PERSPECTIVES IN ZEBRAFISH RESEARCH

EDITED BY: Maria Caterina Mione, Patrick Blader, Filippo Del Bene, Eirini Trompouki and Isaac Henry Bianco

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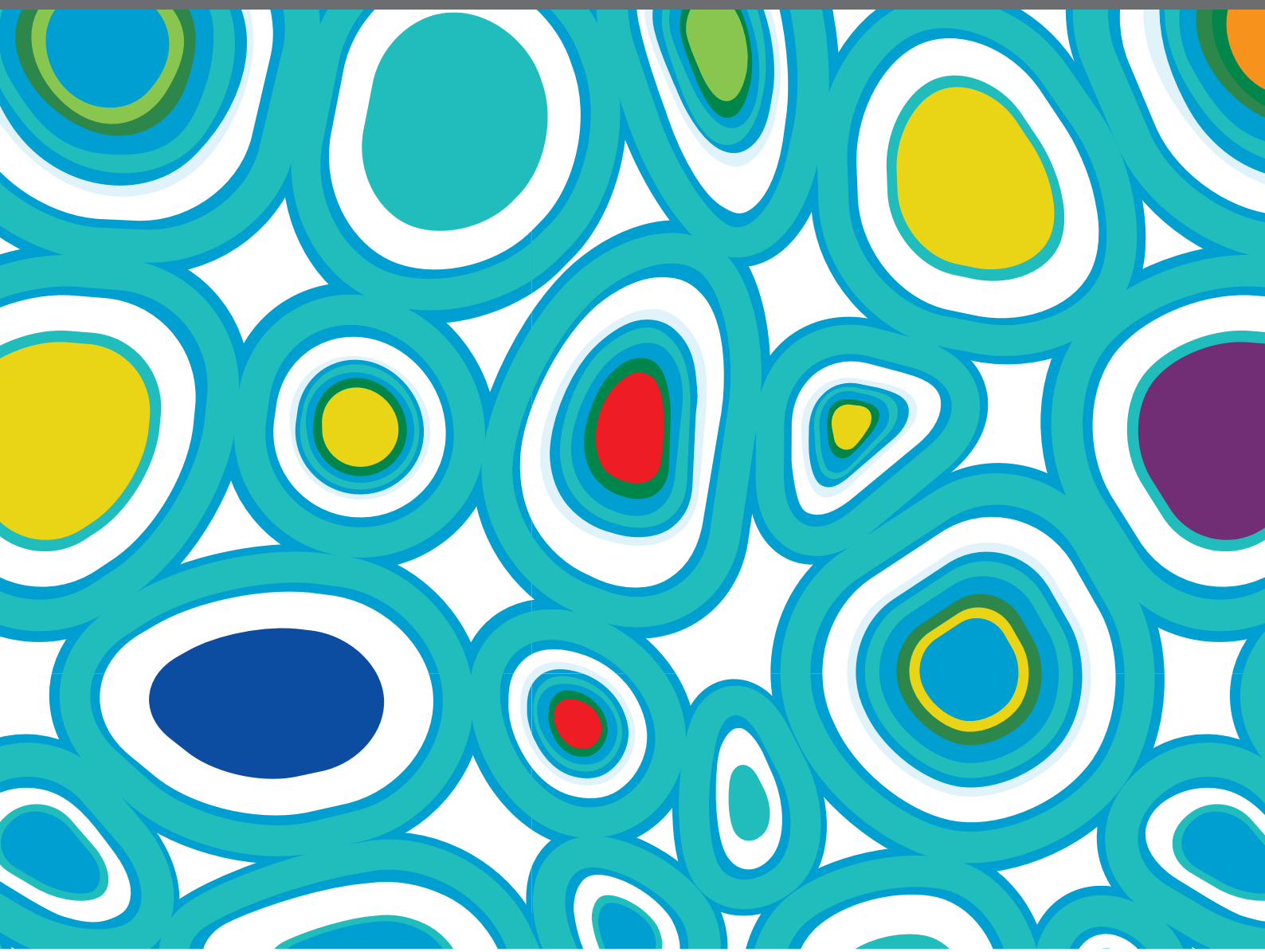

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PERSPECTIVES IN ZEBRAFISH RESEARCH

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In the past decade, a small tropical vertebrate fish, zebrafish, has rapidly gained the interest of research laboratories worldwide as a model system. This topic will provide updated perspectives on all fields of zebrafish research from experts gathering at the 5th Zebrafish Principal Investigators Meeting in Trento, 20-23 March 2018. The community of researchers using zebrafish is rapidly expanding, necessitating a clear plan for how to tackle central questions that remain a challenge in the field and providing inspiration for future studies. This is the aim of the workshop and the Frontiers Research Topic will provide a platform for dissemination of novel ideas arising from this meeting.

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ORIGINAL RESEARCH
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Genome Editing Reveals Idiosyncrasy of CNGA2 Ion Channel-Directed Antibody Immunoreactivity Toward Oxytocin

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Presynaptic cGMP-gated ion (CNG) channels positively or negatively modulate neurotransmitter secretion as well as the strength of synaptic transmission. Zebrafish cGMP-gated ion channel, CNGA2a (a.k.a. CNGA5), was previously reported to be specifically enriched in synaptic terminals of zebrafish oxytocin (OXT) neurons. This conclusion was based on immunoreactivity of a monoclonal antibody (mAb) clone L55/54, which was directed against the carboxy terminal tail of the CNGA2a. To study the role of CNGA2a in oxytocin neurons function, we generated zebrafish mutants of *cnga2a*, *cnga2b* and *oxt* genes using clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing. We show that mAb L55/54 specifically recognizes CNGA2a protein when expressed in heterologous cell culture system. Surprisingly, anti-CNGA2a immunoreactivity was not eliminated following knockout of either *cnga2a*, *cnga2b* or both. However, knockout of *oxt* resulted in total loss of anti-CNGA2a mAb immunoreactivity despite the lack of sequence and structural similarities between OXT and CNGA2a proteins. Our results provide a noteworthy lesson of differences in antibody immunoreactivity, which could only be revealed using specific genetic tools.

Keywords: neuropeptide, cGMP-gated ion channel, neurohypophysis, monoclonal antibody, oxytocin

INTRODUCTION

The hypothalamo-neurohypophyseal system (HNS) is an important neuroendocrine structure that coordinates brain neuronal circuits with peripheral organs responses to maintain body homeostasis (Gutnick and Levkowitz, 2012). The structure and function of the HNS is conserved in all vertebrates. It is composed of hypothalamic neurosecretory cells that produce the cyclic nonapeptides oxytocin (OXT) and arginine vasopressin (AVP). These peptides are packed into large dense-core vesicles, transported along axons that terminate in the posterior pituitary lobe (neurohypophysis) and are released to influence the function of target cells throughout the body (Burbach et al., 2001; Engelmann and Ludwig, 2004; Knobloch and Grinevich, 2014; Wircer et al., 2017). In addition to acting as neuroendocrine hormones, both OXT and AVP also act as neurotransmitters in the central nervous system (CNS) where they modulate social affiliation, stress, learning, and memory functions (Murphy et al., 2012). Notably, both neuroendocrine and

CNS functions of OXT and AVP are conserved in evolution (Wircer et al., 2016). Given the central roles of OXT and AVP in animal physiology, deciphering the mechanisms underlying their neurosecretion has been the subject of many studies for over 60 years (Leng et al., 2015).

CNGA5 protein belongs to a family of cyclic-nucleotide gated (CNG) channels representing a family of cation channels shown to mediate cAMP and cGMP signaling in sensory neurons (Kaupp and Seifert, 2002; Podda and Grassi, 2014). CNG channels play roles in a number of activity-dependent modulatory and adaptive changes in neurons, in the regulation of a voltage-independent mode of Ca^{2+} entry, modulation of neurotransmitter release from presynaptic terminals and modification of synaptic strength (Podda and Grassi, 2014). CNG channels were shown to modulate transmitter release in retinal cone synapses and in the olfactory bulb as well as GnRH neuropeptide release in the hypothalamus (Bradley et al., 1997; Vitalis et al., 2000; Charles et al., 2001; El-Majdoubi and Weiner, 2002). Surprisingly, it was reported that a zebrafish CNG subunit, denoted CNGA5, exhibits restricted brain-specific expression pattern with only weak expression in the olfactory bulb (Tetreault et al., 2006). A subsequent study by Khan et al. (2010) employed a monoclonal anti-CNGA5 antibody and concluded that CNGA5 protein is enriched in synaptic terminals of zebrafish OXT neurons. This inspired us to examine the role CNGA5, which was recently renamed CNGA2a, in the modulation of OXT presynaptic activity.

To study the role of CNGA5 in the regulation of OXT function, we first performed analysis of the above-mentioned anti-CNGA5 L55/54 mAb immunoreactivity in larval and adult zebrafish in combination with genetic ablation of *cnga2a/b* and *oxt* genes. This analysis revealed unexpected discrepancy between *in vitro* and *in vivo* antibody reactivity that could only be shown using specific genetic tools.

RESULTS

Anti-CNGA2a Antibody Immunoreactivity *in vivo* and in Heterologous Cell Culture

Three mammalian cGMP-gated ion channel alpha subunits (CNGA), termed, CNGA1, CNGA2, and CNGA3 are widely expressed in the brain and play roles in visual and olfactory receptor neurons (Podda and Grassi, 2014). Tetreault et al. (2006) reported that a novel CNG isoform, which they named CNGA5, is specifically expressed in the brain. We performed phylogenetic analysis of zebrafish CNGA proteins using the current zebrafish genome database (GRCz10/danRer10) and Phylogeny.fr software (Dereeper et al., 2008). This analysis indicated that the previously named zebrafish *cnga5* (ZFIN ID: ZDB-GENE-061005-1) and *cnga2* (ZFIN ID: ZDB-GENE-050307-2) genes are both close homologs to human and mouse *CNGA2* genes (**Figure 1A** and **Supplementary Figure S1**). We therefore suggest that zebrafish CNGA5 is a paralog of CNGA2, which arose from the ancient ray-finned fish genome duplication. In concurrence with the nomenclature committee of the Zebrafish Information Network (ZFIN), we re-named the *cnga5* gene as *cnga2a* (ZFIN ID: ZDB-GENE-061005-1) and *cnga2* (ZFIN ID: ZDB-GENE-050307-2) as *cnga2b*.

Khan et al. (2010) generated a monoclonal antibody (mAb L55/54) which was raised against CNGA5/CNGA2a carboxy terminal tail. Based on the immunoreactivity of this antibody they concluded that CNGA2a is enriched in synaptic terminals of zebrafish OXT neurons. Using the zebrafish transgenic OXT reporter line, Tg(*oxt*:EGFP) (Blechman et al., 2011; Gutnick et al., 2011), we were able to confirm that mAb L55/54 display strong immunoreactivity which co-localized with anti-OXT immunofluorescence in 6-day-old EGFP-labeled larval hypothalamic neurons and their hypothalamo-neurohypophyseal axonal termini (**Figure 1B**). Similarly, colocalization of mAb L55/54 and anti-OXT immunoreactivity was observed in hypothalamic neuronal perikarya, projecting axons, and neurohypophyseal termini of the adult Tg(*oxt*:EGFP) zebrafish (**Figure 1C**). However, despite the above results we failed to detect *cnga2a* and *cnga2b* mRNA in zebrafish OXT neurons by *in situ* hybridization (**Supplementary Figure S2**). Notably, lack of antibody staining of OXT in the posterior EGFP-labeled OXT neuronal cluster (**Figure 1B**) is in line with our previous published findings (Wircer et al., 2017).

To confirm that mAb L55/54 recognizes the CNGA2a epitope we have transiently expressed the full-length *cnga2a* cDNA in HEK293T cell line and performed Western blot analysis. In this assay, the mAb L55/54 antibody detected two protein bands with an apparent molecular weight of around 72–80 kDa in *cnga2a*-transfected cell lysates but not in the empty vector-transfected control (**Figure 2A**). These protein bands corresponded to the expected molecular weight of the presumably glycosylated zebrafish CNGA2a protein. Additional lower molecular weights protein bands appearing in all transfections, including the vector alone-transfected cell lysate, suggested possible cross-reactivity to other proteins. mAb L55/54 also displayed positive immunofluorescent staining (IFS) of *cnga2a*-transfected HEK293T monolayer cell culture but not of control cells that were co-transfected with empty vector and EGFP expression plasmids (**Figure 2B**).

We conclude that the CNGA2a-directed mAb recognizes CNGA2a protein *in vitro*, when the latter is expressed in heterologous cell culture.

Knockout and Knockdown of CNGA2 Orthologs Do Not Affect Anti-CNGA2a Immunoreactivity

The highly localized immunoreactivity of anti-CNGA2a in OXT neurons and their projecting axons prompted us to examine the role of CNGA2a in the functionality of OXT neurons. We therefore employed the CRISPR/Cas9 gene targeting method to generate germline-transmitted mutant zebrafish lines of *cnga2a* and its paralogous gene *cnga2b*. For each gene, we have generated two types of mutant alleles (**Figure 3A**). Thus, CRISPR-mediated *indel* of 2 bp in *cnga2a* gene resulted in a nonsense mutation leading to the premature stop codon, we termed *cnga2a*-stop (**Figures 3B,C** and **Supplementary Figure S3B**). In a similar

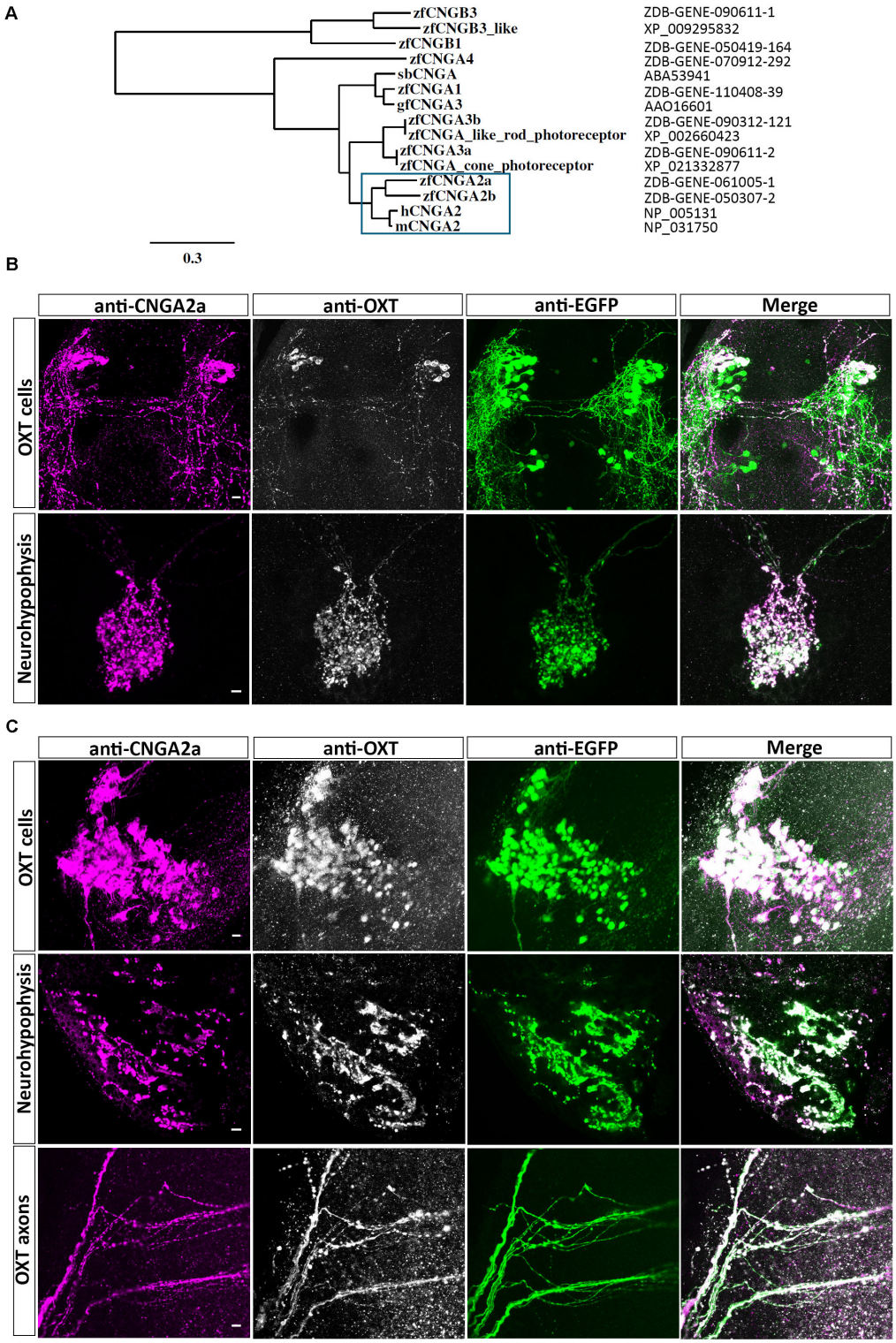

FIGURE 1 | Immunoreactivity of anti-CNGA2a mAb in larval and adult zebrafish. **(A)** Phylogram of the CNG channels protein sequences. Comparison of the CNGA2 protein homologues from zebrafish, human, and mouse species is enclosed in the blue box. The scale bar indicates 30% amino acid residues substitution. **(B,C)** Confocal images showing representative labeling of the OXT perikarya, neuronal projections, and neurohypophyseal axonal termini with anti-CNGA2a mAb (magenta) and anti-OXT Ab (gray scale) in the context of the EGFP-positive OXT-ergic population in *oxt:egfp* reporter (green). Immunohistochemical analysis show colocalization of EGFP+, OXT+, and CNGA2a+ moieties in the cell bodies, axons, and nerve termini in the neurohypophysis of 6-day-old larva (n = 30/30) **(B)** and dissected brain and pituitary from 3-month-old adult zebrafish (n = 3/3) **(C)**. Scale bars: 10 μm.

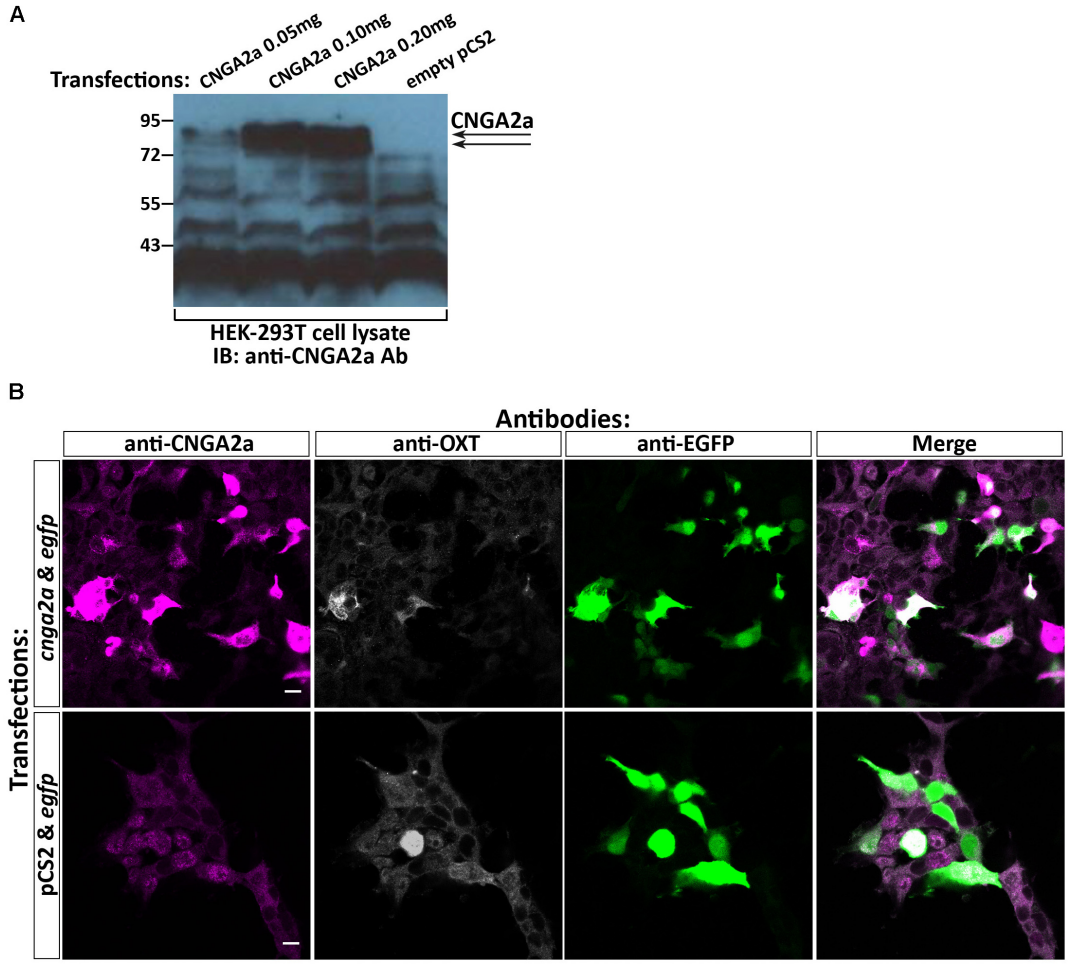

FIGURE 2 | Specificity of anti-CNGA2a mAb in heterologous expression system. **(A)** Western blot analysis of HEK293T cells transfected with *cnga2a* cDNA. HEK293T cells were transiently transfected with different amounts of *cnga2a* cDNA or a mock plasmid and were harvested 48 h post-transfection. Western blot analysis of equal amounts of protein extracts were performed using anti-CNGA2a mAb. The correct position of the doublet CNGA2a protein bands are marked by arrowheads (n = 2/2). **(B)** Confocal images of HEK293T cells transfected with *cnga2a* cDNA. HEK293T cells were transiently co-transfected with combinations of *egfp* cDNA either with *cnga2a* cDNA or an empty pcS2 plasmid. Forty-eight hours post-transfection the monolayer cultures were fixed in 3% paraformaldehyde (PFA), permeabilized with 0.5% Triton-X100/3% PFA, washed in PBS and fluorescently co-stained with anti-CNGA2a (magenta), anti-OXT (gray scale), and anti-GFP (green) antibodies (n = 4/4). Scale bars: 10 μm.

manner, we generated 17 bp *indel* mutation in *cnga2b* gene, which should lead to a truncated protein at amino acid residue 247 (*cnga2b-stop*) (**Figures 3B,C** and **Supplementary Figure S3D**). In addition, we used double-guided CRISPR strategy to generate two large in-frame deletions in exon6 of *cnga2a* and *cnga2b* encoding to amino acids 360–561 (*cnga2a-del*) and 249–431 (*cnga2b-del*), respectively. These large deletions abolish known functional domains attributed to CNG channels (**Figures 3B,C** and **Supplementary Figures S3A,C**).

Because the mAb L55/54 we used is directed to a 106 amino acids peptide that corresponds to the cytoplasmic C-terminal tail of CNGA2a (Khan et al., 2010) we expected reduced or no immunoreactivity in *cnga2a* mutants. Surprisingly, both *cnga2a-stop* and *cnga2a-del* mutant alleles retained mAb L55/5 immunoreactivity (**Figure 4A**). Similarly, knockdown of *cnga2a* by injecting antisense morpholino oligonucleotide (MO) had no effect on mAb L55/54 immunoreactivity (**Figure 4B**). To exclude the possibility that the retained anti-CNGA2a is due to cross-reactivity with CNGA2b paralog we also generated *cnga2a/b* double homozygous mutant (CNGA2a-del/CNGA2b-stop) and demonstrated that it still retained mAb L55/54 immunoreactivity (**Figure 4A**). These results suggested that mAb L55/54 immunoreactivity, which was detected in zebrafish OXT neurons by Khan et al. (2010), is due to an antigenic moiety that is different from the CNGA2a protein.

