which leads to mechanistic hypothesis generation and gives a quantifiable measure of network perturbation.

We are now sequencing the transcriptomes of zebrafish larvae treated with imidacloprid. These data will be used to score the neurotoxicity network to determine how well the computational model can predict changes measured in behavioral and proteomic experiments. Mechanistic insight gained from computational scoring combined with phenotypical and proteomic analyses can provide a comprehensive method for linking molecular events to organ toxicity.

KEYNOTE LECTURE 2

# Visualizing cancer growth in zebrafish

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As the zebrafish gains consideration as a powerful system to study diseases, its use in cancer research steadily increases. Cancer occurs naturally in zebrafish and can be induced with a variety of toxicological, environmental and genetic methods, making this small vertebrate an extraordinary tool in cancer research. Indeed, no other vertebrate model offers the molecular toolbox, the superior imaging capabilities and the discovery power of genetic, chemical and suppressor screens as the zebrafish. We study early events in cancer, focusing on molecular and cellular aspects of transformation, clonal expansion and exploitation of host resources that characterize cancer-initiating cells and cancer stem cells. We have developed powerful systems to initiate cancer in different organs and stages of development, with the goal of understanding the mechanisms of transformation, visualize transformed cells in vivo and establish platforms for genetic and chemical screens. A number of projects have been developed using these approaches and will be presented here, including our latest discoveries in melanoma, uveal melanoma and glioma models.