Anti-CNGA2a Immunoreactivity Is Lost in OXT Knockout Zebrafish

The nearly complete overlap of mAb L55/54 and anti-OXT immunoreactivity in OXT-ergic perikarya and axons inspired us to examine whether mAb L55/54 detects the OXT

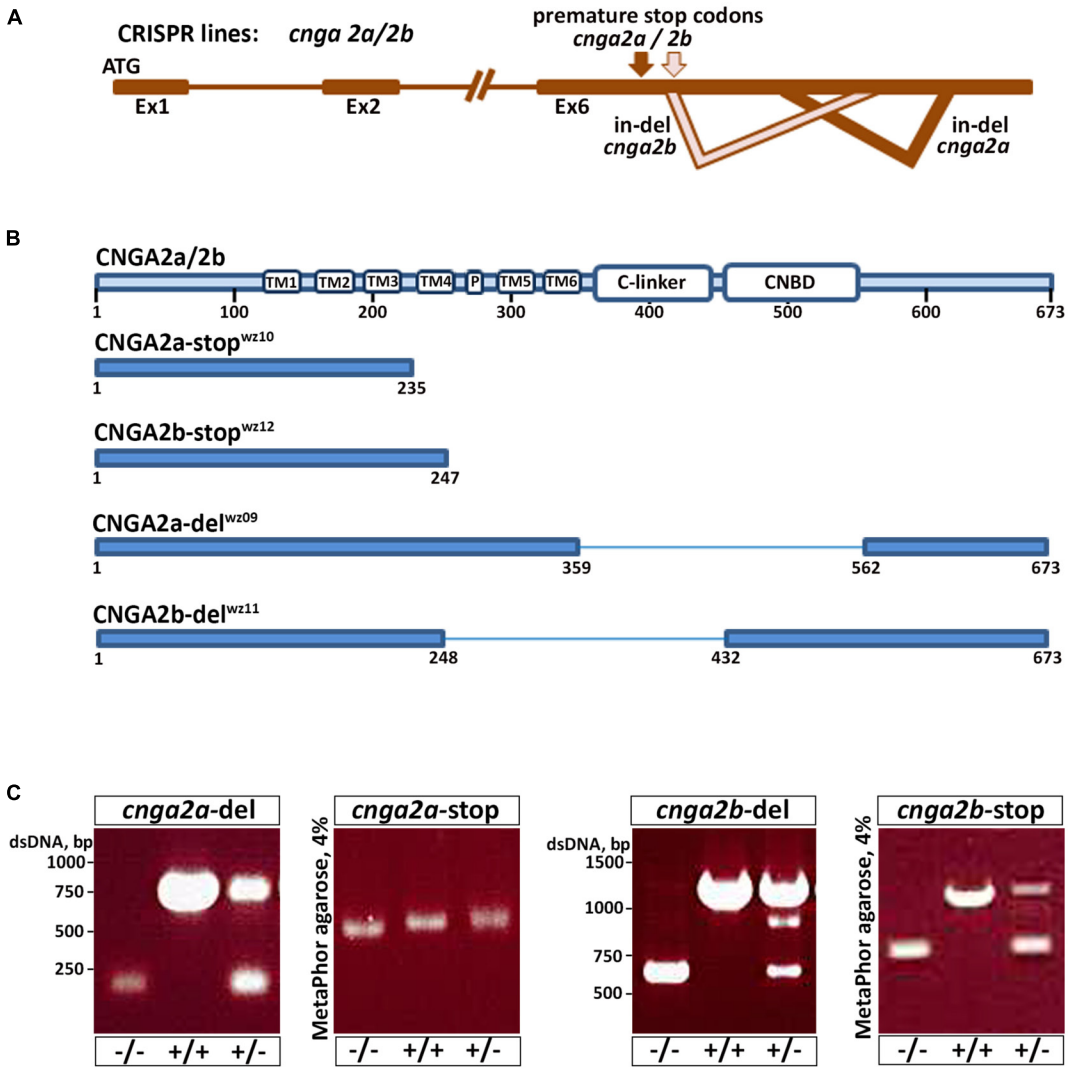

FIGURE 3 | *cnga2* isoforms genome editing using CRISPR/Cas9. **(A)** Schematic representation of the genetic structure of *cnga2* isoforms. *cnga2a* and *cnga2b*. **(B)** Schematic representation of the predicted CNGA2a and CNGA2b protein products following sequence analysis of germline transmitted CRISPR-induced mutation alleles. **(C)** Embryos and adult fish were screened by PCR for germline transmission using gene-specific primers. PCR products were resolved in 1% LE agarose gels for *cnga2a*-del and *cnga2b*-del progenies and in 4% MetaPhor gel for *cnga2a*-stop and *cnga2b*-stop progenies.

neuropeptide. To this end, we generated CRISPR-mediated germline transmitting OXT mutant harboring a 7 bp deletion in the second exon of the zebrafish *oxt* gene (**Figure 5A** and **Supplementary Figure S3E**). This CRISPR-mediated indel mutant lead to a frameshift mutation, which is predicted to abolish the expression of the OXT neuropeptide (**Figures 5A,B**). Indeed, no anti-OXT immunoreactivity was detected in the brains and neurohypophysis of homozygous *oxt* −/− mutant fish (**Figure 5C**). In accordance with our hypothesis that the anti-CNGA2a mAb L55/54 binds to OXT neuropeptide, mAb L55/54 immunoreactivity was not detected in *oxt* −/− mutant fish (**Figure 5C**). To verify that the lack of immunoreactivity in the *oxt* −/− mutant was not as a result of OXT neuronal cell loss we performed anti-OXT and anti-CNGA2a staining of *oxt* −/− mutant which was crossed with the OXT transgenic reporter, Tg(*oxt*:EGFP-*oxt*3′*UTR*). This experiment showed that the loss of OXT expression in the *oxt* −/− mutant fish had no effect on EGFP-positive OXT neurons survival and/or their neurohypophyseal projecting axons (**Figure 5C**).

Taken together, our results showed that although mAb L55/54 detects the CNGA2a protein *in vitro*, it's *in vivo* immunoreactivity is due to an unpredicted antibody cross-reactivity toward OXT neuropeptide.

DISCUSSION

The report of CNGA2a expression in OXT neurons is of great interest as cGMP-gated ion channels play important roles in the modulation of neuronal activity, including the regulation

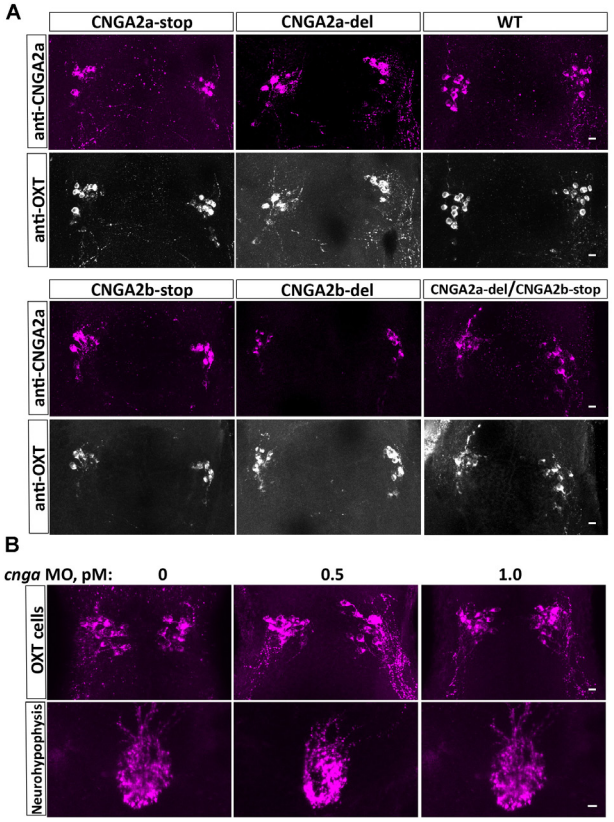

FIGURE 4 | Anti-CNGA2a immunoreactivity is not affected following KO of *cnga2a* and/or *cnga2b*. **(A)** Immunostaining and confocal imaging of 6-day-old larva NPO of the wild type zebrafish (n = 30/30), *cnga2a* (n = 20/20), *cnga2b* (n = 16/16), and double *cnga2a/2b* (n = 6/6) zebrafish mutant variants with anti-CNGA (magenta) and anti-OXT (gray scale) antibodies. **(B)** Anti-CNGA2a mAb immunostaining and confocal imaging of the hypothalamus and neurohypophysis of 6-day-old larva injected with different concentrations of *cnga2a* missense morpholino oligonucleotide (n = 10/10). Scale bars: 10 μm.

of voltage-independent Ca^{2+} entry, neurotransmitter release from presynaptic terminals and synaptic strength (Zufall et al., 1997; Podda and Grassi, 2014). Thus, the possible involvement of CNGA2a in the regulation of OXT function is intriguing. However, the suggested localization of CNGA2a protein in OXT synaptic termini is based on anti-CNGA2a immunoreactivity in OXT neurons. Here, we tested the *in vitro* and *in vivo* immunoreactivity of a mAb L55/54, which was directed to CNGA2a. We show that while anti-CNGA2a-directed mAb recognizes the correct epitope *in vitro*, it has a surprisingly strong *in vivo* cross-reactivity toward the structurally unrelated oxytocin neuropeptide.

Notably, we were able to confirm the previous findings of Khan et al. (2010) who reported that mAb L55/54 recognizes an antigenic moiety, which is expressed in OXT-ergic synaptic termini. Nevertheless, the use of genome editing tools that were not available at the time of the original publication revealed that mAb L55/54 immunoreactivity was not eliminated following knockout of zebrafish *cnga2a* and *cnga2b* genes. It is possible that the CRISPR-mediated *cnga2a* and *cnga2b* and in particular, the internal deletions retained residual antigenic activity. However, knockout of the oxytocin gene resulted in the total loss of immunoreactivity. Moreover, previous studies reported prominent expression of CNGA2 mRNA in the olfactory placode (Barth et al., 1996; Sato et al., 2005). However, both Khan et al. (2010) as well the present study show that mAb L54/55 exclusively stain oxytocin neurons but failed to detect mAb L54/55 immunoreactivity in the olfactory placode or other brain areas. Thus, the immunoreactivity of mAb L55/54 toward OXT neurons is likely due to recognition of the oxytocin neuropeptide.

Monoclonal antibodies mostly demonstrate monospecificity, to a definite protein epitope, however, the cases of cross-reactivity, i.e., recognition of unrelated protein compounds, have been reported (Valentino et al., 1985; Breiteneder and Mills, 2006; Van Regenmortel, 2014). Such cross-reactivity could be due to antibody recognition of either a stretch of continuous amino acid sequence that forms a linear epitope, or a conformational epitope, namely a folded secondary structure that resulted from proximity of distant amino acids (Wilson and Stanfield, 1994; Dall'antonia et al., 2014). We showed that anti-CNGA2a mAb reacts specifically with both the unfolded denatured CNGA2a protein observed in the Western blot analysis as well as with the presumably native protein when overexpressed in heterologous cell culture. However, we found no amino acid sequence identity or similarity between the OXT precursor polypeptide and the C-terminal antigen of CNGA2a, to which mAb L55/54 was directed (**Supplementary Figure S1**). This suggests that the specific mAb L55/54 antigenic sequence within oxytocin peptide or its precursor is not represented by a continuous amino acid segment. Another possibility explaining the observed cross-reactivity of anti-CNGA2a mAb with OXT could be the recognition of a mimotope in the OXT structure that shows limited or even no sequence similarity to the protein immunogen but could mimic the shape or charge of the CNGA2a immune epitope (Knittelfelder et al., 2009; Huang et al., 2014).

Our results raise the question of whether CNGA2a is expressed in OXT neurons. Loss of anti-CNGA2a immunoreactivity in OXT-KO zebrafish may be explained by OXT-dependent CNGA2a expression or stability. However, we failed to detect expression of *cnga2a/b* mRNA by *in situ* hybridization.

Taken together, our results provide a noteworthy lesson of differences in antibody immunoreactivity, which could only be revealed using specific genetic tools. We submit that the specificity of mAb immunoreactivity *in vivo* should always be controlled by the genetic deficiency of the target protein.

MATERIALS AND METHODS

Animals and Antibodies

Zebrafish were raised and bred according to standard protocols. All experimental procedures were approved by the Weizmann Institutional Animal Care and Use Committee (IACUC).

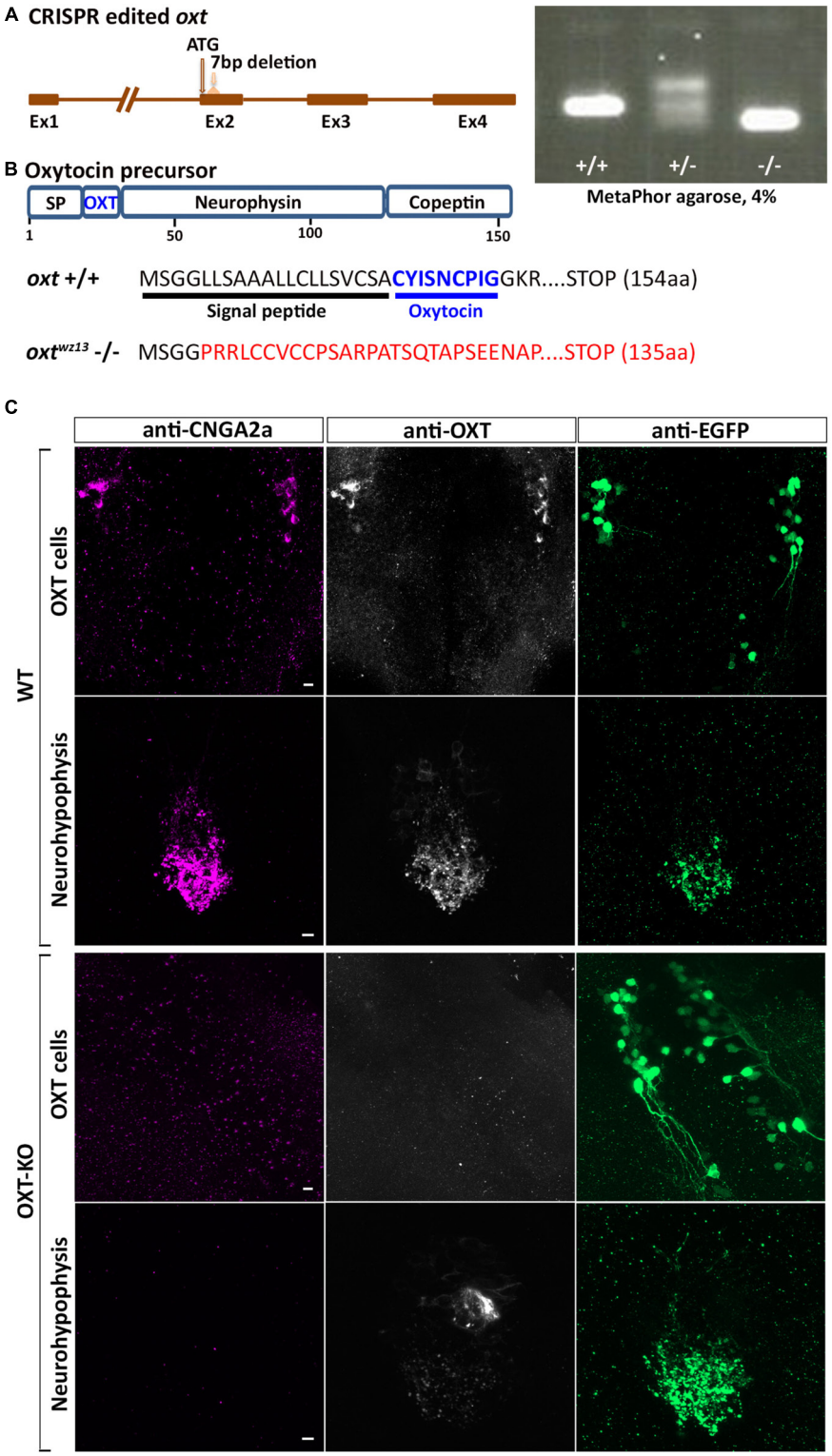

FIGURE 5 | Anti-CNGA2a immunoreactivity is lost following KO of oxytocin. **(A)** Schematic representation of oxytocin (*oxt*) gene. OXT translation start site and sgRNA target site are both indicated with arrows. Embryos and adult fish were screened by PCR for germline transmission using gene-specific primers following PCR products separation in 4% MetaPhor gels. DNA sequence of mutant allele transmitted through the germline contained a 7 bp deletion. **(B)** Schematic representation of predicted translated products from *oxt* +/+ and *oxt* –/– alleles. **(C)** Confocal images showing representative anti-CNGA2a (magenta) and anti-OXT (gray scale) labeling of OXT cells and their neurohypophyseal axonal termini of OXT-KO 6-day-old larva in the background of a transgenic Tg(*oxt:egfp-3′utr*) reporter (green) (n = 12/12). Scale bars: 10 μm.

Zebrafish transgenic line Tg(*oxt*:EGFP) contains a 644 bp upstream region of *oxt* promoter (Blechman et al., 2011; Gutnick et al., 2011), Tg(*oxt*:EGFP-*oxt3′UTR*) contains the abovementioned *oxt* promoter region combined with 600 bp downstream region of *oxt* gene.

Guinea pig polyclonal antibody directed to the oxytocin peptide was purchased from Bachem (Bachem California, Torrance, CA, United States, Cat. T-5021.0050). Rabbit anti-EGFP (A-11122; Life technologies/Thermo Fisher, Waltham, MA, United States) was used to detect transgenic EGFP expression. Anti-CNGA5 mAb was a generous gift of Dr. J. Trimmer (UC Davis, United States). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, United States).

Genome Editing Using CRISPR

Cas9 protein was produced by the Weizmann Institute of Science Protein purification unit using the pET-28b-Cas9-His (Alex Schier Lab Plasmids, Addgene, Cambridge, MA, United States) as a template. CRISPR sgRNAs were designed using CRISPR direct design[1] and are listed in the **Supplementary Table S1**. CRISPR protocol was performed as described in Gagnon et al. (2014). Oligonucleotide containing the T7 promoter sequence upstream of specific target sites was annealed with a constant oligonucleotide bearing Cas9 binding site. sgRNA were generated by *in vitro* transcription using a T7 RNA polymerase MEGA short script T7 kit (Life Technologies, United States) and purified using miRNeasy kit (Qiagen, Germantown, MD, United States). Cas9 protein (600 ng) and sgRNA (200–400 ng) were co-injected at the one-cell stage, and at least five pooled embryos were used to evaluate the genomic mutation of the targeted genes by PCR analysis. Deletion mutants of *cnga2a* and *cnga2b* were generated by using two sgRNAs that resulted in large genomic deletions in the genes. Germline transmitting zebrafish mutants were generated using sgRNAs directed to the *oxt*, *cnga2a*, and *cnga2b* genes (**Supplementary Table S1**).

Genotyping

Embryos or fin-clips of adult fish were placed in PCR tubes, with 50 μl of lysis buffer (50 mM NaOH) and incubated at 95 °C for 30 min. The samples were then neutralized by the addition of 5 μl of 1 M Tris–HCl (pH 7.5) and 2 μl were taken for 25 μl of PCR mix. WT, heterozygous and homozygous *oxt*^{delta7} and *cnga2a/2b-stop* animals were identified by high-resolution analysis of PCR reaction using 4% MetaPhor agarose (Lonza, Rockland, ME, United States) gels. *cnga2a/2b-del* animals were identified by the analysis of PCR using 1.5% SeaKem LE agarose (Lonza, Rockland, ME, United States) gels. For genotyping primer sequences see **Supplementary Table S1**.

Microinjection of Morpholinos

Antisense morpholino (MO) oligonucleotide directed to *cnga2a translation start site* (Gene Tools, LLC, Corvallis, OR, United States) was used as described previously (Blechman et al., 2007). The stock solution of the translation blocking MO (5′-AACAACAGTTGACAGGTCATCCTGC -3′) was prepared by dissolving in distilled water at 1 mM concentration and embryos were micro-injected with an amount of 1 or 2 ng/embryo at the one-cell stage and allowed to develop at 28.5°C.

Transient Transfection, Immunoblot and Immunofluorescent Staining

For *cnga5* expression in cell culture the *cnga5* open reading frame was cloned into the pcDNA3 vector containing DYK-tag to generate pcDNA3-DYK-cnga5 plasmid. HEK293 cells were grown either on glass coverslips or directly in 12-well plates and were thereafter transfected (at 60% confluence) with a total amount of 1.0 μg/well of either the pcDNA3-*Dyk-cnga5* expression vector or control pcDNA3 together with pcDNA3-*egfp* expressing vector (Addgene, Cambridge, MA, United States) using a standard calcium phosphate transfection method. Cells were harvested 48 h post-transfection in 150 μl of hot SDS sample buffer and 15 μl of the crude protein extract was fractionated by 8% SDS-PAGE followed by immunoblotting with an affinity-purified anti-CNGA5 antibody. Fixation and immunofluorescent staining (IFS) of cell monolayers expressing CNGA5 and EGFP proteins was performed as described previously (Nakamura et al., 2000) using anti-CNGA5, anti-OXT, and anti-GFP primary antibodies.

Immunofluorescent Staining of Zebrafish

Embryos were collected at 6 days post-fertilization and fixed in 4% PFA/PBS. Brains and pituitaries were dissected from 3-month-old zebrafish and subjected to fixation in 4% PFA/PBS. For IFS, PFA-fixed larvae and adult tissues were washed in PBS, dehydrated using methanol 100% and stored at −20°C overnight. IFS was performed according to the protocol described in the zebrafish brain atlas (**RRID**:SCR_000606)[2]. The rehydrated samples were blocked in 500 μL of blocking solution (PBS + 10% goat serum + 1% DMSO + 0.3% Triton X100) that was then replaced with 200 μL of fresh blocking solution with commercial primary antibodies at 1:200 concentrations or 2 μg/ml of anti-CNGA5 mAb and incubated overnight at 4°C. Samples were washed and treated with 200 μL of corresponding secondary antibodies in blocking solution at 1:200 concentrations overnight at 4°C. Then, samples were washed and transferred to 75% glycerol. Embryos were mounted ventrally after removal of the jaws. Adult dissected pituitaries and brain tissues were whole-mounted.

Imaging

Images of fluorescently labeled samples were obtained by using Zeiss LSM 800 inverted confocal microscope with 488, 561, and 647 nm lasers and oil immersion X 40 lenses. Maximum intensity projection images of the whole Z-stacks or subset of Z-stacks were generated using the Zen software (Zeiss). Processing of multiple channel images (i.e., linear adjustments of brightness, contrast and levels) was performed on individual channels using Photoshop CS7 Extended (Adobe).

[1] http://crispr.dbcls.jp

[2] http://zebrafishbrain.org/protocols.php

AUTHOR CONTRIBUTIONS

JB and GL designed the study and wrote the manuscript. JB generated constructs and CRISPR mutants, and performed immunostaining, cell culture assays, Western blot, and confocal imaging. SA contributed to the initial conceptualization of the study and optimized the CRISPR/Cas9 method and genotyping. GM provided the affinity-purified anti-CNGA5 antibody, and shared his unpublished zebrafish *cnga2/3/5* EST sequences and bioinformatic analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00117/full#supplementary-material

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METHODS
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Fast Dynamic *in vivo* Monitoring of Erk Activity at Single Cell Resolution in DREKA Zebrafish

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Precise regulation of signaling pathways in single cells underlies tissue development, maintenance and repair in multicellular organisms, but our ability to monitor signaling dynamics in living vertebrates is currently limited. We implemented kinase translocation reporter (KTR) technology to create DREKA ("**d**ynamic **r**eporter of **Erk a**ctivity") zebrafish, which allow one to observe Erk activity *in vivo* at single cell level with high temporal resolution. DREKA zebrafish faithfully reported Erk activity after muscle cell wounding and revealed the kinetics of small compound uptake. Our results promise that kinase translocation reporters can be adapted for further applications in developmental biology, disease modeling, and *in vivo* pharmacology in zebrafish.

Keywords: Zebrafish (*Danio rerio*), signaling pathway activation, ERK activity dynamics, *in vivo* imaging, *in vivo* pharmacology, wounding

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INTRODUCTION

Signaling pathways underlie cellular behavior during development, repair and disease, but fully understanding the function of any one pathway requires one to follow its dynamic activity within the context of single cells in tissues of a whole living organism. An essential pathway for cell proliferation and differentiation is the evolutionarily conserved mitogen activated protein kinase (MAPK) pathway, consisting of a three kinase phosphorylation relay cascade (e.g., RAF/MEK/ERK) (Krens et al., 2006). Dysregulation of the MAPK pathway can lead to severe developmental abnormalities and diseases (Kim and Choi, 2010; Rauen, 2013; Burotto et al., 2014). Hyperactivating mutations in the RAS/MAPK pathway underlie a group of developmental disorders like Costello or Noonan Syndrome, commonly termed RASopathies, and also occur in many types of cancer, including melanoma (BRAFV600E mutation) and colon cancer (K-RASG12D/G12V) (Forrester et al., 1987; Davies et al., 2002; Rauen, 2013). The RAS/RAF/MEK/ERK signaling cascade has therefore become a major drug target in various cancers and inhibitors for RAF, MEK and ERK are available (Girotti et al., 2015).

The need to understand MAPK signaling activation in normal and disease states has led to the development of live reporters for visualization of kinase activity. Sensors based on Förster resonance energy transfer (FRET) provide great insights by visualizing ERK activity in cultured cells and more recent also in mice and zebrafish, but are difficult to implement and fail to accurately report the downregulation of activity (Vandame et al., 2013; Regot et al., 2014; Depry et al., 2015; Hirata et al., 2015; Hiratsuka et al., 2015; Sari et al., 2018). Regot et al. recently introduced an alternative kinase activity reporter termed kinase translocation reporter (KTR) and demonstrated its high sensitivity *in vitro* (Regot et al., 2014). This technology translates a phosphorylation event into a nucleo-cytoplasmic shuttling event of the synthetic reporter, which can easily be observed

by fluorescence microscopy. We reasoned that transferring KTR technology to zebrafish would result in novel vertebrate kinase activity reporters with unprecedented temporal resolution and sensitivity. Due to its transparency and external development, zebrafish is ideally suited for *in vivo* fluorescence microscopy investigations. Current zebrafish pathway reporters like the FGF reporter *Tg(Dusp6:d2EGFP)*^{pt6} strain are based on expression of destabilized fluorescent proteins with a half-life of ∼2 h (Molina et al., 2007). Still, fast and dynamic changes in signaling activity cannot be visualized by such reporters. Here, we generated a KTR-based Erk activity reporter zebrafish strain (DREKA) and successfully demonstrated its ability to visualize fast Erk signaling dynamics in a wound response and its possible application for *in vivo* pharmacology.

MATERIALS AND METHODS

Maintenance of Fish

Zebrafish (*Danio rerio*) were maintained at standard conditions (Kimmel et al., 1995; Westerfield, 2000) according to the guidelines of the local authorities under licenses GZ:565304/2014/6 and GZ:534619/2014/4.

Plasmid Construction

The DREKA transgenesis vector #260 (pDESTubi:ERK-KTR-CloverpATol2) was generated by Gateway® recombination using p5'ubiquitin, pSR1835 containing (pENTR)ERK-KTR-Clover (addgene #59138), p3'pA and pDestTol2pA4 vectors.

The T55L/T62L reporter was created using Gibson assembly (NEBuilder Hifi DNA assembly cloning kit, E5520, New England BioLabs GmbH, Frankfurt, Germany) of PCR fragments amplified from #260 using the following primer pairs:

639_ubiERK-TtoL1R: ACGTGGCTTCTTCGATGGcagCGCTG/

513: CATTTGGACAATTTTGCTGCAGGTAAAATGC and

640_ubiERK-TtoL2-F: tgCCATCGAAGAAGCCACGTctgCCATC/

516: TCGCCCTTGCTCACCATACTAGTGGA and ligated into #260 opened by PstI/SpeI digest (New England BioLabs GmbH, Frankfurt, Germany).

The T55V/T62V reporter was created using Gibson assembly (NEBuilder Hifi DNA assembly cloning kit, E5520) of PCR fragments amplified from #260 using the following primer pairs:

641_ubiERK-TtoV1-R: ACGTGGCTTCTTCGATGGcacCGCTG/

513: CATTTGGACAATTTTGCTGCAGGTAAAATGC and

642_ubiERK-TtoV2-F: tgCCATCGAAGAAGCCACGTgtgCCATC/

516: TCGCCCTTGCTCACCATACTAGTGGA

and ligated into #260 opened by PstI/SpeI digest.

The T55D/T62D reporter was created using Gibson assembly (NEBuilder Hifi DNA assembly cloning kit, E5520) of PCR fragments amplified from #260 using the following primer pairs:

517:ubiERK-TtoD1-R: ACGTGGCTTCTTCGATGGGTCCGCTG/513: CATTTGGACAATTTTGCTGCAGGTAAAATGC and

518: ubiERK-TtoD2-F: acCCATCGAAGAAGCCACGTgacCCATC/

516: TCGCCCTTGCTCACCATACTAGTGGA and ligated into #260 opened by PstI/SpeI digest.

In vitro Transcription of RNA

RNA for microinjection was transcribed *in vitro* using the Invitrogen™ mMessage mMachine™ SP6 transcription kit according to the manufacturer's recommendations (Ambion, AM1340, Waltham, MA, USA).

Microinjection for Transient Assays and Generation of Transgenic Strains

DNA/RNA injection was performed using injection capillaries (glass capillaries GB100F-10, with filament, Science Products GmbH, Hofheim, Germany) pulled with a needle puller (P-97, Sutter Instruments, Novato, USA) mounted onto a micromanipulator (M3301R, World Precision Instruments Inc., Berlin, Germany) and connected to a microinjector (FemtoJet 4i, Eppendorf, Hamburg, Germany).

For transient assays, fertilized Sanger AB Tübingen (SAT) eggs were injected with 25 pg pDESTubi:ERK-KTR-CloverpATol2 and 20 pg H2B-CFP mRNA.

MAPK pathway activation experiments were carried out by co-injecting KalTA4 mRNA (20 pg), H2B-CFP:UAS:HRASG12V (20 pg), and pDESTubi:ERK-KTR-CloverpATol2 (25 pg) at the one cell stage.

To create transgenic zebrafish, 20 pg Tol2 mRNA and 25 pg pDESTubi:ERK-KTR-CloverpATol2 were injected into fertilized SAT eggs at the one cell stage.

mClover expressing embryos were raised to adulthood and screened for germline transmission.

Chemical Inhibition

Transiently ERK-KTR-Clover expressing SAT or DREKA zebrafish embryos were dechorionated and incubated in the following compounds from 29 to 48 hpf: PD98059 (30 μM), vemurafenib (10 μM), PD0325901 (5 μM), trametinib (10 μM), and ulixertinib (1 μM). Stock solutions of compounds were in DMSO and control experiments were carried out in 0.1% DMSO. All compounds were purchased via MedChemTronica, Stockholm, Sweden with the respective ordering numbers HY-12028, HY-12057, HY-10254, HY-10999, HY-15816. Images were recorded at 48 hpf and Erk activity status was analyzed ($n = 5$ embryos each condition, except for vemurafenib $n = 4$ embryos).

Leptomycin B (Cat No. L2913, Sigma Aldrich, Saint Louis, USA) treatment was carried out from 26 hpf for 24 h at 92 μM. Leptomycin B stock solution was in 70% methanol and control experiments were carried out in 0.7% methanol/E3.

Imaging

Zebrafish embryos were prepared for imaging as described previously. (Distel and Köster, 2007). In brief, zebrafish embryos were dechorionated, anesthetized using 1x tricaine in E3 medium (0.16 g/l tricaine (Cat No. E1052110G, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), adjusted to pH 7 with 1M

Tris pH 9.5, in E3), and embedded in 1.2% ultra-low gelling agarose (Cat. No. A2576-25G, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in a glass bottom imaging dish (D35-14-1.5-NJ, Cellvis, Mountain View, CA, USA). Images and time-lapse movies were recorded on a Leica SP8 X WLL confocal microscope system.

Image and Movie Rendering

Images were rendered using Photoshop CS6 (Adobe), Leica LAS X software, Quicktime Pro and Fiji.

Needle Induced Wounding

Zebrafish embryos were anesthetized in 1x tricaine/E3, embedded in 1.2% ultra-low gelling agarose in an imaging dish and manually wounded by introducing a small puncture using an injection needle.

Laser Induced Wounding

Zebrafish embryos were embedded for imaging as described above. A laser-inflicted wound was introduced using the Leica SP8 X FRAP module and laser lines 405, 458, 476, and 488 nm simultaneously at ∼75% laser power. A region of interest was selected manually and was illuminated for 80 to 90 s. After ∼40 s a wound started to appear.

Compound Kinetics Experiments

To investigate compound uptake kinetics, zebrafish embryos (55 hpf) were embedded in 1.2% ultra-low gelling agarose containing 10 μM trametinib. Embedded embryos were covered with 10 μM trametinib in 1x tricaine /E3/PTU (Cat. No. P762925G, Sigma- Aldrich GmbH, Steinheim, Germany) and imaged continuously on a Leica SP8 X WLL system.

Quantification of ERK Signaling Activity

To quantify Erk signaling activity, Clover intensity was measured in the nucleus and in the cytoplasm of cells by selecting the respective region using the intensity v time monitor tool of the time series analyzer plugin for Fiji (J. Balaji, UCLA). The cytoplasmic to nuclear intensity ratio was calculated using Microsoft Excel.

Immunofluorescence

Wounded and control zebrafish embryos (48 hpf) were fixed in 4% paraformaldehyde/PBS (Cat. No 15710-S, Electron Microscopy Sciences, Hatfield, PA, USA) for 4 h at room-temperature. Afterwards zebrafish embryos were transferred into 100% methanol and were incubated at −20°C overnight. Then embryos were transferred into acetone (7 min at −20°C) and incubated in H_2O (room-temperature for 1 h). After washing with PBST (PBS with 0.1%Tween20) embryos were incubated in 150 mM TrisHCl (pH 9) (70°C for 15 min). After washing in PBST embryos were blocked in 10% normal goat serum (NGS) in PBST. Samples were incubated in p-ERK antibody (Cell Signaling technology, Cat. No. #4370) at 1:400 in 10% NGS in PBST overnight at 4°C. The primary antibody was removed and embryos were washed in PBST (6 × 15 min). A secondary antibody, Alexa Fluor 568 goat anti-rabbit antibody (Cat. No. A-21069, Invitrogen) was used at 1:2,000 together with DAPI in 10% NGS in PBST for overnight incubation at 4°C. After this incubation step embryos were washed in PBST (6 x 15 min) and imaged.

RESULTS

In order to generate a highly dynamic reporter for Erk activity, we aimed to adapt KTR technology for its application in zebrafish. The ERK reporter ERK-KTR-Clover consists of an ERK docking site fused to nuclear localization (NLS) and nuclear export signals (NES) and the fluorescent protein mClover. Upon phosphorylation of phospho-acceptor sites within the NLS the export signal overrides the import signal and the green fluorescent reporter localizes from the nucleus to the cytoplasm, hereby visualizing ERK activity (see **Figure 1B**; Regot et al., 2014).

We first investigated *in silico*, if zebrafish Erk1/2 would be able to bind to the reporter construct, which carries the minimal ERK specific docking site (F-site) of mouse Elk1 (FQFP), which is also present in *Danio rerio* Elk1 (Jacobs et al., 1999). ERK1/2 is generally well conserved between human, mouse and zebrafish and the F-site recruitment site (FRS), which binds to the F-site, is present in zebrafish, suggesting that the synthetic KTR construct will likely be able to report ERK activity in zebrafish (**Figure S1**) (Roskoski, 2012; Busca et al., 2016). To test this, we next placed ERK-KTR-Clover under control of the zebrafish *ubiquitin* promoter and transiently expressed the reporter in zebrafish embryos (**Figure 1A**; Mosimann et al., 2011; Regot et al., 2014). In cells of these embryos, ERK-KTR-Clover was found either in the cytoplasm sparing the nucleus, in the cytoplasm and nucleus or predominantly in the nucleus as confirmed by co-expression with the nuclear marker histone2B–CFP (H2B-CFP), indicating various degrees of Erk activity (Distel et al., 2010). We also observed cell type specific differences in reporter localization, e.g., skin epithelial cells showed dynamic and generally higher reporter intensity in the cytoplasm, whereas muscle cells showed stronger reporter signal in the nucleus at 48 hpf ($n = 385$ cells, 10 embryos) (**Figures 1C,C′**). Control reporter constructs, where threonines within the NLS were replaced by either leucine (T55L/T62L) or valine (T55V/T62V) were found in nuclei of all cell types investigated and a phosphomimetic reporter variant T55D/T62D localized to the cytoplasm ($n =$ 132 muscle cells, 3 embryos), indicating that the localization of the reporter is indeed regulated by phosphorylation in zebrafish (**Figure S2**).

We next tested if the ERK-KTR reporter responds to inactivation and activation of the MAPK pathway in skin epithelial and muscle cells. Applying a MEK inhibitor (30 μM PD98059) for 17 h resulted in nuclear localization of the reporter in the majority of cells at 48 hpf ($n = 300$ cells, 5 embryos) (**Figures 1D,D′**). In contrast, stimulation of Erk signaling by co-expression of a constitutively active RAS (H-RAS^{G12V}) shifted reporter localization to the cytoplasm of muscle cells at 48 hpf ($n = 235$ cells, 5 embryos) (**Figures 1E,E′**). These results suggested that ERK-KTR-Clover faithfully reports Erk activity in skin epithelial and muscle cells in living zebrafish embryos.

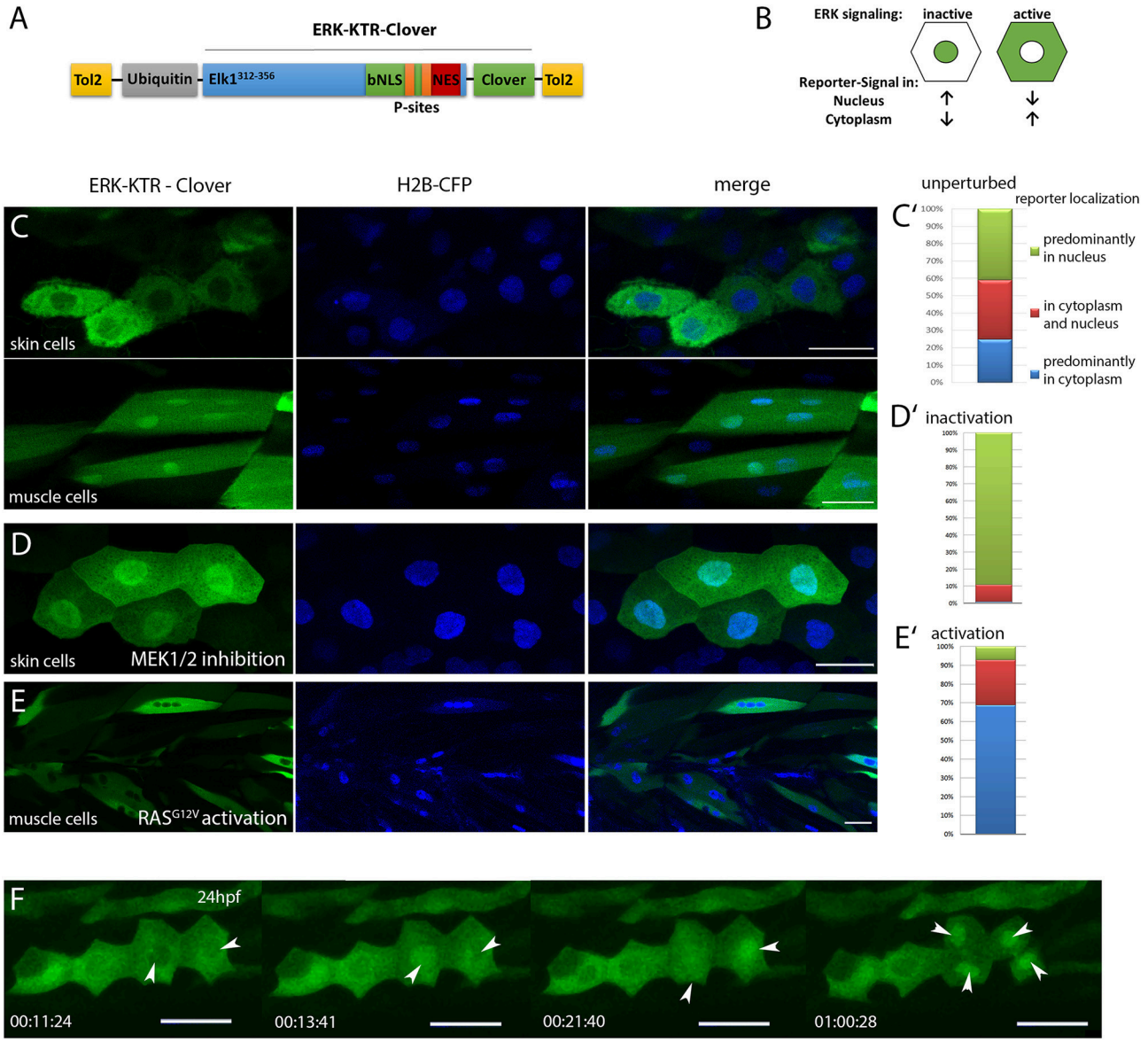

FIGURE 1 | Characterization of ubi:ERK-KTR-Clover in zebrafish embryos. **(A)** Schematic illustration of the DREKA transgenesis vector pDESTubi:ERK-KTR-CloverpATol2. (Tol2, Tol2 recombinase recognition sites; ubiquitin, ubiquitin promoter; Elk1$^{312-356}$, ERK docking sites from Elk1; bNLS, bipartite nuclear localization signal; P-sites, phosphorylation sites; NES, nuclear export signal; modified after Regot et al., 2014) **(B)** Schematic depiction of the ERK-KTR reporter principle. ERK signaling inactive: reporter is mainly localized in the nucleus; ERK signaling active: reporter is localized in the cytoplasm sparing the nucleus. **(C)** pDESTubi:ERK-KTR-CloverpATol2 and H2B-CFP mRNA co-injected wildtype zebrafish embryos express ERK-KTR-Clover in a mosaic manner. mClover fluorescence is mainly found in the cytoplasm of skin epithelial cells, indicating active Erk signaling (upper panel) and in the nucleus of muscle cells indicating absence of Erk signaling activity (lower panel). **(C′)** Quantification of ERK-KTR localization in skin epithelial cells and muscle cells at 48 hpf. Green, mClover fluorescence in the nucleus; Red, mClover fluorescence distributed throughout the cell; Blue, mClover fluorescence in the cytoplasm sparing the nucleus ($n = 385$ cells, 10 embryos at 48 hpf). **(D)** Skin epithelial cells of co-injected embryos incubated with MEK1/2 inhibitor PD98059 (30 μM) overnight show mClover fluorescence in the nucleus at 48 hpf indicating the reporter responds to Mek inhibition. **(D′)** Quantification of mClover localization shows reporter shuttling to the nucleus after MEK1/2 inhibition ($n = 300$ cells, 5 embryos at 48 hpf). **(E)** Muscle cells expressing constitutively active HRAS (zebrafish injected with pDESTubi:ERK-KTR-CloverpATol2, H2B-CFP:UAS:HRASG12V, and KalTA4 mRNA Distel et al., 2009) show active Erk signaling at 48 hpf. **(E′)** Quantification of mClover localization shows constitutively active HRAS induced reporter shuttling to the cytoplasm ($n = 235$ cells, 5 embryos at 48 hpf). **(F)** Mitotic skin epithelial cells of pDESTubi:ERK-KTR-CloverpATol2 injected zebrafish at 24 hpf. Dividing skin epithelial cells (white arrowheads) show dynamic Erk signaling with a sudden change from cytoplasmic to nuclear reporter localization before cytokinesis [compare time points 11:24 min/13:41 min (left arrowhead) and 13:41 min/21:40 min (right arrowhead)]. After cytokinesis the reporter remains in the nuclei of both daughter cells (arrowheads at 1:00:28 h). Images taken from a time-lapse movie (**Movie 2**) are maximum projections of several planes. All scale bars are 25 μm. All images were recorded on a Leica SP8 X WLL confocal microscope and rendered using Photoshop CS6.

Finally, we probed the temporal resolution of the reporter by recording time-lapse movies of ERK-KTR-Clover injected embryos. This revealed changes in Erk activity within minutes in skin epithelial cells over time (**Movie 1**). Interestingly, dividing cells showed a stereotypical pattern of the reporter shuttling to the nucleus right before cytokinesis and staying in the nuclei of both daughter cells afterwards, indicating low Erk activity after cell division (**Figure 1F** and **Movie 2**).

With these transient assays being successful, we next generated transgenic zebrafish *[Tg(ubi:ERK-KTR-Clover)^{vi1}]*, expressing ERK-KTR-Clover under control of the rather weak, but ubiquitous *ubiquitin* promoter in order to be able to investigate Erk signaling over longer periods of time in a non-mosaic manner (Mosimann et al., 2011). We named *Tg(ubi:ERK-KTR-Clover)^{vi1}* "DREKA" for "**d**ynamic **r**eporter of **E**r**k** **a**ctivity." DREKA zebrafish were viable, showed no obvious morphological defects and were fertile (now in F4), indicating that the reporter does not negatively affect endogenous Erk signaling. F2 DREKA were confirmed to report changes in Erk activity by applying different inhibitors of the MAPK pathway. As expected MEK inhibitors trametinib (10 μM) or PD0325901 (5 μM) and ERK inhibitor ulixertinib (1 μM) decreased Erk signaling activity in skin epithelial cells of DREKA embryos, but vemurafenib (10 μM) a type I BRAF inhibitor specific for mutant BRAF (BRAF^{V600E}) did not (**Figure S3**). These results confirmed that DREKA report changes in Erk activity upon external manipulation of the MAPK pathway.

In cells of DREKA zebrafish, changes in reporter localization lead to changes in mClover fluorescence intensity in the cytoplasm (C) and the nucleus (N). Calculating the C/N intensity ratio is thus a way to visualize and quantify relative changes in Erk activity over time on the single cell level. In muscle and skin epithelial cells, the C/N ratio ranged approximately from 0.6 to 1.5 in untreated DREKA zebrafish. Shuttling of the reporter to the cytoplasm by nuclear export is believed to be Exportin dependent. Indeed, inhibiting Exportin 1 by leptomycin B treatment (92 μM for 24 h) led to accumulation of the reporter in the nucleus reaching C/N intensity ratios of up to 0.23–0.27 in muscle and skin cells (typically 0.6–0.7 in untreated zebrafish) (**Figure S4**; Kudo et al., 1999). This shows that the reporter concentration is not reaching saturation in the nucleus in these cell types in untreated DREKA fish.

We next aimed to apply DREKA to assess the temporal dynamics of Erk signaling in a biological process under experimental conditions with control over an external stimulus eliciting Erk signaling and turned to a wounding assay.

Upregulation and correct temporal orchestration of ERK activity is necessary for proper wound healing across species. In *Xenopus* embryos, two temporally distinct phases of wound healing have been observed: an early and fast phase with high Erk activity and a second slow phase with high PI3K activity (Li et al., 2013).

We asked if Erk signaling dynamics are similar in zebrafish embryos after wounding muscle cells. To verify that wounding activates Erk signaling, we punctured muscle tissue of 48 hpf DREKA embryos using a glass needle. Indeed, muscle cells close to the wound showed Erk signaling activation whereas muscle cells further away remained unaltered (n = 6 embryos; **Figures 2A,A′**). Erk signaling activity in muscle cells surrounding the wound was independently confirmed by immunofluorescence for phosphorylated Erk (n = 10 embryos; **Figures 2B,B′**). We next switched to a laser-induced wounding assay, which enabled us to follow Erk signaling changes continuously from the moment the wound was introduced. We detected an almost immediate response in neighboring cells after wounding of zebrafish muscle by high power laser illumination for 80–90 s (**Figures 2C,D**). The intensity of mClover in the cytoplasm increased steadily with the cytoplasmic/nuclear intensity ratio becoming >1 between 2 and 3 min and peaking around 4 min after wounding in fast responding cells (n = 7 cells, 3 embryos) (**Figures 2C,D** and **Movie 3**). Directly neighboring cells (**Figure 2C**, red arrow) responded first and subsequently Erk signaling activity also increased in cells further away (**Figure 2C**, purple arrow) from the wound. Spreading of Erk activity within one cell type (epithelial cells) was reported previously in mouse (Hiratsuka et al., 2015). Here, we observed two cell types, muscle cells and skin epithelial cells, relaying Erk signaling (**Figures 2C,E** and **Movie 3**, **4**). After ∼45 min muscle cells more distant to the wound started to become inactive for Erk signaling again and cells adjacent to the wound followed around 1 h after wounding (n = 6 embryos; **Figures 2E,F**). Intriguingly, we observed wounds, which spontaneously ruptured a second time after Erk signaling had already decreased, inducing another rapid, and simultaneous activation of Erk signaling in surrounding cells (**Figure 2F** and **Movie 4**). In such cases, live monitoring of Erk activity is of tremendous advantage as second rupture events would have likely been missed with current methods (e.g., immunofluorescence or Western Blotting), complicating the interpretation of the Erk signaling pattern (**Movie 4**). Wounding muscle cells in the presence of MEK1/2 inhibitors trametinib (10 μM, **Movie 5**) or TAK-733 (10 μM, **Movie 6**) did not elicit a translocation of the reporter as observable in 0.1% DMSO control experiments (**Movie 7**) (n = 4 embryos each), confirming that changes in reporter localization are MAPK dependent in our muscle wounding assay.

We next assessed the use of DREKA for *in vivo* pharmacology. Zebrafish is a popular model organism for small compound screening due to the ease of compound administration to the water. However, the kinetics of compound uptake, although of great importance are often unknown. To reveal uptake kinetics DREKA fish were exposed to 10 μM trametinib (MEK 1/2 inhibitor) and Erk activity was continuously monitored in skin epithelial cells, which showed predominantly active Erk signaling in their unperturbed state (**Figure 3A**). Ten minutes after treatment Erk signaling activity was still strong, however already after ∼20 min a significant reduction in activity was detected with full inhibition being visible after around 50–60 min (n = 6 embryos) (see **Figure 3** and **Movie 8**). Only mitotic skin cells maintained active Erk signaling, a finding that is consistent with previous research describing the insensitivity of mitotic cells to Mek inhibition (Hiratsuka et al., 2015). This indicates the potential of DREKA zebrafish and KTR technology for *in vivo* pharmacology at cellular resolution.

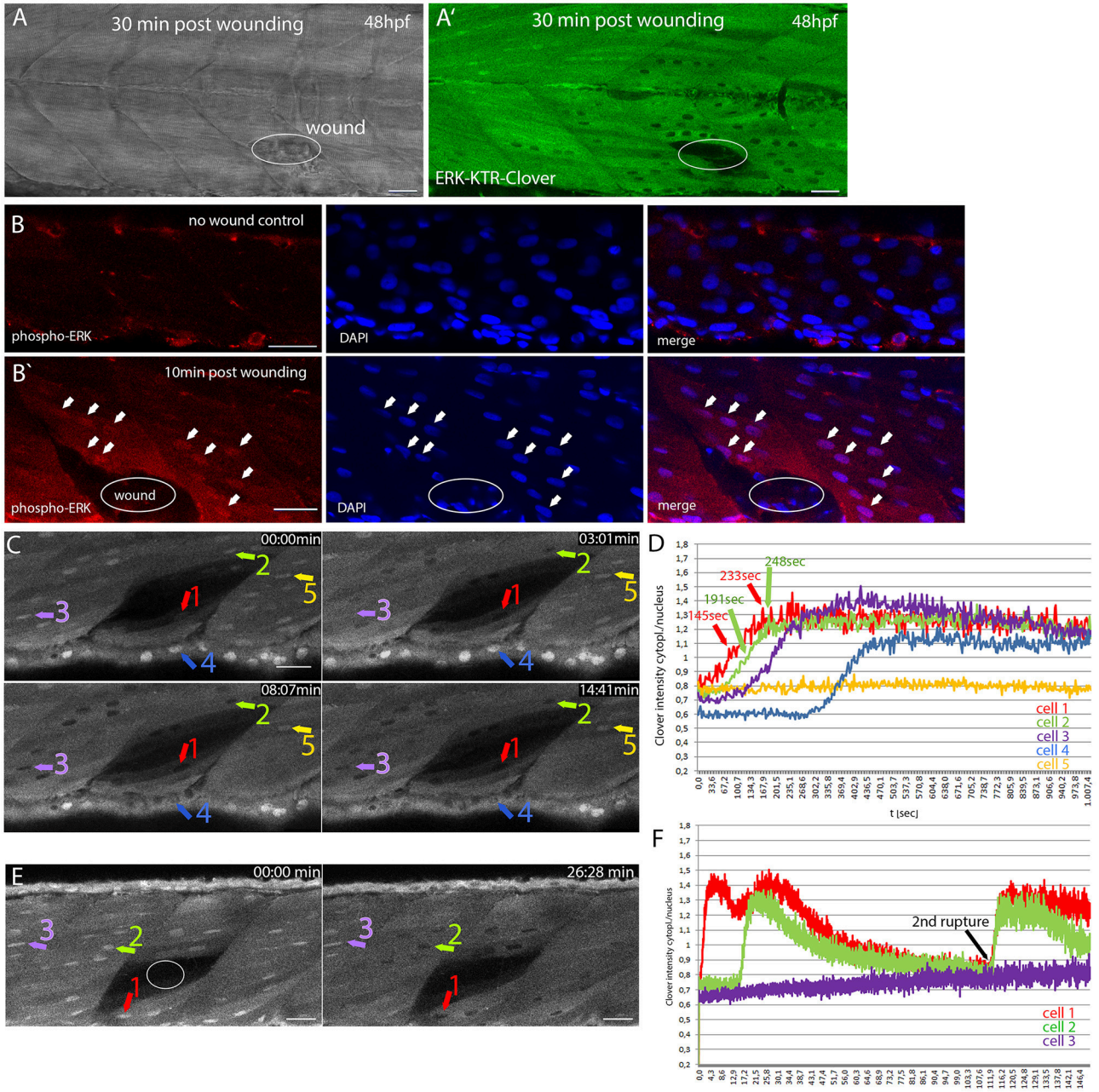

FIGURE 2 | Monitoring dynamic Erk activity in DREKA zebrafish **(A)** Brightfield image and fluorescence image **(A')** of a DREKA embryo wounded with a glass needle at 48 hpf. Images were taken 30 min after wounding. Active Erk signaling was observed in muscle cells around the wound ($n = 6$ embryos). **(B)** Immunofluorescence staining for phosphorylated Erk (red) in muscle cells of control and zebrafish embryos wounded with a glass needle 10 min post-wounding **(B')**. DAPI staining is shown in blue. Arrows mark some of the cells with phosphorylated Erk being visible in the nucleus surrounding the wound (circle). **(C)** Erk signaling activation in muscle and skin epithelial cells after laser induced wounding in 54 hpf DREKA. Four time points (0, 3, 8, and 14 min) taken from time-lapse **Movie 3** show the fast activation of Erk signaling. **(D)** Quantification of Erk activity in 5 cells as depicted in **(C)** starting ~65 s post-wound appearance. Erk activity is shown as cytoplasmic/nuclear ratio of mClover intensity over time (seconds). Cells close to the wound [#1(red) and #2(green)] show a fast response with higher reporter concentrations in the cytoplasm compared to the nucleus around 145 s (cell #1) and 191 s (cell #2) post-wound appearance and peaking after ~233 s (cell #1) and 248 s (cell #2), respectively. Cells further away respond later [#3(purple)] or remain inactive [#5(yellow)]. In addition to muscle cells, Erk signaling activation was also observed in skin epithelial cells [#4(blue)]. **(E)** Erk signaling activity in response to laser induced wounding of muscle cells in 72 hpf DREKA. mClover fluorescence (shown in gray) at 0 and 26 min after the wound (circle) has been introduced (see **Movie 4**). After wounding mClover starts to localize from the nucleus to the cytoplasm in cells directly adjacent to the wound, indicating Erk signaling activation (cell #1). Muscle cells further away from the wound become active for Erk signaling at later time points (cell #2) or remain inactive (cell #3). **(F)** Quantification of Erk activity in 3 cells as shown in **(E)** over 2.5 h starting ~40 s post-wound appearance (see **Movie 4**). Erk signaling activity is shown as cytoplasmic to nuclear mClover intensity over time (minutes) for one muscle cell close to the wound (#1 red), one muscle cell further away (#2 green), and one non-responding muscle cell (3# purple) (colored arrows in **E)**. Note the second rupture event leading to a rapid activation of Erk signaling. All scale bars are 25 μm. All images were recorded on a Leica SP8 X WLL confocal microscope and rendered using Photoshop CS6.

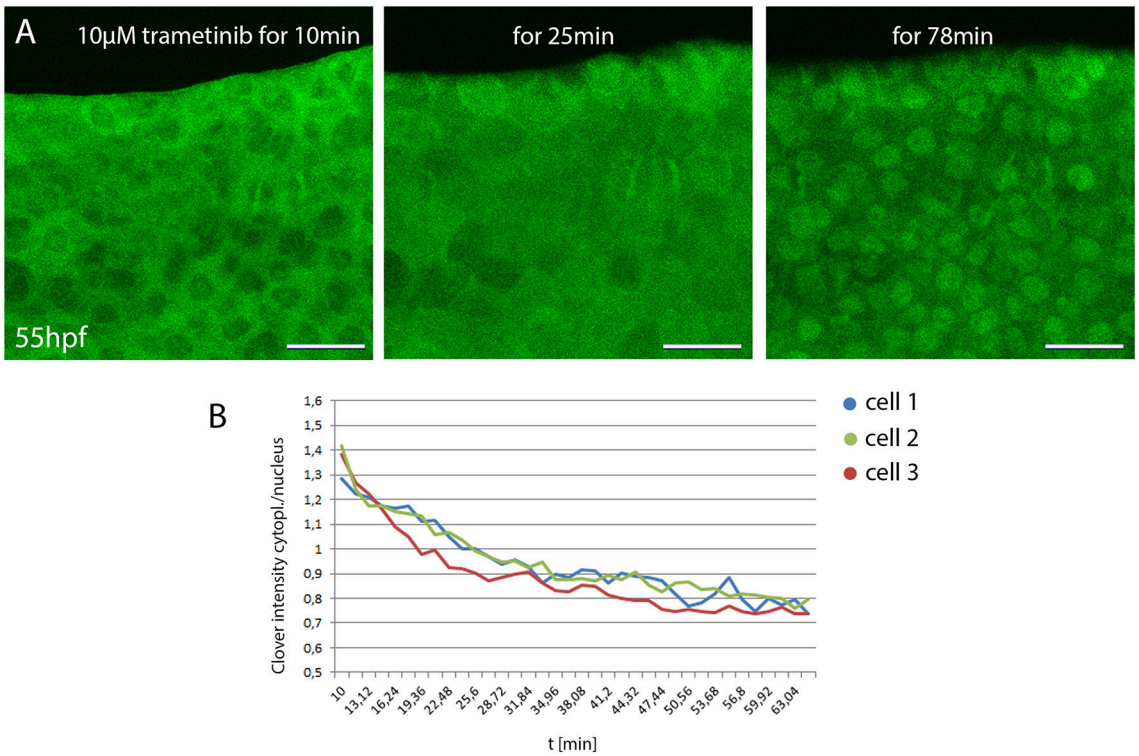

FIGURE 3 | Monitoring small compound uptake kinetics in DREKA zebrafish **(A)** 55 hpf DREKA embryos were embedded in agarose containing 10 μM trametinib to investigate compound uptake kinetics in skin epithelial cells. After 10 min skin epithelial cells still show active Erk signaling. Around 20 min Erk activity is visibly decreased and by 50–60 min cells show full Erk signaling inhibition. **(B)** Quantification of Erk activity over time. Cytoplasmic to nuclear mClover intensity over time in three representative skin epithelial cells in DREKA zebrafish treated with 10 μM trametinib at 55 hpf. All scale bars are 25 μm. All images were recorded on a Leica SP8 X WLL confocal microscope and rendered using Photoshop CS6.

DISCUSSION

Recently, KTRs were introduced to report kinase activity with higher sensitivity and higher temporal resolution than commonly used kinase fusion or FRET based reporters *in vitro* (Regot et al., 2014). KTRs were also successfully applied in *C. elegans* (de La Cova et al., 2017). Here, we demonstrate that KTR technology can be transferred to a vertebrate model organism for real-time kinase activity monitoring. We created the zebrafish Erk activity reporter *tg(ubi:ERK-KTR-Clover)*^{vi28} (DREKA), containing an ERK-KTR, reported to not cross-react with p38 or JNK (Regot et al., 2014). DREKA zebrafish are viable, show no morphological abnormalities and are fertile (now in F4), indicating that ERK-KTR does not have adverse effects on zebrafish development when expressed under the *ubiquitin* promoter. We show that ERK-KTR faithfully reports changes in Erk activity in zebrafish skin epithelial cells and muscle cells by chemical and genetic perturbation as well as using phosphomimetic and "phospho-dead" reporter constructs. The dynamic range of this KTR appears well suited for measurements in muscle and skin cells with a relatively large cytoplasm. The cytoplasmic to nuclear (C/N) reporter intensity ratio, used as readout for Erk activity, typically ranges from 0.6 to 1.5, allowing one to monitor a broad range of Erk activity without saturation in skin and muscle cells. Whether this holds true for all cell types in the developing zebrafish needs further investigation as observed minimal and maximal C/N intensities vary between different cell types. In fact, in contrast to *C. elegans*, in zebrafish neural cells within the CNS, the reporter localized predominantly to the cytoplasm at all investigated time points. However, (T55L/T62L) and (T55V/T62V) reporter variants were present in nuclei of neural cells within the CNS (**Figure S2**). This suggests that either due to its high sensitivity ERK-KTR is already reporting very weak Erk activity in neural cells or that the C/N baseline ratio of reporter localization is shifted in this cell type. Multiple factors including cellular concentrations of nuclear import/export machinery proteins (e.g., importins, exportins, and Ran proteins) as well as phosphatases and cell morphology (cytoplasmic to nuclear volume ratio) can influence the localization and the relative intensity of the reporter in the respective compartment and thus the C/N ratio. Therefore, optimizing the NLS for reporter usage in neural cells in zebrafish, similar to modification attempts for *C. elegans* could improve the ERK activity reporter for this cell type (de La Cova et al., 2017). In addition, second generation constructs including a nuclear marker being co-expressed with the KTR can be used to solely measure changes in nuclear reporter intensity (de La Cova et al., 2017).

Although no absolute values of Erk activity can currently be stated, the generated transgenic Erk reporter strain (DREKA) offers unprecedented temporal resolution for monitoring changes in Erk activity in specific zebrafish cell types. State-of-the-art reporter strains are based on expression of destabilized fluorescent proteins. Thus, they suffer from delays reporting the onset and offset of signaling activity and are not capable of reporting fast dynamic signaling processes. In contrast, a readout based on nucleo-cytoplasmic shuttling of the fluorescent KTR reporter is not subject to limitations imposed by protein expression and stability rates.

In our wounding assay we observed an immediate increase (within seconds) of the cytoplasmic to nuclear reporter distribution, indicating Erk signaling activation with a peak after ∼4 min. Previously, it had been reported that the Ras/Erk signaling module acts as a high-bandwidth and low-pass filter with the need for an external stimulus to persist for at least 4 min to activate the Ras/Erk module *in vitro* (Toettcher et al., 2013). If the faster response in our assay is due to actual differences between the *in vitro* and *in vivo* situation or if activation dynamics are cell type or stimuli specific, needs further investigations. Sensitivity differences of the used ERK reporter might also play a role. Consistent with possibly faster responses, Erk activity was found to be active 2 min after wounding in a *Xenopus* embryo wound assay based on Western blot analysis of Erk phosphorylation (Li et al., 2013). In *Xenopus*, Erk remained active between 30 and 60 min matching our observation in DREKA fish. This suggests that Erk activity dynamics might be similar in response to wounding across different cell types and developmental time points in *Xenopus* and zebrafish.

As pilot experiments indicated the feasibility of differential Erk activity analysis in selected cells during zebrafish development, we envision that DREKA fish will be useful for developmental biologists to decipher when and where Erk activity is needed for proper tissue formation. Here, we have also generated an UAS:ERK-KTR variant to be readily combined with available Gal4 zebrafish strains for tissue specific analysis of Erk activity. Moreover, KTR-enabled monitoring of Erk signaling activity in various mutant zebrafish and disease models, including cancer models, will reveal potentially altered signaling dynamics. Here, the development of software tools for automated analysis will soon be required due to the large datasets created by such experiments.

DREKA zebrafish and KTR technology also promise to be useful for pharmacological applications. For example effects of single or combination of compounds targeting Erk signaling can be investigated as we have shown by applying MEK, ERK, or RAF inhibitors (see **Figure 3** and **Figure S3**). Off target effects of compounds, which are not primarily directed at the MAPK pathway will also be revealed. Although zebrafish is widely used in drug screening, the understanding of pharmacokinetics in zebrafish is currently lacking behind. A recent study applied liquid chromatography-mass spectrometry (LC-MS) based methods to determine paracetamol concentrations in zebrafish larvae (Kantae et al., 2016). Complementing such rather laborious approaches, DREKA offer a direct means to determine the time small compounds added to the water need to accumulate inside a cell at a concentration able to inhibit Erk activity as we demonstrated for trametinib (**Figure 3**). The ability to measure an effect on single cells within an intact organism in real time will also be beneficial to understand how drugs work *in vivo* and why they might fail. In comparison to conventional pharmacology approaches at tissue or organ level, single cell *in vivo* pharmacology is likely to enhance drug development (Vinegoni et al., 2015).

Finally, kinase translocation technology can be used to create reporters for various kinases, including JNK, p38, PKA, or AKT (Regot et al., 2014; Maryu et al., 2016). Multiplexing possibilities arise, which will allow one to dissect the interplay of various signaling pathways in a cell type specific way *in vivo*. This capability will likely have impact on our understanding of vertebrate development and disease.

ETHICS STATEMENT

All procedures involving animals were carried out according to EU guidelines and Viennese legislation (licenses: GZ:565304/2014/6 and GZ:534619/2014/4).

AUTHOR CONTRIBUTIONS

VM and CS performed experiments, analyzed data, and wrote the manuscript. MS performed experiments. SG generated reporter constructs. MD designed and performed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00111/full#supplementary-material

Figure S1 | *In silico* cross-species analysis of Erk binding to ERK-KTR. **(A)** Alignment of zebrafish (*Danio rerio*) and mouse (*Mus musculus*) Elk1. The conserved FQFP motif of the F-site (ERK docking site) is shown in red. **(B)** Alignment of human, mouse and zebrafish Erk2. Amino acids M199, L200, Y233, L234, L237, and Y263 (human numbering), which form the F-site recruitment site are shown in red (Roskoski, 2012). Alignments were performed on ensembl.org.

Figure S2 | Localization of ERK-KTR control constructs T55V/T62V and T55D/T62D. 48 hpf zebrafish embryos expressing either ERK-KTR-mClover or T55V/T62V and T55D/T62D control reporter constructs. **(A)** Neural cells within the

CNS expressing ERK-KTR-mClover. The reporter localizes to the cytoplasm. **(B)** The T55V/T62V control reporter is also found in the nucleus of neural cells within the CNS. **(C)** Muscle cells expressing ERK-KTR-mClover. **(D)** The phosphomimetic T55D/T62D reporter construct localizes predominantly to the cytoplasm when expressed in muscle cells. All scale bars are 25 μm. Images were recorded on a Leica Sp8 X WLL confocal system and rendered with Adobe Photoshop CS6.

Figure S3 | Effects of different inhibitors on Erk activity in DREKA zebrafish. Compounds were applied to F2 DREKA embryos at 29 hpf and quantification was performed on skin epithelial cells at 48 hpf. Percentage of cells with active Erk signaling shown as box plot from left to right: untreated control; ERK1/2 inhibitor: ulixertinib (1 μM); MEK1/2 inhibitor: trametinib (10 μM), PD0325901 (5 μM); B-RAF inhibitor: vemurafenib (10 μM); [each dot represents one embryo; box plot was created using R (r-project.org)].

Figure S4 | Effects of nuclear export inhibition by leptomycin B. 26 hpf DREKA zebrafish larvae were treated with the nuclear export inhibiting compound leptomycin B at 92 μM for 24 h. **(A,C,E)** Treated DREKA, **(B,D,F)** untreated control DREKA. Muscle cells **(A,B)**, neural cells in the hindbrain **(C,D)**, and cranial ganglion cells **(E,F)** all show increased reporter signal in the nucleus in treated embryos compared to untreated siblings. Scale bars in **(A,B,D)** are 50 μm, in **(C,E,F)** 25 μm. Images were recorded on a Leica Sp8 X WLL confocal system using a 25x objective. Images were rendered using Adobe Photoshop CS6.

Movie 1 | DREKA reveal dynamic Erk signaling in skin epithelial cells during development. Time-lapse movie of transiently ERK-KTR-Clover (green) expressing zebrafish embryos from ∼48 to 54 hpf. Dynamic Erk activity can be observed as nuclear cytoplasmic shuttling of mClover in skin epithelial cells (see arrow). Z-stack images were recorded approximately every 3.5 min on a Leica SP8 X WLL confocal microscope using a 40x objective. Z-stacks are shown as maximum projections.

Movie 2 | Erk activity in dividing cells. Time-lapse movie of transiently ERK-KTR-Clover (green) expressing zebrafish embryos from ∼24 to 32 hpf. Dividing cells (arrows) show a stereotypical pattern of Erk signaling activity. Z-stack images were recorded approximately every 1 min on a Leica SP8 X WLL confocal microscope using a 40x objective. Z-stacks are shown as maximum projections.

Movie 3 | Fast Erk signaling response in muscle and skin epithelial cells surrounding a wound. 54 hpf DREKA embryos were wounded by laser illumination. Erk signaling becomes active in muscle (white arrows) and skin epithelial cells (yellow arrow) surrounding the wound (circle). Single plane images were recorded starting ∼65 s after wound appearance every 2.58 s on a Leica SP8 X WLL confocal microscope using a 40x objective.

Movie 4 | Erk signaling in cells surrounding a wound. 72 hpf DREKA embryos were wounded by laser illumination (white circle). mClover fluorescence is shown in gray scale. Erk activity was monitored for 2.5 h after wounding. Spreading of Erk activity from the wound to muscle cells further away is visible. Note that both, muscle cells (red and green arrows) and skin cells (yellow arrow) react to wounding. After ∼30–40 min Erk signaling activity decreases. Around 1 h 54 min the wound breaks open again activating Erk signaling in many cells simultaneously. Images were recorded starting ∼40 s after wound appearance every 2.58 s on a Leica SP8 X WLL confocal microscope using a 40x objective.

Movie 5 | Wounding response in presence of 10 μM trametinib. 26 hpf DREKA embryos were treated with 10 μM MEK1/2 inhibitor trametinib for 3 h and then embedded into ultra-low gelling agarose containing 10 μM trametinib. Larvae were locally wounded by laser illumination and mClover fluorescence in muscle cells was recorded. Erk activity was monitored in a time-lapse movie for at least 15 min after wounding. No Erk activation can be observed. Images were recorded on a Leica SP8 X WLL confocal microscope using a 40x objective.

Movie 6 | Wounding response in presence of 10 μM TAK-733. 26 hpf DREKA embryos were treated with 10 μM TAK-733 for 3 h and then embedded into ultra-low gelling agarose containing 10 μM TAK-733. Larvae were locally wounded by laser illumination and mClover fluorescence in muscle cells was recorded. Erk activity was monitored in a time-lapse movie for at least 15 min after wounding. No Erk activation can be observed. Images were recorded on a Leica SP8 X WLL confocal microscope using a 40x objective.

Movie 7 | Wounding response in presence of DMSO. As control to **Movies 5**, **6** 26 hpf DREKA embryos were treated with 0.1% DMSO for 3 h and then embedded into ultra-low gelling agarose containing 0,1% DMSO. Larvae were locally wounded by laser illumination and mClover fluorescence in muscle cells was recorded. Erk activity was monitored for 20 min after wounding. After 90 s Erk activity is visible and spreading to neighboring muscle cells. Images were recorded on a Leica SP8 X WLL confocal microscope using a 40x objective.

Movie 8 | Small compound uptake kinetics revealed by DREKA. 55 hpf DREKA embryos were embedded in agarose containing 10 μM trametinib to investigate compound uptake kinetics in skin epithelial cells. Approximately 10 min later a time-lapse movie of Erk activity was recorded. Initially, skin epithelial cells are still active. Around 25 min after compound administration Erk activity is visibly decreased and by 50 min cells show Erk signaling inhibition. Z-stack images were recorded approximately every 2 min for 1 h on a Leica SP8 X WLL confocal microscope using a 40x objective. Z-stacks are shown as maximum projections.

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Bloody Zebrafish: Novel Methods in Normal and Malignant Hematopoiesis

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Hematopoiesis is an optimal system for studying stem cell maintenance and lineage differentiation under physiological and pathological conditions. In vertebrate organisms, billions of differentiated hematopoietic cells need to be continuously produced to replenish the blood cell pool. Disruptions in this process have immediate consequences for oxygen transport, responses against pathogens, maintenance of hemostasis and vascular integrity. Zebrafish is a widely used and well-established model for studying the hematopoietic system. Several new hematopoietic regulators were identified in genetic and chemical screens using the zebrafish model. Moreover, zebrafish enables *in vivo* imaging of hematopoietic stem cell generation and differentiation during embryogenesis, and adulthood. Finally, zebrafish has been used to model hematopoietic diseases. Recent technological advances in single-cell transcriptome analysis, epigenetic regulation, proteomics, metabolomics, and processing of large data sets promise to transform the current understanding of normal, abnormal, and malignant hematopoiesis. In this perspective, we discuss how the zebrafish model has proven beneficial for studying physiological and pathological hematopoiesis and how these novel technologies are transforming the field.

Keywords: zebrafish, hematopoiesis, next generation sequencing, hematopoietic (stem) cells, technology

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INTRODUCTION

Over the past three decades, zebrafish has been established as an important model to study various biological processes during development and homeostasis, including hematopoiesis. Many attractive features underpin the success of zebrafish as a model for vertebrate hematopoiesis. Cell-intrinsic and -extrinsic signaling mechanisms in hematopoiesis are well conserved between zebrafish and mammals, with the exception of a few hematopoietic niche components (Liao et al., 1998; Murayama et al., 2006; Bertrand and Traver, 2009; Paik and Zon, 2010; Goessling and North, 2011; Zhang and Liu, 2011; Zhang et al., 2013; Frame et al., 2017; Nik et al., 2017; Gore et al., 2018). Moreover, zebrafish embryos are small and transparent so they are ideal for imaging and easy to manipulate, at low cost. Additionally, genetic manipulation is easy and population studies can be easily performed in zebrafish. Thus, zebrafish have become invaluable vertebrate models for robust large-scale genetic screens (Mullins et al., 1994; Driever et al., 1996; Amsterdam et al., 1999) and, more recently, high-throughput chemical compound screens (North et al., 2007; Yeh et al., 2009). However, there are certain disadvantages in the zebrafish model. For example, zebrafish is not a mammal, but rather a poikilothermic animal in which the development of embryos occurs outside of the animal body and without placenta. That may lead to many metabolic and other differences between zebrafish and mammals, including drug action and utilization. Finally, the

zebrafish genome is duplicated and thus many genes have paralogs and homologs that make the otherwise easy genetic manipulation complicated (Glasauer and Neuhauss, 2014).

Embryonic hematopoiesis in zebrafish is a multistep process occurring in a spatially restricted manner in three distinct waves. During the intraembryonic primitive wave, the medial and anterior lateral mesoderm give rise to erythroid and myeloid cells, respectively. Erythro-myeloid progenitors (EMPs) form in the posterior blood island (PBI) during a transient intermediate wave. Finally, during the definitive wave, hematopoietic stem cells (HSC) with multilineage capacity originate in the aorta-gonad-mesonephros (AGM) region. The HSCs then translocate to and expand in the caudal hematopoietic tissue (CHT), which is followed by the colonization of the kidney and the thymus (**Figure 1**). Interestingly, it was recently discovered that HSC-independent T-cells can originate from the AGM and PBI during the embryonic and larval stages of development (Tian et al., 2017).

Zebrafish has been extensively used for modeling human hematopoietic disease, including anemia, thrombocytopenia, bone marrow failure syndromes, leukemia, and lymphoma (Taylor and Zon, 2011; Kwan and North, 2017; Potts and Bowman, 2017; Gore et al., 2018). The first transplantable leukemia modeled in zebrafish was T-cell acute lymphoblastic leukemia (T-ALL), which was induced by T cell-specific c-Myc overexpression (Langenau et al., 2003). Thereafter, several models of myelodysplastic syndromes and myeloproliferative neoplasms have been described (Le et al., 2007; He et al., 2014; Gjini et al., 2015; Peng et al., 2015).

Although for many years zebrafish was mainly used to study embryonic and larval developmental hematopoiesis, recent technological advances have transformed the field. In this perspective, we will briefly discuss how research using zebrafish genetic models in combination with chemical screens, high-end imaging, and genome-wide molecular, metabolics and proteomics approaches has contributed to our understanding of hematopoiesis.

HEMATOPOIETIC GENERATION, LINEAGE TRACING, AND DIFFERENTIATION

Imaging the Origin of Hematopoiesis

The transparency and accessibility of zebrafish embryos was pivotal to collect evidence showing that hematopoietic stem and progenitor cells (HSPCs) emerge from the ventral wall of the dorsal aorta *in vivo* (Bertrand et al., 2010; Kissa and Herbomel, 2010). Moreover, high-end imaging techniques in zebrafish embryos uncovered the mechanisms of thymus development (Hess and Boehm, 2012) and revealed that HSPCs are amplified and interact with endothelial cells in the CHT (Tamplin et al., 2015). Multiple signaling pathways and cell-interactions affect HSPC emergence. For instance, inflammatory signaling provided by neutrophils is required for HSC generation (Espin-Palazon et al., 2014; Li et al., 2014; Sawamiphak et al., 2014; He et al., 2015). These unique properties of zebrafish allowed to uncover the role of macrophages in HPSC mobilization and definitive hematopoiesis (Travnickova et al., 2015). Where transient and rapid cell-interactions occur, light-sheet microscopy, SPIM (selective plane illumination) or spinning disk microscopy can be used to visualize these processes *in vivo* in embryos and adults, because these systems record time-lapse 3D fluorescent images 100–1000x faster than conventional confocal microscopy (Inoue and Inoue, 1996; Huisken et al., 2004; Arrenberg et al., 2010). Moreover, transparent adult zebrafish models (White et al., 2008) have enabled the imaging of adult hematopoiesis, thereby opening the way to research exploring different HSC niche components and HSC-niche interactions in the adult kidney marrow, thymus, and spleen.

Lineage Differentiation: The Impact of Single-Cell RNA Sequencing

The development of single-cell RNA-sequencing (scRNA-seq) revolutionized the way we understand hematopoiesis. As most cellular compartments have a certain degree of heterogeneity, with bulk RNA-seq one cannot distinguish between a small transcriptional difference in many cells, and a large transcriptional difference in a few cells. Several insightful reviews describe the different methods used for single-cell RNA-seq (Kolodziejczyk et al., 2015; Ziegenhain et al., 2017; Dal Molin and Di Camillo, 2018).

In zebrafish, one of the first methods used to characterize the transcriptome of single cells was massive parallel qPCR, where up to 96 transcripts could be analyzed in great sequencing depth using the Fluidigm system. This method revealed two distinct sub-populations of HSPCs in the CD41-GFP low-expressing stem cell compartment of the adult kidney marrow. Moreover, by using this technique and genetic ablation of T cells, a previously uncharacterized hematopoietic cytotoxic T/NK cell population in zebrafish was uncovered (Moore et al., 2016).

Recent technological advances in scRNA-seq have enabled analyses without restriction to specific transcripts. A re-examination of the CD41-GFP^{low} population revealed four HSPC sub-populations with different cellular characteristics and potential novel markers for HSCs were uncovered. Importantly, some cells in these subpopulations expressed the thrombocyte differentiation program long before they would have been characterized as thrombocytes, showing that there is an early lineage bias (Guo et al., 2013; Buenrostro et al., 2015; Paul et al., 2015; Drissen et al., 2016; Grover et al., 2016; Nestorowa et al., 2016; Olsson et al., 2016; Alberti-Servera et al., 2017; Velten et al., 2017; Villani et al., 2017; Buenrostro et al., 2018; Dahlin et al., 2018). In addition, scRNA-seq analyses in various transgenic lines revealed that ribosomal genes and lineage regulators control hematopoietic differentiation (Athanasiadis et al., 2017) and uncovered several novel hematopoietic populations, including two new types of NK cells (Tang et al., 2017). Finally, elegant comparative evolutionary studies on LCK-GFP transgenic zebrafish and mammals showed that membrane proteins are less conserved in NK cells than in T cells (Carmona et al., 2017). In the pathological context, scRNA-seq analysis of Myc-induced T-ALLs demonstrated that few cells expressed an immature stem cell program, suggesting that only a small proportion of leukemia

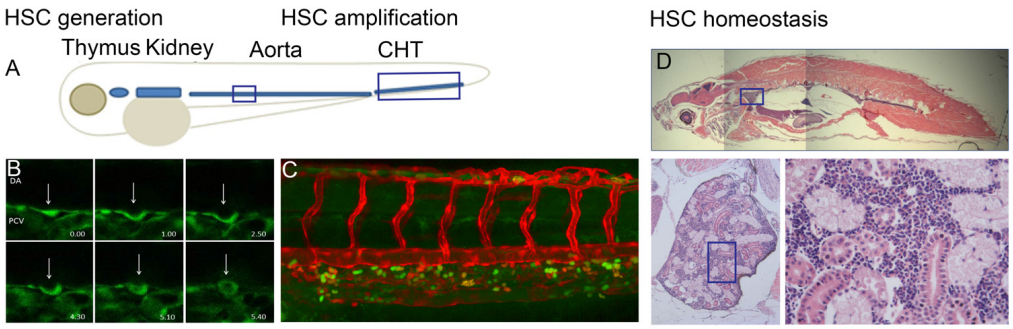

FIGURE 1 | (A) Overview of definitive hematopoietic sites in the developing embryo where HSPCs are born from hemogenic endothelial cells of the dorsal aorta (DA). HSPCs are amplified in the caudal hematopoietic tissue (CHT) and migrate to the kidney and thymus. **(B)** Endothelial-to-hematopoietic transition (EHT) event imaged in a Tg(fli:GFP) embryo between 32 and 40 hpf. **(C)** CHT region in Tg(flt:RFP)/Tg(CD41:GFP) embryo at 56 hpf indicating erythroid myeloid progenitors (EMP) in green and definitive HSPCs in yellow as they originate from the artery and retain RFP at this timepoint. **(D)** Sagittal section through an adult zebrafish where the head kidney is indicated with enlargement showing the hematopoietic cells in between the kidney tubules.

cells promote the disease. This is remarkable as a single transgenic approach was used to initiate leukemogenesis and all leukemia cells overexpress Myc (Moore et al., 2016).

Lineage Tracing

Zebrafish has traditionally been utilized to lineage-trace differentiation during embryonic stages by labeling single cells with dyes and following them throughout development, until the dye fades or dilutes. However, the recent development of complex genetic models has removed this time restriction and enabled lineage tracing from the embryo into adulthood. For instance, HSPCs generated from the hemogenic endothelium of the aorta have been lineage-traced by using the multicolor transgenic labeling system "blood bow" (Henninger et al., 2017) in combination with high-end imaging and fluorescence-activated cell sorting (FACS). Additionally, labeling with CRISPR/Cas9 scarring in embryos and tracing of unique hematopoietic clones into adulthood has revealed that the hematopoietic system is only generated from a handful of cells present at dome stage (Alemany et al., 2018). This study claimed that all clones contribute to all blood lineages, a subject that is controversial in mammalian studies (Yamamoto et al., 2013; Notta et al., 2016; Pei et al., 2017).

A different approach for lineage-tracing cells consists of performing high-throughput scRNAseq at various developmental stages and then mapping similarities in transcriptional profiles across a pseudo timescale of differentiation (Macosko et al., 2015). By using this method in early embryogenesis, two independent studies have described gradually divergent differentiation patterns for specific lineages and uncovered signaling networks required for zebrafish development (Farrell et al., 2018; Wagner et al., 2018).

Future studies combining scRNAseq with lineage tracing will be paramount to advance our understanding of the developmental origins of hematopoietic populations. However, this approach has the important caveat that scRNA-seq does not provide topographic information for each individual cell. To overcome this limitation, the Van Oudernaarden and Bakkers laboratories have developed RNA-tomography (TOMOSEQ), a method that combines traditional histological techniques with low-input RNA sequencing and mathematical image reconstruction (Junker et al., 2014).

IDENTIFICATION OF NOVEL REGULATORY MECHANISMS OF NORMAL AND MALIGNANT HEMATOPOIESIS

Chemical Screens to Identify Regulators of Normal and Abnormal Hematopoiesis

Zebrafish is an ideal vertebrate model system to conduct bio-reactive compound screens (Zon and Peterson, 2005; Cusick et al., 2012; Tamplin et al., 2012; Veinotte et al., 2014; Rennekamp and Peterson, 2015; Deveau et al., 2017). The animals are small-sized and lay hundreds of eggs that develop very rapidly, thereby allowing the monitoring of compound activity and biotoxicity *in vivo* across development. Such screens have led to the identification of prostaglandin E2 as a compound that increases HSC production (North et al., 2007). Prostaglandin E2 is currently being investigated for HSC expansion applications in human and non-human primates (Goessling et al., 2011; Cutler et al., 2013).

Important insights into the molecular regulation of T-ALL came from zebrafish studies where immature T cells served as models for T-ALL cells. By screening small molecules for an effect on immature T cells using LCK-GFP transgenic zebrafish, a novel compound, 1H-indole-3-carbaldehyde quinolin-8-yl-hydrazone, named Lenaldekar, was identified with the potential to specifically attack T-ALL cells (Ridges et al., 2012). Lenaldekar also has a potential effect against autoimmune diseases such as multiple sclerosis, as they are caused by an off-target activity of T cells (Cusick et al., 2012). Currently there are ongoing clinical trials to study the effectiveness of this promising compound. These examples highlight the power of zebrafish models for screening novel chemical compounds affecting normal, abnormal or malignant hematopoiesis (Shafizadeh et al., 2004; Yeh et al., 2009;

Paik et al., 2010; Gutierrez et al., 2014; Arulmozhivarman et al., 2016).

Future studies addressing malignancy heterogeneity may combine chemical screens with scRNAseq to identify therapy-resistant cells and explore the mechanisms underpinning resistance to treatment in individual cells, a fundamental unresolved question in the cancer research field.

Effects of Perturbations in Embryonic HSC Generation and Adult Hematopoiesis

Several acute myeloid leukemia (AML) predisposition syndromes are caused by innate mutations in transcription factors that affect embryonic hematopoiesis, such as Gata2 and Runx1 (Babushok et al., 2016), suggesting that perturbations in embryonic hematopoiesis affect the adult HSC compartment. As the effects of alterations in embryonic hematopoiesis can be easily monitored in zebrafish throughout development, as well as during adulthood, this is an excellent system to study AML predisposition syndromes. Until the recent development of targeted gene editing, manipulating the zebrafish genome to create specific mutations for making knockout and knockin animals was challenging. Although TILLING (Targeting Induced Local Lesions in Genomes) was a significant advancement, this is a costly method that requires thousands of fish to search for a STOP codon in the gene of interest. Moreover, TILLING is rather limiting as it does not allow to induce specific mutations (Wienholds et al., 2003; Draper et al., 2004). Targeting the zebrafish genome with zinc-finger nucleases was the beginning of a new era in zebrafish biology, as selected genes could finally be specifically targeted for genome editing (Amacher, 2008; Foley et al., 2009). Shortly after this technology was introduced, TALENS (Dahlem et al., 2012; Hwang et al., 2014; Huang et al., 2016; Liu et al., 2016), and more recently, CRISPR/Cas9 (Hruscha et al., 2013; Irion et al., 2014; Shah et al., 2015; Li et al., 2016; Liu et al., 2017) were developed. Whilst it is relatively easy to generate knockouts and large deletions with these gene-targeting techniques, making knockin animals remains challenging. Nevertheless, several laboratories have successfully created knockin animals by using CRISPR/Cas9 and co-injecting a repair template to facilitate homology-directed repair (Hruscha et al., 2013; Auer et al., 2014; Albadri et al., 2017; Kesavan et al., 2017). Additionally, Cre/lox, Flp/FRT, and ΦC31 systems are also currently being used in zebrafish for precise genome editing (Mosimann et al., 2013; Felker and Mosimann, 2016; Carney and Mosimann, 2018). Importantly, tissue-specific expression of Cas9 in the hematopoietic system can be performed in zebrafish to enable conditional manipulation of hematopoietic cells (Ablain et al., 2015). A major caveat in both perturbing the zebrafish genome and comparing the zebrafish with the mammalian transcriptome in the context of clinical translation, is, as previously mentioned, the Teleost genome duplication (Glasauer and Neuhauss, 2014). As a result most genes are present twice with (partially) redundant biological roles. This means that for a complete perturbation of a mammalian gene, the zebrafish counterparts have to be removed both, complicating genetic crossings and analyses.

Epigenetic Regulation of the Hematopoietic System

Chromatin conformation is essential for controlling gene expression, and deregulation of this process may cause malignant transformation (Groschel et al., 2014). Zebrafish is an excellent system to explore the mechanisms underlying chromatin regulation and to evaluate the effects of chromatin-modifying drugs *in vivo*. Gene regulatory elements can be identified in zebrafish using chromatin immunoprecipitation combined with sequencing (ChIP-seq), however, the technique is limited by the low number of zebrafish-specific antibodies currently available and the large amount of input material required (Havis et al., 2006; Trompouki et al., 2011; Bogdanovic et al., 2013). ChIP-seq has been mostly used in early zebrafish embryos (Paik et al., 2010; Vastenhouw et al., 2010; Bogdanovic et al., 2012; Xu et al., 2012; Winata et al., 2013; Nelson et al., 2017; Meier et al., 2018). Antibodies against histone marks, which are highly conserved between species, have been successfully utilized in zebrafish erythrocytes to describe the potential locus control region (LCR) regulating globin expression (Ganis et al., 2012). Moreover, given the functional conservation of these genes, zebrafish is useful to functionally validate enhancers identified in mouse and/or human models (Tijssen et al., 2011; Chiang et al., 2017).

Other techniques for identifying gene regulatory elements are based on the detection of open chromatin, for instance, assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq). ATAC-seq requires much less input material than ChIP-seq, and has even been used successfully with single cells (Fernandez-Minan et al., 2016; Doganli et al., 2017). This method allowed the identification of endothelial enhancers (Quillien et al., 2017) and revealed the role of cohesin in rearranging the genomic architecture during the transition of maternal to zygotic transcription in early embryos (Meier et al., 2018). Combining scRNA-seq with ATAC-seq and immune phenotypic analysis is a powerful approach to integrate our understanding of lineage differentiation with the regulatory elements involved in that process (Buenrostro et al., 2018). DNA methylation studies can also be used to understand chromatin accessibility, although more material is needed in these methods. Methylation experiments have been conducted in zebrafish albeit not specifically in the hematopoietic system (Lee et al., 2015; Kaaij et al., 2016). Since many tissue-specific fluorescent lines exist in zebrafish, future research should aim to identify enhancers and promoters in specific cell types, rather than using whole embryos.

Despite the advantages of ATAC-seq and methylation analyses, these approaches cannot offer the same information as ChIP-seq. Thus, improved ChIP-seq protocols, such as the high sensitivity indexing-first chromatin immunoprecipitation approach (iChIP) developed in Ido Amit's laboratory, should be adapted to zebrafish (Gury-BenAri et al., 2016). Moreover, it would be important to unravel chromatin interactions in active enhancer and promoter regions during hematopoiesis. However, although chromatin conformation has been studied in early zebrafish embryos (Gomez-Marin et al., 2015; Fernandez-Minan et al., 2016), to date no studies have addressed this question specifically in zebrafish hematopoiesis. It is important to mention

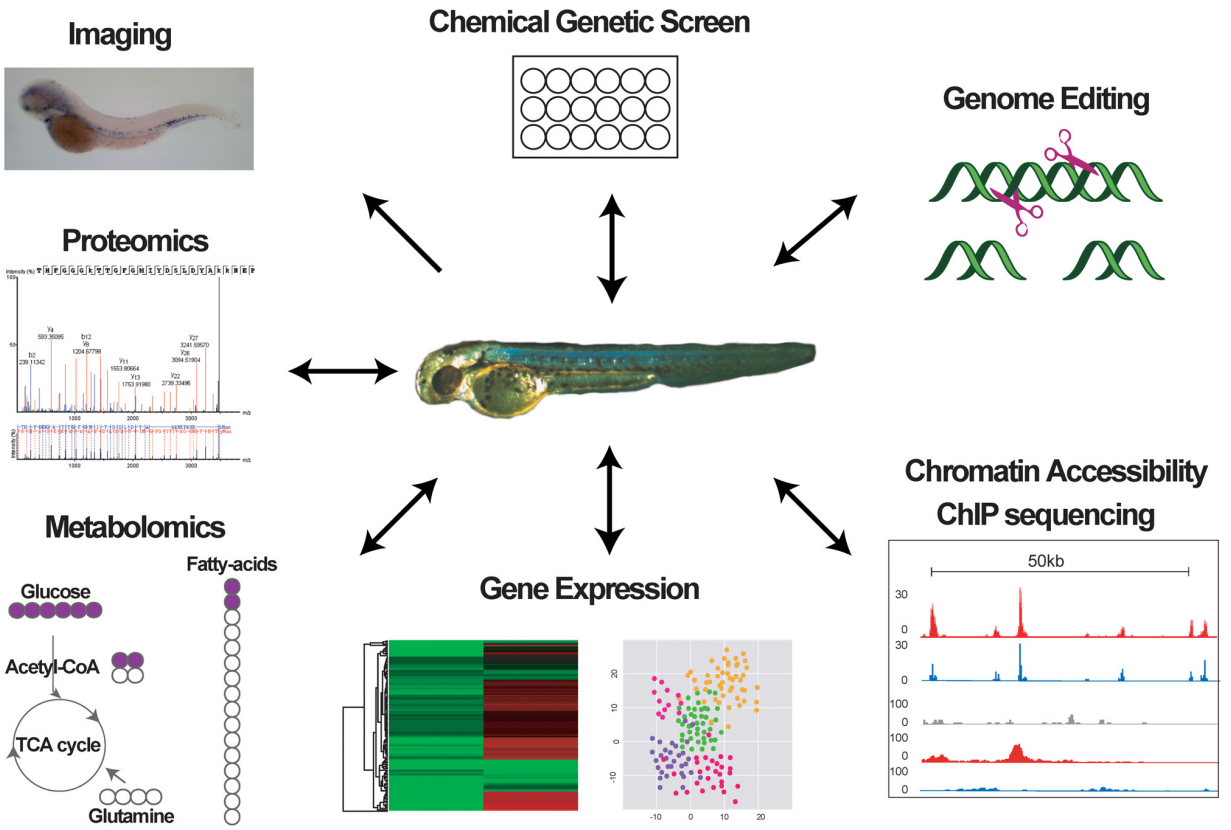

FIGURE 2 | Graph indicating different methods used to study zebrafish hematopoiesis.

the combined effort of many groups to collate all available genome-wide data in zebrafish in the DANIO-CODE Data Coordination Center[1] (Tan et al., 2016). This recently launched database will provide an easy access to high-quality genome data to all scientists.

Proteomics and Metabolomics Studies

In the era of genome-wide technology, gene expression studies should be complemented with proteomic studies, as transcriptional and translational outcomes can sometimes differ. Additionally, the extension of these analyses to metabolomics may uncover another layer of regulation critical for hematopoiesis. Indeed, it was recently shown that dormant stem cell populations have low metabolic activity, and this is required to maintain the hematopoietic system during aging and periods of intense stress (Cabezas-Wallscheid et al., 2017). Although proteomics and metabolomics methods have not yet been extensively explored in zebrafish, particularly in the hematopoietic system, some studies have reported differences between transcript and protein levels in multiple genes by using proteomic analyses either in whole zebrafish embryos or in specific cell populations during regeneration (Alli Shaik et al., 2014; Baral et al., 2014; Rabinowitz et al., 2017). Metabolomics has proven useful to understand the neurological damage resulting from chemical perturbations in zebrafish embryos (Ong et al., 2009; Rabinowitz et al., 2017; Roy et al., 2017). Finally, as mass spectrometry analyses are constantly improving, the sensitivity of these methods will likely overcome the current problem of heterogeneous and low cell-number populations.

CONCLUSION

The zebrafish has become an invaluable model system for understanding how HSCs form and are maintained, and how hematopoietic cell differentiation is regulated during embryogenesis and in adulthood. The unique advantages offered by this model system over traditional mouse models regarding the use in chemical screens and the accessibility during embryonic stages allowing easy manipulation and visualization and tracing into adult stages, in combination with recent new technologies (**Figure 2**), have opened the way for novel exciting hypotheses on the mechanisms promoting hematopoietic diseases, the role of the niche in normal and malignant hematopoiesis, and the effect of chemical compounds on malignant cells. The high conservation between the zebrafish and human hematopoietic systems means that discoveries in fish may have strong translational potential and important clinical implications for the treatment of hematopoietic diseases.

[1]https://danio-code.zfin.org

AUTHOR CONTRIBUTIONS

EdP and ET conceived and wrote this manuscript.

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Setting Eyes on the Retinal Pigment Epithelium

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The neural component of the zebrafish eye derives from a small group of cells known as the eye/retinal field. These cells, positioned in the anterior neural plate, rearrange extensively and generate the optic vesicles (OVs). Each vesicle subsequently folds over itself to form the double-layered optic cup, from which the mature eye derives. During this transition, cells of the OV are progressively specified toward three different fates: the retinal pigment epithelium (RPE), the neural retina, and the optic stalk. Recent studies have shown that folding of the zebrafish OV into a cup is in part driven by basal constriction of the cells of the future neural retina. During folding, however, RPE cells undergo an even more dramatic shape conversion that seems to entail the acquisition of unique properties. How these changes occur and whether they contribute to optic cup formation is still poorly understood. Here we will review present knowledge on RPE morphogenesis and discuss potential mechanisms that may explain such transformation using examples taken from embryonic *Drosophila* tissues that undergo similar shape changes. We will also put forward a hypothesis for optic cup folding that considers an active contribution from the RPE.

Keywords: morphogenesis, eye development, squamous epithelial cell, zebrafish (*Danio rerio*), optic cup

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INTRODUCTION

All the organisms of the animal kingdom that bear a visual sensing organ share the need of protecting the light-receptive cells with a pigmented structure (Martinez-Morales, 2016). In the vertebrate eye, this structure is represented by the retinal pigment epithelium (RPE). The RPE consists of a monolayer of cells positioned at the back of the neural retina. One of its prominent features is the production and accumulation of pigment granules in specialized organelles, the melanosomes. These organelles are responsible for quenching the excess of light that may otherwise damage the photoreceptors (Strauss, 2005), the retinal cells in charge of sensing and processing light input. A second feature of the RPE cells is a highly elaborated apical membrane with extensions that surround and closely interact with the outer segment of the photoreceptors. This organization is essential for other functions that the RPE bears on photoreceptor homeostasis. These include the cyclic and circadian rhythms-dependent phagocytosis of the photoreceptor outer segment; the recycling of water and ions generated during the high photoreceptor metabolic activity; the secretion of growth factors and an active participation in visual phototransduction (Strauss, 2005; Letelier et al., 2017). Thus, RPE-photoreceptor dependence is such that they can be considered as a single functional unit. This is also reflected by the pathological consequence that RPE impairment has on the function and survival of photoreceptors. This occurs, for example, in genetically or

environmentally triggered degenerative diseases that lead to partial or total vision loss, such as different forms of Retinitis Pigmentosa (Letelier et al., 2017).

The RPE is a neuroectodermal derivative. In zebrafish, its specification begins in cells that occupy the dorso-medial portion of the optic vesicle (OV), which is the first morphologically recognizable primordium of the eye (Kwan et al., 2012). All OV cells are initially alike and express a small network of regulatory genes – such as the transcription factors Otx2, Pax6, Rx, Six3, Six6, and Lhx2 – essential for the acquisition of eye identity (Fuhrmann, 2010; Beccari et al., 2013). Inductive signals initiate the specification of the targeted OV cells into three derivatives: the RPE, the neural retina and the optic stalk (Fuhrmann, 2010; Fuhrmann et al., 2014). The regulatory mechanisms driving neural retina patterning and its morphogenesis are fairly well known (Martinez-Morales et al., 2017). RPE specification, which entails an important morphological and functional divergence from a "neural" phenotype, is instead less well understood. Similarly, the impact that RPE specification has on eye morphogenesis has been poorly addressed. Here, we will discuss these issues focusing on the RPE of the zebrafish, a species in which RPE cells undergo an extreme transformation from a neuroepithelial to a squamous morphology.

CURRENT VIEW OF ZEBRAFISH EYE MORPHOGENESIS

The adult zebrafish eye shares strong similarities with that of other vertebrates but its initial morphogenesis occurs with slightly different mechanisms. In amniotes, eye progenitors are bilaterally positioned in the anterior neural plate (Inoue et al., 2000) and protrude to form the OVs as polarized neuroepithelial cells. In the zebrafish instead, progenitors are specified in the center of the anterior neural plate as a single cohesive group of cells (Martinez-Morales and Wittbrodt, 2009; Fuhrmann, 2010; Martinez-Morales et al., 2017). These cells acquire neuroepithelial and polarized characteristics whilst progressively organizing into OVs (Rembold et al., 2006; Ivanovitch et al., 2013). Zebrafish OVs are flat and composed of a folded continuous layer of neuroepithelial cells, so that the apical surface of the two layers, connected by a rim region, face one another separated only by a virtual lumen (**Figure 1**). This organization differs from the balloon-shaped OV of the chick and mouse embryos. All OV progenitors have the potential to acquire RPE, neural retina or optic stalk identities. However, zebrafish fate map studies have shown that the ventral/lateral layer (abutting the lens ectoderm) originates the neural retina (Li et al., 2000b), whereas the dorsal/medial layer (averting the lens ectoderm) contributes to both the neural retina and the RPE (**Figure 1**). Indeed, a good proportion of medial layer cells undergo "epithelial flow" around the rim (also called rim involution, **Figure 1**), emitting dynamic lamellipodia that attach to the extracellular matrix (ECM) and generate the necessary force to rotate into the lateral layer (Li et al., 2000b; Kwan et al., 2012; Heermann et al., 2015; Sidhaye and Norden, 2017). So far, there is no evidence of a similar type of flow during mammalian or avian eye morphogenesis. Nevertheless, in teleost, this event results in the imbalance of cell number between the two layers, possibly contributing to the concomitant modifications of cell shape the two layers undergo. Indeed, cells of the lateral layer undertake basal constriction mediated by actomyosin contractility (Martinez-Morales et al., 2009; Nicolas-Perez et al., 2016). The result is cone-like cells with a reduced basal but enlarged apical surface (Sidhaye and Norden, 2017). This rearrangement, together with apico-basal elongation and lateral compaction of the prospective retinal cells, promotes the inward folding of the OV and the formation of the optic cup (OC; **Figure 1**; Bogdanović et al., 2012; Heermann et al., 2015; Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017).

As the ventral/lateral layer expands and undergoes shape changes, the remaining cells of the dorsal/medial OV layer begin to acquire RPE identity and transform their appearance (**Figure 1**). Similar to the other OV cells, these cells are initially organized as a pseudostratified epithelium but soon align their nuclei and reduce their height along their apico-basal axis to form a cuboidal monolayer of cells. In amniotes, this is the final shape of the RPE (Bok, 1993) but, in teleost, cuboidal cells further flatten and originate an array of squamous polygonal cells (**Figure 1**; Li et al., 2000b). One of the yet unanswered questions is whether this cellular transformation is a "passive" process. That is, RPE cells could change shape and stretch in response to external forces exerted by the expanding apical surface of the neural retina and possibly by other surrounding tissues. This is a possibility given that few medial layer cells (future RPE) need to overlay and match the extension of the apical neural retina surface, in virtual absence of RPE cell proliferation (Cechmanek and McFarlane, 2017). In a different and not mutually exclusive view, the RPE could instead be programmed to stretch cell autonomously, as a consequence of regulated and active rearrangements of its own cytoskeleton and adhesive properties. So far, this possibility has not been directly tested but in the *Drosophila* embryo there are examples of tissues undergoing a similar conversion. The underlying mechanisms have been studied and could provide clues regarding RPE flattening, as we detail below.

FROM A PSEUDOSTRATIFIED EPITHELIUM TO A SQUAMOUS MONOLAYER OF CELLS

The *Drosophila* imaginal disks are among such examples. These epithelial sac-like structures present in the larva originate most of the adult appendages during metamorphosis (Whittle, 1990). Initially, the entire imaginal disk is composed of cuboidal cells that, through a differential re-arrangement of microtubules (Tang et al., 2016), differentiate into a layer of columnar and elongated cells, or disk proper, and a squamous peripodial epithelium (**Figure 2A**; McClure and Schubiger, 2005). The acquisition of the peripodial morphology involves Decapentaplegic (Dpp) (McClure and Schubiger, 2005), a member of the TGF-beta family of signaling factors. Suppression of Dpp signaling (McClure and Schubiger, 2005) or RNAi-mediated interference with the peripodial expression of the transcription factor Yorkie/YAP (Fletcher et al., 2018), an effector of the Hippo

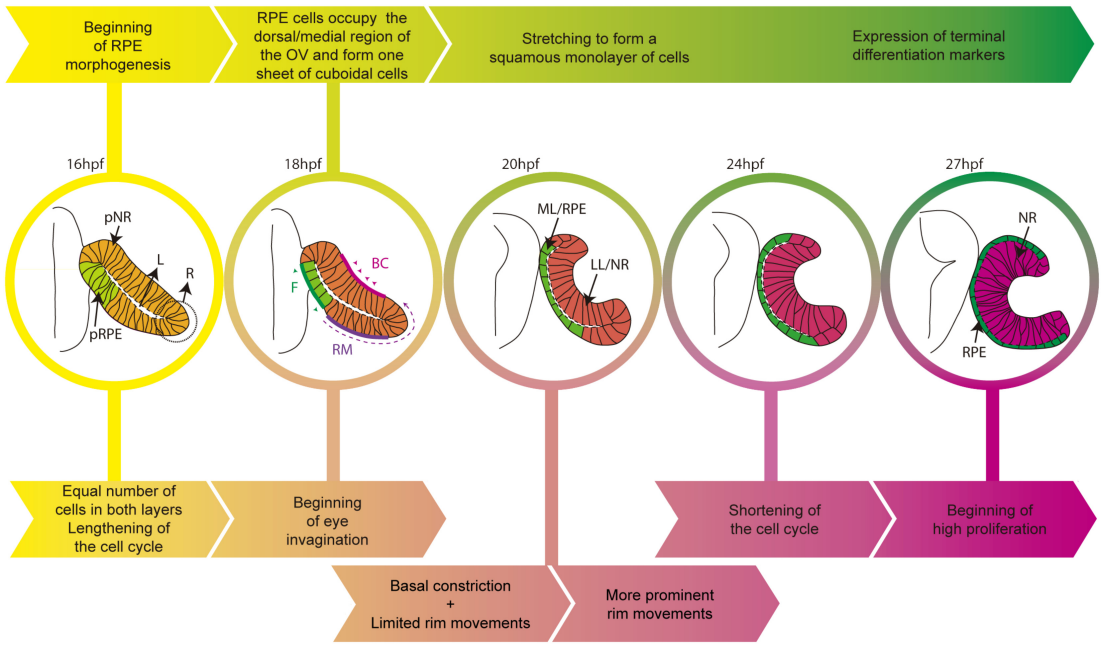

FIGURE 1 | Schematic diagram of the time line of zebrafish eye morphogenesis. The top arrows (yellow to green) indicate the salient events in RPE development whereas bottom arrows (yellow to magenta) those related to the entire eye primordium. Eye structures are color coded: progenitors, light yellow; lateral/neural retina layer, dark pink; medial/RPE layer, green. BC, basal constriction; F, flattening; L, virtual lumen; LL, lateral layer; ML, medial layer; NR, neural retina; pNR, prospective neural retina; R, rim region; RM, rim movements; RPE, retinal pigment epithelium; pRPE, prospective RPE.

pathway, is sufficient to prevent stretching of the peripodial membrane, suggesting that this is an active process, regulated at the transcriptional level. Similar results were obtained with Yorkie/YAP manipulation in the *Drosophila* ovarian follicular epithelium (Fletcher et al., 2018), which undergo dramatic flattening during growth of the egg chamber (Kolahi et al., 2009). In these cells, decreased concentration at the apical membrane of upstream Hippo pathway components – i.e., the Crumbs-Expanded and Merlin-Kibra protein complexes – or inactivation of kinase Warts, leads to Yorkie/YAP nuclear localization, which is required to actively promote further cell flattening (**Figure 2B**; Fletcher et al., 2018).

These results are not surprising given that the transcriptional regulators YAP and the highly related TAZ are emerging as pivotal mediators of the cell self-perception within its environment, so that mechanical inputs from the surrounding promotes YAP/TAZ nuclear localization and, thus, a context-dependent gene expression response (**Figure 2C**; Totaro et al., 2018). On the other hand, in *yap* medaka fish mutants the entire body lacks tension as also observed in a YAP-deficient human RPE cell line (Porazinski et al., 2015). This is because YAP – but not TAZ that seems to control only cell proliferation in medaka fish – is required for the expression of ARHGAP18, a RhoGAP that suppresses F-actin polymerization. As a consequence, cells have less cortical actomyosin and do not deposit properly fibronectin fibrils in the surrounding ECM, making all tissues prone to collapse (Porazinski et al., 2015). Thus, YAP/TAZ has a double and likely feed-forward role, not necessarily dependent on Hippo pathway activation (Totaro et al., 2018). It acts as a mechano-transducer of the environmental tension by shuttling from the cytoplasm to the nucleus (**Figure 2C**), as well as a determinant of tissue tension by directly or indirectly controlling the expression of regulators of cytoskeletal dynamics.

In this scenario, YAP/TAZ appear appropriate candidates to mediate RPE cell flattening. However, loss of *Yap* function in mouse is embryonic lethal before the OV begins to form (Morin-Kensicki et al., 2006), whereas conditional inactivation of *Yap* in the optic primordia induces the prospective RPE territory to adopt a neural retina-like identity (Kim et al., 2016). Notably, the latter phenotype is also observed in mouse embryos after genetic inactivation of *Otx1/2*, *Mitf* and β-catenin, genes considered as RPE determinants (Nguyen and Arnheiter, 2000; Martinez-Morales et al., 2001; Westenskow et al., 2009). So far it is unclear if the function of YAP in the mouse RPE is linked to Hippo, or related to Wnt-β-catenin signaling, as observed in other contexts (Totaro et al., 2018), or perhaps both or none. Nevertheless, the expression of Otx2 and of two RPE terminal differentiation markers, Sox9 and Ezrin, is missing in the prospective RPE of *Yap* mutants. This indicates that YAP acts rather upstream in the regulatory network controlling RPE specification (Kim et al., 2016) and makes it difficult to determine its possible implication in RPE flattening. A window of opportunity to address this question may come from the zebrafish. In this species, the combined abrogation of *yap* and *taz* alleles lead to an eye that lacks the RPE, although it is still unclear if their progenitors are initially formed and then fail to undergo differentiation or are, for example, incorporated in the neural retina. Embryonic transplanted cells null for *yap* and *taz* do not contribute to

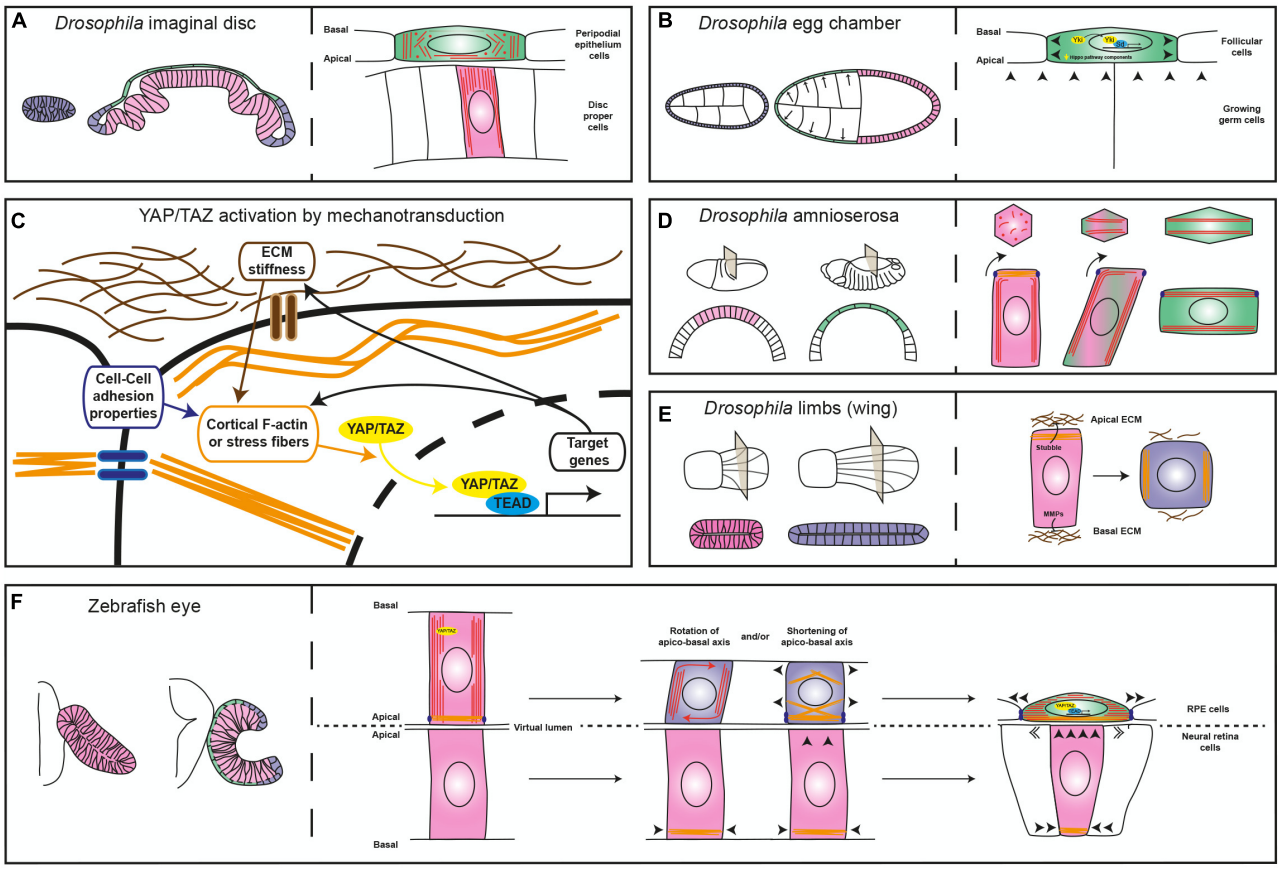

FIGURE 2 | Diagrams comparing different mechanisms of epithelial flattening. **(A)** Left, the drawing depicts the immature *Drosophila* imaginal disk composed of a homogeneous layer of cuboidal cells (blue) and the mature disk composed of a columnar epithelium, the disk proper (pink) and a squamous layer, the peripodial epithelium (green). Right, the enlarged view depicts the differential microtubule (red lines and dots) organization of the two layers. **(B)** The left drawing depicts the development of the *Drosophila* egg chamber, which is surrounded by the follicular cells. The egg chamber is initially composed of a homogeneous cuboidal epithelium (blue) that then becomes flat in the anterior region (green) and columnar in the posterior one (pink). The right drawing depicts the contribution of Yorkie (Yki) to follicular cell flattening. **(C)** Schematic representation of the relation between sources of cell tension and YAP/TAZ cellular location (cytoplasmic vs. nuclear). **(D)** On the left, view of the *Drosophila* embryo at two different stages of development and relative cross sections showing the changes the amnioserosa undergoes (columnar in pink and squamous in green). The right panel depicts epithelial flattening of the amnioserosa cells driven by perpendicular rearrangement of the long axis of the cell (modified from Pope and Harris, 2008). **(E)** Left, view of two developmental stages of the *Drosophila* limb (the wing) and relative cross sections showing cell shape changes from columnar (pink) to cuboidal (blue). The right panel depicts ECM degradation and reorganization of myosin-II, which promote cell shortening. **(F)** Left, schematic representation of the zebrafish optic vesicle and optic cup, with the RPE in green, the neural retina in pink and the rim in blue. The right panel depicts two possible mechanisms that could contribute to RPE flattening, as discussed in the text. In all panels, arrowheads indicate mechanical forces; microtubules are represented in red, acto-myosin filaments in orange, ECM in brown and junctions in violet.

RPE formation, whereas forced expression of *yap* in neural retinal progenitors induce the formation of pigment granules, indicating that Yap/Taz act cell-autonomously imposing RPE characteristic. This activity seems to depend on their nuclear interaction with Tead transcription factors (Miesfeld et al., 2015). In contrast, mutants in *yap* alone develop an RPE that, however, is discontinuous (Miesfeld et al., 2015). The patchy RPE appearance can be interpreted as a failure of RPE specification only in a subset of progenitors but it might also reflect poor cell-to-cell adhesion or alterations in cytoskeletal dynamics, as observed in *yap* medaka mutants. Whether the latter interpretation is correct needs further investigation but there is evidence that cytoskeletal dynamics during epithelial morphogenesis may be linked to the same mechanisms that promote fate specification, as observed during the first lineage separation the mammalian morula undergoes. This occurs at the transition from 8 to 16 blastomere stage during which, individual blastomeres acquire the capacity to interpret their relative position with respect to the inside-outside of the embryo (Mihajlovic and Bruce, 2017). Outside blastomeres are specified as trophectoderm (future placenta) and adopt a flat epithelial conformation, whereas inside blastomeres form the inner cell mass (the origin of the future embryo). This lineage bifurcation depends on the differential activation of the Hippo pathway. In prospective trophectodermal cells, the Hippo pathway is inhibited and Yap/Taz accumulates in the nucleus forming Yap/Taz/Tead complexes that activate the expression of lineage specific genes, such as *Cdx2* (Nishioka et al., 2009). At the same time, Yap/Taz lead to the acquisition of

a well-defined apico-basal polarity, a differential distribution of adherens junction and cytoskeletal components with respect to the inner cell mass, thereby enabling cell flattening (Mihajlovic and Bruce, 2017). Thus, specification and stretching of both trophectoderm and RPE require Yap/Tead activity, suggesting functional parallelisms.

Besides the possible involvement of YAP/TAZ as drivers of RPE morphogenesis, changes in cytoskeletal organization and modifications of cell-to-cell contacts are additional active mechanisms that could promote RPE cell flattening. These mechanisms have been observed in the conversion of the amnioserosa cells of the gastrulating *Drosophila* embryo from columnar to squamous epithelium (**Figure 2D**). This conversion occurs thanks to a 90° rotation of their cellular components – including microtubule bundles, centrosome, nucleus and endoplasmic reticulum, which changes the long axis of the cell perpendicular to its initial position. This phenomenon seems to be initiated autonomously by the bending of the growing microtubules that, in the columnar shaped amnioserosa, find resistance to their growth in the apical accumulation of actin filaments. Notably, microtubule rotation is accompanied by remodeling of the adherens junctions dependent on myosin contraction (**Figure 2D**; Pope and Harris, 2008). At the moment, microtubule dynamics during zebrafish OC formation has been analyzed only in the context of epithelial flow at the rim, without finding a significant contribution to this process (Sidhaye and Norden, 2017). However, thin sectioning of the zebrafish OV shows the possible rotation of the nuclear longitudinal axis in the prospective RPE cells as they undergo flattening (see **Figure 1B** in Li et al., 2000b). Thus, the amnioserosa flattening model (Pope and Harris, 2008) may be relevant for explaining zebrafish RPE morphogenesis. Modifications may be nevertheless needed as the RPE cells transit through an intermediate cuboidal shape absent during amnioserosa transformation. This intermediate shape may depend on ECM remodeling, as observed in morphogenetic elongation of the epithelium of the *Drosophila* limbs (**Figure 2E**). In the limbs, the transcriptionally regulated expression of matrix proteases releases the epithelium from apical and basal ECM constraints and allows the re-localization of myosin-II from the apical to the lateral membranes. The result is a columnar to cuboidal transition and a consequent expansion of the tissue surface (Diaz-de-la-Loza et al., 2018). In theory, a similar mechanism could explain how the RPE matches the apical surface of the folding neural retina.

In the above-mentioned examples of epithelial transformations, there seems to be only a marginal or no contribution of cell proliferation and cell death. Both processes are likely dispensable or marginally involved also in RPE differentiation. Cell death is negligible at early steps of zebrafish eye formation and there is an increase in the length of the progenitor cell cycle during the period spanning from OV to OC transition (Li et al., 2000a). Thus, the total number of cells barely changes as the RPE flattens. Furthermore, pharmacological inhibition of cell proliferation causes small but properly patterned eyes (Kwan et al., 2012; Heermann et al., 2015; Cechmanek and McFarlane, 2017; Sidhaye and Norden, 2017).

A MODEL OF OPTIC CUP FORMATION BASED ON TISSUE COLLABORATION

In the first section, we have indicated that the basal constriction of neural retinal cells and cell flow around the rim act as motors of OC invagination (Bogdanović et al., 2012; Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017). Both processes contribute to a notable expansion of the neural retina apical surface, which may be sufficient to generate pressure on the closely apposed apical surface of the future RPE. This conclusion comes from interference studies performed by bathing the entire embryo with drugs that inhibit cytoskeletal organization (Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017). However, in these conditions the prospective RPE cytoskeleton is also compromised, making it difficult to exclude its possible contribution to the failure of OC folding. Similar consideration applies to the analysis of the interaction of the basal surface of the prospective neural retina cells with laminin, an ECM component deposited at the basal surface of both OC layers (Bryan et al., 2016; Sidhaye and Norden, 2017). Knock-down of laminin subunits reduces the contractility of the basal surface of the retinal layer and perturbs cell translocation at the rim, thereby impairing OV folding (Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017). Whether there are concomitant RPE morphological alterations remains to be carefully analyzed.

With these considerations in mind, folding of the zebrafish OV into a cup could be modeled as a morphogenetic process that requires the contribution of the dorso/medial and ventro/lateral layers. The expansion of the apical retinal layer may be an initial trigger that exerts tension on the overlying layer, thereby forcing YAP nuclearization and the activation of Tead-mediated transcription of tissue determinants (i.e., *Otx* genes) as well as a YAP-related control of cytoskeletal dynamics (**Figure 2F**). Changes in cytoskeletal dynamics, in turn, may promote prospective RPE flattening either by simply shortening the long axis of the neuroepithelial cell or by favoring the perpendicular rotation of its long axis (**Figure 2F**). These changes may increase RPE stiffness, which feed-back into the retina, promoting the flow of ventro/medial cells through the rim, as previously proposed (Heermann et al., 2015). The rim flow seems in fact independent from the neural retina basal constriction, as it is still present in the medaka *opo* mutants (Bogdanović et al., 2012), in which a mutation in a transmembrane protein implicated in retinal basal adhesion prevents OC folding (Martinez-Morales et al., 2009). In contrast, expansion of the RPE surface driven by cell stretching and cytoskeletal-mediated increased stiffness may "push" rim cells into the retina (Heermann et al., 2015).

Other studies support an active role of the RPE in OC morphogenesis. The RPE can bend autonomously in culture (Willbold et al., 1996). In eye-organoids derived from mammalian embryonic stem cells (Eiraku et al., 2011; Nakano et al., 2012), significantly high levels of phospho-myosin accumulation make the RPE stiffer than the neural retina as determined by atomic force microscopy (Eiraku et al., 2011). This and other observations lead to a proposal that the RPE forms a rigid shell around a softer retina and this differential

tension together with the apical constriction of rim cells induces OC formation (Eiraku et al., 2012). Furthermore, in absence of Wnt signaling, the mouse RPE does not extend and the OV does not fold, suggesting that the RPE has to reach a sufficient size to sustain the curvature of the OC (Carpenter et al., 2015). Besides, in the zebrafish *otx* morphants and *Otx* null mice, in which the RPE does not form, the neural retina is everted (Martinez-Morales et al., 2001; Lane and Lister, 2012), indicating that the RPE is needed for neural retina invagination.

Thus, it seems reasonable to propose that tension and extension of the RPE can be crucial for proper OC folding just as much as basal constriction of the retinal cells or epithelial flow at the rim. This hypothesis needs experimental verification.

WHAT COMES NEXT

4D analysis of the embryonic zebrafish has provided a wealth of information on eye morphogenesis, although full comprehension of RPE formation is lagging behind and many questions remain open. For example, there are still uncertainties on how many of the movements sustaining zebrafish eye morphogenesis can be fully applied to the formation of the amniote eye. Meanwhile, details of the gene regulatory network leading to zebrafish RPE specification are unclear. In mice, this network includes Wnt-βcatenin signaling and the activity of the transcription factors Yap, Otx, Mitf followed by Pax6 and Sox9, which both contribute to maintain RPE identity (Nguyen and Arnheiter, 2000; Martinez-Morales et al., 2001; Masuda and Esumi, 2010; Raviv et al., 2014; Kim et al., 2016). In zebrafish, *otx* genes have a similarly essential role in RPE specification but *mitf* genes seem to be dispensable (Lane and Lister, 2012), whereas *yap* inactivation gives a phenotype that differs from that of the mouse, as already discussed. These differences might be explained by the zebrafish genome duplication or the presence of additional related genes that have adopted their function as proposed in the case of *mitf* (Lane and Lister, 2012). Alternatively, they may derive from species specific adaptations of the RPE gene regulatory network. Identification of such adaptations may help to understand if there is any functional significance in the different shape of the vertebrate RPE: cuboidal in amniotes but squamous in teleosts.

AUTHOR CONTRIBUTIONS

TM-M, FC, and PB conceived and wrote the manuscript.

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OPINION
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Zebrafish—A Model Organism for Studying the Neurobiological Mechanisms Underlying Cognitive Brain Aging and Use of Potential Interventions

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Classically, the zebrafish model organism has been used to elucidate the genetic and cellular mechanisms related to development since the embryo forms and grows externally following fertilization. This provides insight into the genetic control of developmental processes in humans because their genomes are similar. Also, unlike other animal models, the genes of zebrafish can be manipulated quite easily by using reverse genetic screens tools such as morpholinos, which transiently silence target genes of interest or systems such as the transposon-mediated insertional mutagenesis or CRISPR-Cas9. Moreover, one pair of fish will provide up to 300 offspring, which means that if there is a gene of interest that is manipulated, then it can be transmitted to a large population of fish. What is beginning to emerge is that similar to other mammals, adult zebrafish have an integrated nervous system, which is proposed to contain homologous brain structures to those found in humans, as well as equivalent cellular and synaptic structure and function. Moreover, like humans, zebrafish exhibit age-related declines in cognitive functions, and a convergence of evidence has indicated that subtle changes in cellular and synaptic integrity underlie these changes. Therefore, the zebrafish is a powerful model organism for studying the neurobiological consequences of aging-related behavioral and biological changes, which offers the potential to identify possible interventions that would promote healthy aging. In what follows, we present and discuss recent findings and advances along these directions.

BEHAVIORAL TASKS AND ABILITIES ALTERED IN AGED ZEBRAFISH

The zebrafish is a promising model for studying age-related changes in cognition and perception. Early behavioral studies date back to 1960s and the characterization of zebrafish behavior has accelerated since 2000 (Kalueff et al., 2013). They have been suggested to reflect the evolutionarily conserved nature of many behaviors and to resemble those of other species (Kalueff et al., 2014; Stewart et al., 2014; Orger and de Polavieja, 2017). A rich repertoire of behavioral phenotypes has been identified for cognitive functioning, perceptual processes, and associated disorders (Stewart and Kalueff, 2012). Using different behavioral assays (e.g., inter- and intra-trial habituation, T-maze, conditioned place preference paradigms), previous studies indicated that zebrafish have both simple and relatively complex forms of learning, and also display good

performance on cognitive tasks dependent on short-term and long-term memory (Blaser and Vira, 2014; Gerlai, 2016). There is also growing interest in other aspects of zebrafish behavior which significantly depend on perception, low-level discrimination, and sensitivity (Neuhauss, 2010). For instance, the basic components of the zebrafish visual system, the visual processing hierarchy, and pathways are similar to those commonly found in other species (Bilotta and Saszik, 2001). In particular, most of the previous research evaluated visual motion perception and sensitivity through optomotor response and/or optokinetic reflexive eye movements. These behavioral studies point to qualitatively similar visual acuity and contrast sensitivity functions for zebrafish (Rinner et al., 2005; Haug et al., 2010; Tappeiner et al., 2012). It has also been shown that zebrafish perceive first- and second-order motion. They also experience motion illusions commonly used in studies on human vision such as reverse-phi illusion, motion aftereffect, and rotating snakes illusion (Orger et al., 2000; Gori et al., 2014; Najafian et al., 2014). Within the context of visual motion, these studies provide behavioral evidence that mechanisms and principles similar to those of humans and other species underlie zebrafish sensory processing and associated behavior.

Characterizing aging-related changes in zebrafish behavior has important implications for our understanding of cognition and perception. First, aging-related changes in cognition are a part of the normal aging process and common in all the species. Monitoring age-dependent changes in cognition and perception is difficult to perform on the same human subject throughout life. Due to their short lifespan, behavioral assays and paradigms developed, zebrafish provides an ideal model to study cognitive and perceptual performance during aging. Second, when these behavioral studies are combined with already developed molecular and genetic tools on this aging model, we will also have a deeper understanding on the functional links between key synaptic targets, cognition, and perception during neural aging. Previous studies report significant declines in learning and memory in aged zebrafish. Typically, old zebrafish have less performance on tasks relevant with associative learning, avoidance, spatial learning and working memory (Yu et al., 2006; Arey and Murphy, 2017; Brock et al., 2017). Compared to wild-types, mutants with impaired acetylcholinesterase function had better performance in spatial learning, entrainment and increased rate of learning (Yu et al., 2006; Parker et al., 2015). These findings suggest that cholinergic signaling may also play a role in age-related cognitive decline. In terms of perceptual performance, there are studies comparing larvae and adult zebrafish. However, we have limited knowledge on how perceptual performance (and thus perception and sensitivity) changes during neural aging. A challenge for the future is to characterize aging-related changes in perceptual performance and sensitivity of adult zebrafish. As mentioned above, we consider that such studies can provide comprehensive information not only on perception and behavior in general (Owsley, 2016) but also on the cellular and molecular mechanisms underlying specific aspects (e.g., motion) of perception and sensitivity.

AGING-RELATED NEUROBIOLOGICAL ALTERATIONS

Understanding the cellular mechanisms that underlie cognitive decline is important for determining sites of actions for possible interventions that could ameliorate alterations in cognitive function. Early reports indicated that age-related cognitive decline was due to significant cell (Brody, 1955; Devaney and Johnson, 1980; Henderson et al., 1980) and synapse loss (Geinisman et al., 1977; Bondareff, 1979; Curcio and Hinds, 1983; Haug and Eggers, 1991; Shi et al., 2005). However, it has become well accepted that significant cell (Haug and Eggers, 1991; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Peters et al., 1998) and synapse loss does not occur in conjunction with normal aging-related declines in cognitive capacities (Poe et al., 2001; Newton et al., 2007; Shi et al., 2007). Therefore, research studies have been designed at examining markers of cellular and synaptic integrity during the aging process, such as altered neurogenesis rates (Kempermann et al., 1998, Luo et al., 2006) and the levels of key excitatory and inhibitory pre- and post-synaptic proteins (Newton et al., 2007; Shi et al., 2007; Adams et al., 2008), since subtle changes in cellular and synaptic functions likely underlie the aging-related declines in cognitive abilities. Moreover, examining key molecular targets that control these processes will increase our understanding of the cellular and synaptic regulation of behavior across the lifespan.

While these aging-related changes in cellular and synaptic processes could be examined in many different animal species, the zebrafish model organism is well-adapted to studying the cellular and molecular changes with aging because they have similar patterns as mammals with regards to the cellular aging process. Zebrafish on average live approximately three to five years and share a similar genome with humans (Kishi et al., 2003; Howe et al., 2013). Moreover, senescence-associated ß-galactosidase, which is a biomarker of aging, increases with advancing age in zebrafish, and this cellular alteration has been described in humans as well (Kishi et al., 2003; Arslan-Ergul et al., 2016). Finally, zebrafish have continued neurogenesis even into late adulthood (Kizil et al., 2012; Schmidt et al., 2013), they express key excitatory and inhibitory pre- and post-synaptic proteins (Karoglu et al., 2017), and classical cellular synaptic plasticity (i.e., long-term potentiation) is found in their brains (Nam et al., 2004). Recent work in the zebrafish brain has demonstrated that there are age-related declines in genes related to cellular and synaptic structure and growth (Arslan-Ergul and Adams, 2014), neurogenesis (Edelmann et al., 2013; Arslan-Ergul et al., 2016), and synaptic alterations (Arslan-Ergul et al., 2016; Karoglu et al., 2017). Interestingly, as has been shown in mammals, these changes depend on the gender of the animal (Arslan-Ergul and Adams, 2014; Karoglu et al., 2017), and the data are in good agreement with those showing sexually-dimorphic patterns published in young zebrafish brains (Ampatzis et al., 2012). Taken together, these findings indicate that the zebrafish is an appropriate model to study the effects of cellular and synaptic aging and its relationship to cognitive decline.

USE OF INTERVENTIONS TO ALTER AGING-RELATED PROCESSES

A major goal of research related to elucidating the altered cellular and synaptic processes that underlie cognitive aging is to determine possible interventions to restore youthful cellular and synaptic function. As was mentioned previously, mutant zebrafish with lower levels of acetylcholinesterase had better performance in spatial learning, entrainment, and increased rate of learning (Yu et al., 2006; Parker et al., 2015). Therefore, these animals likely have a more youthful cellular and synaptic profile as compared to their wild-type counterparts. Currently, we are investigating this possibility and our data suggest that genetic manipulation of the cholinergic system alters the course of aging-related changes in the synaptic protein levels. We have demonstrated that at old ages as compared to their wild-type siblings, mutants have higher levels of synaptophysin, which is an indicator of presynaptic integrity, and gephyrin, a component of post-synaptic inhibitory transmission, and interestingly these changes are gender-dependent (Karoglu et al., 2018). If we can determine the cellular and synaptic profile of these mutants and how they relate to cognitive aging, it would provide potential targets for drug development to ameliorate the effects of cognitive decline.

Another potential intervention with promise is dietary restriction (DR), which is the only non-genetic intervention that reliably increases both lifespan and healthspan. Numerous studies have shown that a lifelong reduction in caloric intake from *ad libitum* levels increases lifespan (Roth et al., 2001; Lin et al., 2002; Colman et al., 2009). Additionally, DR increases neuronal proliferation and survival (Lee et al., 2002; Kitamura et al., 2006; Park and Lee, 2011; Park et al., 2013). We applied a short-term DR of 10 weeks and observed that this treatment did not prevent an age-related decline in cell proliferation but altered the telomere lengths of these neuronal cells (Arslan-Ergul et al., 2016), thereby DR exerted positive effects by subtly altering the cell cycle dynamics of these neurons. We have tested the timing and duration of short-term DR and a potential DR-mimetic, rapamycin, as the positive effects of DR are thought to be modulating the mammalian target of rapamycin signaling pathway. Our data indicate that a longer duration of both DR and its mimetic is more effective on aging-related changes in synaptic protein levels and transcripts, which might reflect a conserved mechanism of the beneficial effects of DR and rapamycin on life- and healthspan (Celebi-Birand et al., 2018). These studies also have the potential to provide suitable therapeutic targets around which drug development can proceed for ameliorating the devastating effects of cognitive decline.

CONCLUSIONS

The zebrafish is clearly a powerful model organism that can be used to understand the aging-related changes in both cognition and the underlying cellular and molecular processes. As previously mentioned, zebrafish exhibit characteristics that are similar to humans, as well as other mammals, including the fact that these animals age gradually, and they demonstrate aging-related changes across both cognitive and neurobiological spectrums. It clear that both genetic and non-genetic interventions can be applied to alter the course of the aging process and provide potential drug targets that could be manipulated to ameliorate age-related cognitive declines. Therefore, this model will help researchers elucidate the biological mechanisms that underlie aging-related cognitive decline.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Workshop on Germ Cells

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Germ cell research in vertebrates has traditionally been challenging, but recent breakthroughs have overcome technical difficulties, demonstrating and expanding the power of the zebrafish experimental system for their analysis *in vivo*. Exploiting the transparency of the zebrafish embryo, germ cell migration was the first topic that moved the germ cells of this organism into the spotlight of modern research. In recent years, research on teleost germ cells has expanded into additional fields, manifested by a session dedicated to this cell type at the European Zebrafish PI meeting in Trento.

Keywords: zebrafish (Danio rerio), germ cells, fertility, oogenesis and embryonic development, gene editing, germ cell migration, European zebrafish PI Meeting, Trento Italy

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The first talk by **Yaniv Elkouby** (The Hebrew University of Jerusalem, Israel) started with the earliest stages of oogenesis. Addressing these early stages in the adult ovary is challenging and Yaniv established the juvenile zebrafish ovary as an excellent model for early oogenesis (Elkouby, 2017). Using this system, Yaniv pioneered live imaging of follicle formation in zebrafish (Elkouby and Mullins, 2017). In his talk, he showed spectacular movies of chromosomes forming the meiotic *bouquet* arrangement *in vivo,* in which all chromosome telomeres cluster in one location of the inner nuclear membrane. His data showed that the polarity of the *bouquet* predicts the future animal-vegetal axis of the oocyte. He thereby discovered the first cellular markers for oocyte asymmetry during oogenesis (Elkouby et al., 2016), and highlighted the function of the oocyte centrosome in integrating meiosis, oocyte patterning and Balbiani body assembly in the early differentiating oocyte (Elkouby, 2017). Traditionally, zebrafish oogenesis was subdivided into five stages, but Yaniv now identified additional features that characterize the progression of oogenesis. Based on these features, he purified stage-specific oocytes and performed RNA-sequencing, in collaboration with Mary Mullins and Antonio Giraldez. This work identified a differential gene expression signature with dynamic trends in gene ontogeny between stages, providing further molecular insight into early oogenesis.

A recurrent obstacle for studying the role of key regulators during germline development is their genetic depletion. A large proportion of these genes also play crucial roles in the somatic cells or cause early loss of the germline. **Florence Marlow** (Icahn School of Medicine at Mount Sinai in New York, USA) tackled this challenge by establishing a system for germline specific gene editing *via* CRISPR/Cas9. Using specific promoters, she can either activate Cas9 in the entire germline or specifically in the female. As a demonstration of the technology, she recapitulated the *kif5Ba* mutant maternal-effect phenotype previously described by her laboratory (Campbell et al., 2015), but without somatic defects. As this innovative technology allows eliminating somatic side-effects, this interesting approach will have a great impact on future research in the zebrafish germline field.

The process of fertilization has fascinated researchers since its discovery, but the identification of the molecular players in vertebrates has been difficult (Evans, 2012; Miyata et al., 2016). Two talks identifying novel gene functions essential for female fertility open new lines of research

in reproductive biology. While a postdoc in Alex Schier's lab at Harvard University, **Andrea Pauli** previously identified hundreds of novel transcripts that were predicted to encode for short proteins (Pauli et al., 2014). In her talk, Andi presented unpublished results from her own lab (Research Institute of Molecular Pathology (IMP) Vienna BioCenter, Austria) concerning one of those candidate proteins that they named Bouncer (Herberg et al., 2018). Bouncer is a conserved GPI-anchored vertebrate protein that localizes to the egg membrane in fish. *Bouncer* mutant female zebrafish are sterile due to a defect in sperm entry. Remarkably, replacing zebrafish Bouncer with Bouncer of Medaka, an evolutionarily distant fish, allowed Medaka sperm to fertilize zebrafish eggs. Thus, Bouncer acts as gate-keeper of the egg during fertilization by allowing conspecific sperm to enter while keeping heterospecific sperm out. These experiments identify Bouncer as a novel, maternal protein, which mediates species-specific fertilization.

In addition to species-specific recognition mechanisms between sperm and egg, in several species including teleosts, fertilization requires a peculiar opening at the surface of the vitelline membrane that facilitates sperm entry and is called the micropyle. The micropyle mechanically prevents polyspermy by restricting the entry of only a single sperm. It is formed by a single specialized follicle cell, the micropylar cell, that grows much bigger than its neighbors and acquires a typical mushroom-shape during oogenesis. To date, hardly anything is known about the molecular pathways controlling micropyle formation. The lab of **Virginie Lecaudey** (Goethe University of Frankfurt, Germany) discovered that females mutant for the Hippo pathway effector Taz/*wwtr1* are sterile, because their oocytes do not form a functional micropyle (Dingare et al., 2018). Indeed, she showed that a Taz antibody specifically labeled the micropylar cell. Taz therefore provides the first marker to molecularly distinguish the micropylar cell from the other follicle cells that surround the oocyte. Using this marker, they could follow the cytoskeletal changes occurring in the prospective micropylar cell itself and at the oocyte animal pole cortex, with which it remains associated. Through identifying this role for the *taz* gene Virginie's lab has established a molecular foothold to unravel the cellular changes associated with micropyle formation and provided novel insights into the molecular cause of sterility in zebrafish.

Zebrafish germ cells are specified during embryogenesis by a germline-specific RNP-granule termed germ plasm. The lab of **Roland Dosch** (Georg-August University of Goettingen, Germany) previously identified the Bucky ball (Buc) protein as the first vertebrate germ plasm organizer (Dosch et al., 2004; Marlow and Mullins, 2008; Bontems et al., 2009). Importantly, the amino acid sequence of Bucky ball does not provide any information about its biochemical mechanism, but Roland showed that the germ plasm organizer Oskar from *Drosophila* can, like Buc, promote formation of germ cells in zebrafish (Krishnakumar et al., 2018). Thus, although the *Drosophila* and zebrafish proteins show no protein sequence similarities, zebrafish Bucky ball and *Drosophila* Oskar appear to have similar activities in the embryo, which presumably involve interactions with the same germ plasm proteins and RNAs to promote germline fate. These results identify the first protein pair, which has the same function in different organisms in the absence of any sequence homology. These data thus indicate that germline specification might be more conserved than previously anticipated.

Although primordial germ cells differentiate into only one type of cells, the gametes, the germline is considered to be totipotent as it can give rise to the complete organism in the next generation. This totipotent property should be suppressed in primordial germ cells that are exposed to a range of differentiation signals during their migration. Relevant for the control over germ cell fate, the lab of **Erez Raz** (Muenster University, Germany) had previously identified the Dead end protein as a key regulator of zebrafish germline development. It was later discovered that Dead end also plays a crucial role in the mammalian germline, where mutations in Dead end cause depletion of germ cells and teratomas. In his talk, Erez reported remarkable results that upon knockdown of Dead end, zebrafish germ cells cannot maintain their germline fate (Gross-Thebing et al., 2017). While a small proportion of germ cells undergo apoptosis, most of the *dead end* depleted cells differentiate into somatic cell types of the three germ layers. Indeed, Erez showed data, in which his lab reprogrammed Dead end depleted primordial germ cells into endodermal derivatives by overexpressing Taram-a, which is a constitutively-active TGF-β receptor known to induce endoderm in early embryos (Renucci et al., 1996; Peyriéras et al., 1998). In addition to the contribution toward understanding the process of germ cell fate maintenance, these data highlight the possible use of this intriguing cell population in regenerative therapies.

These interesting presentations spurred lively discussions. The session emphasized the recent contribution of the zebrafish to major technical and conceptual advances in germ cell biology, long sought after since postulated by Weismann in 1895. The spectrum of the talks at the workshop demonstrated that the research field is increasingly expanding but made also clear that many aspects of the zebrafish germline such as spermatogenesis should be added to future meetings. This trend was also visible in other sessions at this European Zebrafish PI meeting, where additional studies of germline biology were presented such as the talk of René Ketting (IMB, Mainz, Germany) on the activation of the piRNA pathway in primordial germ cells. Overall, the germ cell workshop demonstrated the potential of the zebrafish model in expanding our knowledge of vertebrate germ cell specification, fate maintenance, and gamete differentiation and function. The positive momentum present at the meeting promises further exciting discoveries in the future, through and beyond the next European Zebrafish PI Meeting in 2021.

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Is Alzheimer's Also a Stem Cell Disease? – The Zebrafish Perspective

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Alzheimer's disease (AD) is the most common neurodegenerative disease and is the leading form of dementia. AD entails chronic inflammation, impaired synaptic integrity and reduced neurogenesis. The clinical and molecular onsets of the disease do not temporally overlap and the initiation phase of the cellular changes might start with a complex causativeness between chronic inflammation, reduced neural stem cell plasticity and neurogenesis. Although the immune and neuronal aspects in AD are well studied, the neural stem cell-related features are far less investigated. An intriguing question is, therefore, whether a stem cell can ever be made proliferative and neurogenic during the prevalent AD in the brain. Recent findings affirm this hypothesis and thus a plausible way to circumvent the AD phenotypes could be to mobilize the endogenous stem cells by enhancing their proliferative and neurogenic capacity as well as to provide the newborn neurons the potential to survive and integrate into the existing circuitry. To address these questions, zebrafish offers unprecedented information and tools, which can be effectively translated into mammalian experimental systems.

Keywords: zebrafish, Alzheimer's disease, neural stem/progenitor cells, regeneration, neurogenesis

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THE RE-RISE OF STEM CELL ASPECT FOR NEURODEGENERATIVE DISEASES

Stem cells are the main reservoir for production of new cells. Understanding the basic biology of how stem cells are specified, maintained and regulated has been an exciting focus of research for many decades. Yet, there are still missing pieces especially on how stem cells could be utilized for neurodegenerative diseases (Jebelli et al., 2015; Tincer et al., 2016; Wyss-Coray, 2016). Stem cells offer great promises for medicine, as they can be the golden way to a "regenerative therapy" (Doetsch and Scharff, 2001; Lopez-Toledano and Shelanski, 2004; Rodriguez and Verkhratsky, 2011; Kizil et al., 2012b; Gage and Temple, 2013; Hong et al., 2014; Lilja et al., 2015). By using the endogenous stem cells, tissue loss could be reverted or the integrity of the existing tissues could be enhanced. Such outcomes would have ramifications in several human diseases but possibly among the most interesting is neurodegeneration (Tincer et al., 2016; Wyss-Coray, 2016). Indeed, since the generic term of "neurodegeneration" denotes the state of losing cells of the nervous system and in particular the neurons, transplantation of stem cells into the brain to get more neurons produced from these stem cells were one of the first treatment options (Dantuma et al., 2010; Mu and Gage, 2011; Rodriguez and Verkhratsky, 2011; van Tijn et al., 2011;

Ager et al., 2015; Lee et al., 2015; Tincer et al., 2016; Wyss-Coray, 2016; Espuny-Camacho et al., 2017), for instance, injection of fetal NSCs for treating Parkinsonism (Baetge, 1993; Dunnett et al., 1997; Svendsen et al., 1997; Brundin and Bjorklund, 1998; Studer et al., 1998). These efforts could not gain spotlight as the transplanted stem cells or progenitors could not survive or could not form the desired cell types. For a couple of decades, the main focus in neurodegenerative diseases has been to prevent the neuronal death and synaptic failure (Carter and Lippa, 2001; Selkoe, 2003; Iqbal et al., 2016; Wyss-Coray, 2016). In Alzheimer's disease (AD) – where the main culprit of the pathology is accumulation of Amyloid plaques and neurofibrillary tangles that lead to the loss of mostly cholinergic innervations in the brain – preventing the loss of synaptic degeneration and reduction in the neurotransmitter acetylcholine was prioritized as a therapy option (Fischer et al., 1987; Tuszynski et al., 1990; Nagele et al., 2002; Park et al., 2012; Gu et al., 2015). Several current drugs on the market for AD are blockers of the enzyme choline acetyltransferase, which degrades the cholinergic neurotransmitters in the brain. These drugs also failed to cure the disease despite causing meager slowdown in the cognitive decline in Alzheimer's patients (Schneider et al., 2014). Similarly, physically destroying the plaques causes a cognitive advantage while does not fully restore the disease-associated symptoms (Takeda and Morishita, 2015). All these hypotheses and failures tell us a lesson: Alzheimer's is not only a neuronal disease but also a complex mixture of malfunctioning in various cell types. An array of different cell types was implicated in the onset and progression of AD (De Strooper and Karran, 2016). These include changes in immune components (Amor et al., 2010; Heneka et al., 2015; Heppner et al., 2015), neurovascular niche (Kirkitadze et al., 2002; De Strooper and Karran, 2016), NSCs (Tong et al., 2015; Tincer et al., 2016), astrocytes (Attems and Jellinger, 2014; Lian and Zheng, 2016), and oligodendrocytes (Bartzokis, 2011; Ettle et al., 2016), suggesting a multifactorial influence on the initiation of AD. It can even be hypothesized that the loss of neurons – which is relatively a late symptom of the disease – might be the consequence of the yet-elusive real cause. When we generate a temporal onset of various symptoms of AD – mostly in animal models – we see that the first changes in the brain are the deterioration of the immune system balance, gliotic response from astrocytes and reduction in neural stem cell proliferation (Aguzzi and Haass, 2003; Selkoe, 2003; Blennow et al., 2006; Harman, 2006; Chai, 2007; Arendt, 2009; Hardy, 2009; Huang and Mucke, 2012; De Strooper and Karran, 2016; Dzamba et al., 2016; Tincer et al., 2016). As Amyloid deposition and neurofibrillary tangles occur, an inflammatory reaction manifests and becomes chronic in time. Concomitant to this reaction, NSCs also reduce their proliferation rate and produce less neurons long before the myelin breakdown, synaptic degeneration and neuronal cell death manifest (Demars et al., 2010; Tincer et al., 2016). Therefore, it is a plausible hypothesis to think that the inflammatory environment is negatively affecting the brain homeostasis in Alzheimer's conditions not only by eliciting a chronic inflammation that is detrimental for synapses on its own but also by reducing the capacity of the brain to produce more neurons – an ability that could have been utilized to replace the lost neurons. These questions seem to have opened a wide research realm focusing on the role of immune system in AD (Akiyama et al., 2000; Heneka et al., 2005, 2015; Wyss-Coray, 2006; Amor et al., 2010; Aguzzi et al., 2013; Heppner et al., 2015; Kizil et al., 2015). Many reports documenting the effects of inflammation on AD pathology and the role of immune cells on the progression of the disease emerged. It is quite likely that coming years will bring important paradigm shifts in the relationship of immune system and the AD. However, a largely overlooked phenomenon in this context is the NSCs. Can NSCs and neurogenesis be the key to the cure for neurodegeneration? (Ziabreva et al., 2006; Waldau and Shetty, 2008; Taupin, 2009; Dantuma et al., 2010; Rodriguez and Verkhratsky, 2011; Tincer et al., 2016). This is where zebrafish could contribute to the answer of this provocative question.

ZEBRAFISH AND THE HOPE FOR STEM CELL-BASED REGENERATIVE THERAPIES

No existing model for AD recapitulates the full spectrum of the disease, and existing mouse models are not exceptions (LaFerla and Green, 2012). These models can be considered at best the tools to study the early onset stages of Alzheimer's (De Strooper and Karran, 2016). Although mouse models provided invaluable information on the pathology of AD, these mammalian models are not ideal to study "regeneration" as they do not have regenerative ability at first place (Goss, 1991). Zebrafish, an animal model that can regenerate its neurons offers unprecedented hope for restoring lost neurons in AD (Kizil et al., 2012b; Cosacak et al., 2015; Tincer et al., 2016; Kizil, 2018).

Mammals fail to regenerate amputated limbs, cardiac tissue, brain or spinal cord due to their restricted and limited regenerative potential (Tanaka and Ferretti, 2009; Poss, 2010). Current studies focus to improve methods or develop novel approach that can induce regenerative programs into the mammalian systems (Antos and Tanaka, 2010; Gemberling et al., 2013; Cosacak et al., 2015). One approach is to induce regeneration by activating endogenous regeneration programs. Zebrafish could serve as a model to understand those molecular cues as many "regeneration" programs were identified in zebrafish and they serve as interesting candidates toward this aim (Raya et al., 2003; Zupanc, 2008; Kizil et al., 2009, 2012a,b,c; Millimaki et al., 2010; Kyritsis et al., 2012; Diotel et al., 2013; Berberoglu et al., 2014; Cosacak et al., 2015; Alunni and Bally-Cuif, 2016; Bhattarai et al., 2016; Katz et al., 2016; Mokalled et al., 2016; Kizil, 2018; Than-Trong et al., 2018). Hence, the remarkable feature of regeneration in zebrafish deserves a closer attention for translational ramifications.

The exciting yet provocative argument of "zebrafish can teach us" could be challenged from another perspective: it could also be argued that the reduced capability of regeneration in rodents makes them better models as they are closer to the human situation. It is surely true that a model, which is as close as possible to human condition, would be ideal to work out reductionist aspects of a disease and indeed the mouse

models offered invaluable knowledge on AD pathology. However, regenerating organisms endow a novel perspective of stem cell plasticity and regenerative ability that might be harnessed for therapeutic ramifications in humans but may not be investigated in mammalian systems. If nature has evolved a set of molecular programs that enable regenerative output of NSCs in AD conditions, zebrafish and other regenerating organisms but not mammals could teach us these programs. In the long run, those programs must be tested in mammals to investigate if they are evolutionarily conserved and whether they are sufficient to elicit a stem cell response similar to that of zebrafish. This could be the step where zebrafish could come in handy: identification of naturally occurring "candidate" programs that might underlie a regenerative touch to the old problem of AD. It is also necessary to mention that the nature of regenerative ability and why it is lost evolutionarily in mammals are still unknowns. Therefore, the applicability of the knowledge from zebrafish to humans needs further studies, which will shed more light onto the extent of parallelism between mammals and zebrafish in disease conditions.

NEURAL STEM CELLS AND NEURONAL REGENERATION

Mammalian nervous system contains NSCs that give rise to newborn neurons during development as well as adulthood (Doetsch et al., 1999; Gage, 2000; Conti and Cattaneo, 2010; Gage and Temple, 2013). The ability of NSCs to form neurons however varies and is still controversial (Kronenberg et al., 2003; Galvan and Jin, 2007; Kempermann et al., 2008, 2018; Ernst et al., 2014; Magnusson et al., 2014; Urban and Guillemot, 2015; Magnusson and Frisen, 2016; Boldrini et al., 2018; Sorrells et al., 2018). During development, NSCs give rise to all neuronal subtypes (Gage, 2000; Temple, 2001; Doetsch, 2003; Kriegstein and Alvarez-Buylla, 2009; Hansen et al., 2010; Pacary et al., 2012; Urban and Guillemot, 2015). But, during the adulthood, the NSCs are restrictive and limited to fewer areas – the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus of the hippocampus (Doetsch and Scharff, 2001; Alvarez-Buylla et al., 2002; Spalding et al., 2013; Kempermann et al., 2018). Though constitutive neurogenesis occurs in these neurogenic regions, upon injury they fail to achieve neuronal repair due to lack of neurogenic inputs (Silver and Miller, 2004; Rolls et al., 2009; Costa et al., 2010). For instance, in case of mammalian traumatic injury model, there is absence of permissive environment for NSCs to react effectively.

Unlike mammals, zebrafish can successfully regenerate the injured part of its brain (Chapouton et al., 2007; Zupanc, 2008; Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kizil et al., 2012a,b,c; Kyritsis et al., 2012; Marz et al., 2012; Barbosa et al., 2015; Cosacak et al., 2015; Bhattarai et al., 2016; Kizil, 2018). This ability is possible because of the stem cell niches and the neurogenic regions that harbors proliferative neural progenitor cells (Adolf et al., 2006; Grandel et al., 2006; Chapouton et al., 2007; Kaslin et al., 2009). However, there is more to it. The regenerative ability after neuronal loss in zebrafish brain relies on the activation of specific molecular mechanisms that do not exist in normal homeostatic state or even during development of those structures (Zupanc, 2008; Kaslin et al., 2009; Fleisch et al., 2010; Kizil et al., 2012b; Cosacak et al., 2015; Alunni and Bally-Cuif, 2016; Kizil, 2018; Shimizu et al., 2018). There is still a long way to understand the complete picture that makes the zebrafish brain special, yet the path is quite promising. Can we understand in zebrafish how new neurons are made and can we harness this information for humans to effectively regenerate our brains when needed – for instance in AD?

ADDRESSING STEM CELL POTENTIAL IN ALZHEIMER'S DISEASE MODEL IN ADULT ZEBRAFISH BRAIN

One of the hallmarks of AD is accumulation of amyloid plaques that are made up of the short peptide Amyloid-beta42 (Aβ42) (Yang et al., 1995; Duff et al., 1996; Younkin, 1998). In mammals, plaques elicit chronic inflammation and together with the plaques lead to synaptic failure, reduced neural stem cell plasticity and neurogenesis (**Figure 1**). We recently developed a microinjection-based method to generate an Aβ42 model in adult zebrafish that displayed AD-like phenotypes (Bhattarai et al., 2016, 2017a,b). Aβ42 aggregation in adult zebrafish brain led to phenotypes reminiscent of human AD pathophysiology: neuronal death, inflammation, synaptic degeneration, memory and learning deficits. In addition, this model also induced regenerative response by activation of NSCs and subsequent neurogenesis to compensate the neuronal insult (**Figure 1**). Therefore, this Aβ42 toxicity model in adult zebrafish offers an opportunity to study the molecular mechanisms how NSCs can be activated to form neurons and induce regeneration in AD condition. Interestingly, this regenerative neurogenesis response upon Aβ42 in adult zebrafish brain was mediated by a crosstalk between the immune system and the NSCs via an unexpected mediator: Interleukin-4 (IL4), an anti-inflammatory cytokine (**Figure 1**). Although the role of IL4 in suppressing the inflammatory response and in turn relieving the suppressive effects of inflammation on the neural stem cell proliferation in mammalian Alzheimer's models were known, the direct regulation of the inflammatory environment on NSCs – which are the only non-immune cell types that express the receptor for IL4 – was a novel finding. Even with known molecules, zebrafish could provide novel understanding and ideas on how crosstalk mechanisms between the neurodegenerative milieu and the NSCs in the adult zebrafish brain could induce regenerative response (Bhattarai et al., 2016; Kizil, 2018). These studies also proposed that neural stem cell activity might be key to a successful recovery from neurodegeneration.

CAN ALZHEIMER'S BE TREATED WITH INCREASED NEUROGENESIS?

The role of neurogenesis in Alzheimer's pathology and whether new neurons could really rescue the symptoms of Alzheimer's is

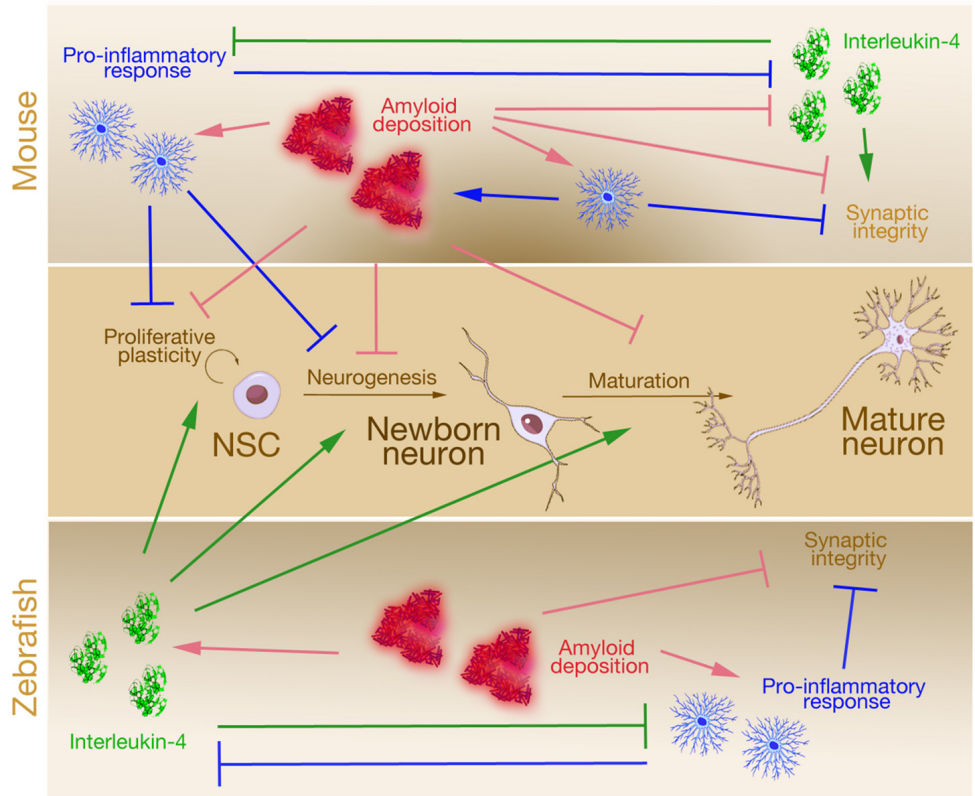

FIGURE 1 | A simplified comparison of the effects of Alzheimer's disease on neural stem cell plasticity in mouse and zebrafish. In mouse, Amyloid deposition initiates pro-inflammatory response that potentiates Amyloid toxicity that impairs neural stem cell proliferation, neurogenesis, neuronal maturation, and synaptic integrity. This chronic inflammation suppresses anti-inflammatory factor Interleukin-4, which is beneficial for neuronal survival and synaptic integrity. In zebrafish, although Amyloid deposition follows a toxicity cascade similar to that of the mouse (activation of pro-inflammatory response and hampered synaptic integrity), Amyloid also leads to induction of anti-inflammatory factor Interleukin-4, which enhances neural stem cell proliferation, neurogenesis, and neuronal maturation. The effects of Interleukin-4 counteracts synaptic degeneration and reduced neural stem cell plasticity.

quite controversial and some researchers are skeptical toward this approach because the effects of Amyloid deposition on stem cell proliferation are beneficial or detrimental in a context dependent manner (Haughey et al., 2002; Lopez-Toledano and Shelanski, 2007; Diaz-Moreno et al., 2013; He et al., 2013; Lee et al., 2013; Bhattarai et al., 2016). Since neurogenesis cannot be equated with functional integration into the circuitry, the need to detect the effects of newborn neurons on circuit integrity has not been met sufficiently (Wen et al., 2004; Yamasaki et al., 2007; Blurton-Jones et al., 2009; Gomez-Nicola et al., 2014). However, when we scrutinize the course of manifestation of AD, we see that the NSCs are affected during the neurodegenerative conditions in all mammalian model systems tested: a progressive decline in neural stem cell pool during the course of neurodegeneration (Haughey et al., 2002; Ziabreva et al., 2006; Waldau and Shetty, 2008; Rodriguez and Verkhratsky, 2011; He et al., 2013; Martinez-Canabal, 2014; De Strooper and Karran, 2016; Dzamba et al., 2016; Tincer et al., 2016). But in case of Aβ42-mediated neurodegeneration in zebrafish, increased neuronal death was followed by increased proliferation of NSCs (Bhattarai et al., 2016, 2017a,b). Zebrafish brain reacted to neurodegeneration by utilizing neuro-inflammatory crosstalk to mediate the regenerative response. It indicates that the molecular mechanism regulating the regenerative response after amyloid-mediated neurodegeneration was pathology-induced plasticity response, and could be helpful to alleviate the symptoms of AD (Kizil, 2018). In fact, supporting evidence to this hypothesis came from comparative studies in a tissue mimetic 3D human NSCs plasticity assays and neuronal cultures as 3D systems are emerging as promising surrogates for human brain disease modeling (Justice et al., 2009; Haycock, 2011; Tang-Schomer et al., 2014; Zhang et al., 2014; Pasca et al., 2015; Ravi et al., 2015; Choi et al., 2016; Murphy et al., 2017; Papadimitriou et al., 2018). To test whether IL4 would act, similarly, in humans during AD – and therefore can be used as a regenerative paradigm, we developed an *in vitro* 3D culture system to grow mature human cortical neurons and networks from human NSCs (Papadimitriou et al., 2018). This system provides an in-vivo like environment including the essential components of the extracellular matrix, which are dynamically produced by the cultured cells and allows experimentation on a wide spectrum of human brain

physiology: from neural stem cell plasticity to neuronal differentiation, from neuronal maturation to integration of neurons into existing networks. Adapting a glycosaminoglycan-based, cell-responsive hydrogel platform, we stimulated primary human neural stem cells (NSCs) from human cortex to form extensive neuronal networks *in vitro*. The 3D cultures exhibited neurotransmitter responsiveness, electrophysiological activity, tissue-specific extracellular matrix (ECM) deposition, and the expression of pro-neural genes and cortical neuronal markers that are undetectable in conventional 2D cultures. Importantly, those cultures formed from primary (human fetal) cortical cells, closely resemble the human physiology, which is critical for any disease modeling or therapeutic drug discovery efforts. The 3D cultures displayed a robust neural stem cell proliferation and neuronal differentiation, which is essential for a self-sustaining germinal niche of the human brain. After being formed *in situ*, our cultures express mature cortical neuronal markers showing a tissue-mimetic development (Papadimitriou et al., 2018). In this system, we modeled Amyloid toxicity as in adult zebrafish brain and found that the 3D culture system nicely recapitulated the major Alzheimer's phenotypes such as the synaptic degeneration, loss of network connectivity, reduced neural stem cell proliferation and Tauopathies in a highly reproducible manner (Papadimitriou et al., 2018). Interestingly, treatment with IL4 under high Amyloid burden restored the neural stem cell proliferation, neurogenesis, network formation and functional integration of neurons into the existing circuitry, suggesting that increasing the neurogenesis in Alzheimer's conditions could rescue the symptoms and might be a plausible way to cure this disease. In fact, a recent *in vivo* study found that increasing adult neurogenesis in Alzheimer's model of mice increases the cognitive abilities and generated a healthier brain microenvironment in AD conditions (Choi et al., 2018), suggesting that the role of neurogenesis in conjunction with inflammation is a charming research realm in AD.

TABLE 1 | Comparison of zebrafish and rodent models in Alzheimer's disease research.

Zebrafish	Rodents
Zebrafish advantegeous over rodents	
Amyloid-mediated neuronal death	No neuronal death
Neuroregenerative capacity	No neuroregenerative capacity
Stem cell plasticity for neurogenesis	Stem cells reduce plasticity and neurogenesis
Cost efficient generation and maintenance	Expensive generation and maintenance
High number of animals testable	Limited number of animals testable
3R strategies developed	3R strategies to be developed
Zebrafish and rodents equal	
Synaptic degeneration	Synaptic degeneration
Cognitive decline with Amyloidosis	Cognitive decline with Amyloidosis
Genetic tools available	Genetic tools available
Does not reflect the entire biology of the human disease	Does not reflect the entire biology of the human disease
Rodents advantegeous over zebrafish	
Non-mammalian physiology	Mammalian physiology
Need for adaptation to preclinical studies	Suitable for preclinical studies
Limited number of models expressing disease-related proteins	Variety of models expressing disease-related proteins

Notwithstanding with the ease of charting this interaction, realizing an immune-stem cell crosstalk in human brains that will lead to a real recuperation seems like a sci-fi novel. However, we know quite a bit on how inflammation is affecting the AD brain (Akiyama et al., 2000; Sastre et al., 2006; Amor et al., 2010; Glass et al., 2010; Aguzzi et al., 2013; Heneka et al., 2013, 2015; Heppner et al., 2015). Chronic phase of inflammation impinges on stem cell plasticity and synaptic integrity while resolution of inflammation provides a relief on the inflammatory burden and affected cell types may regain their potentials. An example of this regulation pertaining to our findings is the effects of Interleukin-4. After experimental models of inflammation, microglial dynamics were shown to be regulated by Interleukin-4 (e.g., pro-inflammatory cytokine release and the extent of initial inflammatory response) and this had an effect on neurogenesis dynamics and neuronal activity (e.g., long term potentiation in hippocampus, neural stem cell proliferation and neuroprotection) (Maher et al., 2005; Nolan et al., 2005; Lyons et al., 2007, 2009; Clarke et al., 2008; Nunan et al., 2014; Barrett et al., 2015). These "beneficial" effects of IL4 was considered to be because of its anti-inflammatory roles. However, in mouse brains, a direct interaction between anti-inflammatory factors and NSCs was not shown. In zebrafish and 3D cultures of human brains, on the other hand, IL4 seems to be directly affecting neural stem/progenitor cells by enhancing their neurogenic output (Bhattarai et al., 2016; Papadimitriou et al., 2018). This proposes an alternative approach to neuroinflammation research where we may need to decouple the microglial inflammation dynamics and direct interaction of immune factors with NSCs, which may be a collateral by-stander effect. In one hypothetical scenario, we may need to investigate which molecules partake in the direct crosstalk between immune system and NSCs in zebrafish and see whether those molecules are able to activate NSCs directly in mammals. Given that even though an immune-related factor would be available in AD brains, its effect is limited to those cells that can receive the signal. The by-stander effects of immune factors could be used to design a stage-specific modulation of NSCs in disease conditions. By a hypothetical scenario, we can appreciate why the immune-related signaling in neuronal compartment and in NSC niche can give us alternative treatment options in humans. For example, in a scenario, an immune factor could turn out to be beneficial for NSC plasticity in AD conditions, but this molecule would be an anti-inflammatory factor (e.g., IL4). Therefore, this factor would prevail only when there is a resolution of inflammation, which is not the case in AD. Therefore, the human NSCs would not be able to increase their proliferation simply due to the stage of the disease (they could otherwise do). Then, a drug can be designed to activate the immune-type signaling in NSCs regardless of the inflammation conditions and this can help elicit a neurogenic contribution from NSCs even if the

inflammation is not resolved. When combined with strategies to increase the survival of newborn neurons, such a "nudge" on NSCs could contribute to the remedy of the disease, which could otherwise not happen naturally. Therefore, understanding the direct interaction of immune system with NSCs by using zebrafish and other appropriate models is important to establish deeper knowledge on the crosstalk between various cell types and NSCs. Additionally, activating the neural stem cell proliferation and neurogenesis in AD conditions must definitely be re-visited as an effective way of tackling this horrendous disease. The neural stem cell aspect of the AD could also provide us new ways for clinical therapies and may help to overcome the inefficient drug discovery efforts for Alzheimer's so far.

LIMITATIONS AND PROMISES AHEAD

Although zebrafish could be an excellent tool from which we could understand how NSCs could be utilized to revert the symptoms of AD, there are experimental and physiological limitations we have to consider (**Table 1**). Zebrafish is a vertebrate and has evolutionary similarities to humans; however, it is still different than the human brains in terms of complexity, molecular structure, and physiology. Given that even mouse models of Alzheimer's cannot be perfect surrogates for human disease, it would be naive to assume that zebrafish brain would fully recapitulate the AD in human brains. This is an aspect where the disease models could be refined in fish and could be made more compatible with human situation. By doing so, zebrafish could also be in part used for early phase pre-clinical studies to test drug efficiency. Additionally, the neurodegenerative disease models should be diversified in zebrafish in order to match the versatility of disease causing proteins. A future perspective for AD modeling in zebrafish could be to generate transgenic animals that display a more chronic and steady accumulation of disease hallmarks that persist throughout the adult stages. Several examples of those efforts are emerging (Malaga-Trillo et al., 2011; Xi et al., 2011; Schmid and Haass, 2013; Cosacak et al., 2017; Lopez et al., 2017; Kizil, 2018). Additionally, using comparative mammalian assays such as organoids or 3D culture systems (Choi et al., 2014, 2016; Fatehullah et al., 2016; Mansour et al., 2018; Papadimitriou et al., 2018) could be a way to check the stringency of conclusion from zebrafish as to whether or not they would hold true in mammalian brains.

Despite its disadvantages listed above, zebrafish holds up well with the handiness of the rodent models of AD in several aspects such as the diversity of genetic tools and the ability of modeling disease hallmarks such as synaptic degeneration and cognitive decline (**Table 1**). Nevertheless, neither mouse models nor zebrafish models can recapitulate the whole pathophysiological biology of AD as in human brains, which suggests that those models are useful insofar as their strengths in particular aspects were found. For instance, zebrafish is quite advantageous over rodents in many aspects (**Table 1**). These include (1) the pathological outcomes that resemble the human brain such as the ability of Amyloid depositions to lead to neuronal death, (2) regenerative ability owing to the capacity of NSCs to respond to tissue loss by enhanced plasticity and neuro-regenerative outcome, (3) cost of experimental studies, (4) number of animals that can be tested in a laboratory setting, and (5) the availability and possibility of 3R-friendly experimentation schemes.

OUTLOOK

Although zebrafish lags behind the mammalian models in certain aspects, it already outperforms in many (**Table 1**). For instance, stem cell biology in zebrafish offers unprecedented information on the molecular programs that enable stem cell-based regeneration. Many research reports contributed to understanding of how NSCs in adult zebrafish brain function, are affected by external cues and respond to loss of neurons through recruitment of diverse signaling pathways including Notch, Wnt, Fgf, Bmp, and chemokine signaling (Mueller et al., 2004; Adolf et al., 2006; Grandel et al., 2006; Chapouton et al., 2007, 2011; Pellegrini et al., 2007; Zupanc, 2008; Diotel et al., 2010, 2013; Kroehne et al., 2011; Rothenaigner et al., 2011; Baumgart et al., 2012; Coolen et al., 2012, 2013; Kishimoto et al., 2012; Kizil et al., 2012a,b,c; Kyritsis et al., 2012; Marz et al., 2012; Alunni et al., 2013; Salta et al., 2014; Barbosa et al., 2015; Rodriguez Viales et al., 2015; Than-Trong and Bally-Cuif, 2015; Alunni and Bally-Cuif, 2016; Bhattarai et al., 2016; Kizil, 2018; Shimizu et al., 2018; Than-Trong et al., 2018). This large repertoire of knowledge will be instrumental in comparing the neuro-regenerative aptitude of zebrafish NSCs to human conditions and would help to find out how a successful proliferation-neurogenesis cascade could be elicited in mammals. Especially in the AD condition, which is the focus of this Perspective Article, such information could be instrumental and be a game-changer by providing an alternative approach to the disease mechanism and to its treatment (**Figure 1**). The role of neurogenesis in the manifestation of AD and its cure is rising to the spotlight again and zebrafish could offer valuable information on how our NSCs could be made "regenerative" using endogenous molecular programs and possibly by tweaking the immune system. Together with chemical/genetic screens, gene targeting and advances in research methodology; zebrafish stands out as an influential model that could drive preclinical findings toward novel clinically relevant discoveries.

AUTHOR CONTRIBUTIONS

CK formulated the perspective. PB and CK wrote the manuscript.

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An Eye on the Wnt Inhibitory Factor Wif1

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The coordinated interplay between extrinsic activating and repressing cell signaling molecules is pivotal for embryonic development and subsequent tissue homeostasis. This is well exemplified by studies on the evolutionarily conserved Wnt signaling pathways. Tight temporal and spatial regulation of Wnt signaling activity is required throughout lifetime, from maternal stages before gastrulation until and throughout adulthood. Outside cells, the action of numerous Wnt ligands is counteracted and fine-tuned by only a handful of well characterized secreted inhibitors, such as for instance Dickkopf, secreted Frizzled Related Proteins and Cerberus. Here, we give an overview of our current understanding of another secreted Wnt signaling antagonist, the Wnt inhibitory factor Wif1. Wif1 can directly interact with various Wnt ligands and inhibits their binding to membrane bound receptors. Epigenetic promoter methylation of Wif1, leading to silencing of its transcription and concomitant up-regulation of Wnt signaling, is a common feature during cancer progression. Furthermore, an increasing number of reports describe Wif1 involvement in regulating processes during embryonic development, which so far has not received as much attention. We will summarize our knowledge on Wif1 function and its mode of action with a particular focus on the zebrafish (*Danio rerio*). In addition, we highlight the potential of Wif1 research to understand and possibly influence mechanisms underlying eye diseases and regeneration.

Keywords: Wif1, Wnt signaling, zebrafish, eye, retina, cancer

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INTRODUCTION

Gene regulatory mechanisms facilitate the fundamental necessity that not all cells express and activate all genes and signaling pathways at the same time. Even in the presence of pathway components within a cell, the temporal control of signaling activity needs to be tightly controlled during development and tissue homeostasis. This can occur outside the cells such that ligands are prevented to bind receptors by secreted inhibitory molecules. These repressor proteins can act by directly binding to the ligand or by interacting with their receptor. Studies of the Wnt signaling cascades have been particularly informative as to the mechanisms of extrinsic control of signal activation by either of the nineteen Wnt ligands in mammals and twenty-six in teleosts (Kawano and Kypta, 2003; Beretta et al., 2011; Cruciat and Niehrs, 2013). The secreted repressors interacting with Wnt ligand receptors that have been identified so far include

Dickkopf (Dkk) (Glinka et al., 1998; Niehrs, 2006), Sclerostin (Sost)/Wise (Sostdc1) (Itasaki et al., 2003; Li et al., 2005; Semenov et al., 2005) and Insulin growth factor binding protein 4 (Igfbp4) (Zhu et al., 2008). Conversely, those that directly bind to Wnt ligands comprise secreted Frizzled Related Proteins (sFRPs) (Hoang et al., 1996), Cerberus (Bouwmeester et al., 1996) and Wnt inhibitory factor 1 (Wif1) (Hsieh et al., 1999). Among these, Wif1 has perhaps received the least recognition. Its function is, however, implicated in various crucial processes during eye development and homeostasis (Hunter et al., 2004; Park et al., 2014), neurogenesis and axon extension (Hunter et al., 2004; Nakaya et al., 2008), lung and anorectal development (Xu et al., 2011; Ng et al., 2014), tooth morphogenesis (Lee et al., 2015), chondrogenesis (Surmann-Schmitt et al., 2009), stem cell maintenance (Ding et al., 2008; Nakatsu et al., 2011), regeneration and cancer (Wissmann et al., 2003; Lim et al., 2017; **Figure 1**). The Wif1 protein structure exhibits intriguing features suggesting that its full spectrum of action and importance has yet to be uncovered. Its expression is largely conserved from teleosts to human. This is a prerequisite for using genetically amenable animal models such as the zebrafish for studying Wif1 function *in vivo* to better understand the pathophysiology of Wif1 linked human diseases needed for therapy development.

WIF1 CHARACTERISTICS

Wif1 was identified as an expressed sequence tag in the human retina and was functionally described as a negative regulator of canonical or Wnt/beta-catenin signaling in 1999 (Hsieh et al., 1999). However, Wif1 is able to physically interact with both canonical and non-canonical Wnt ligands such as Drosophila wingless, and vertebrate Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a, and Wnt11 (Hsieh et al., 1999; Surmann-Schmitt et al., 2009). In addition, Wif1 binding to zebrafish glycoprotein Olfactomedin1 (Nakaya et al., 2008) and to connective tissue growth factor (CTGF/CCN2) was reported *in vitro* (Surmann-Schmitt et al., 2012). The 379 amino acids (aa) Wif1 protein comprises an *N*-terminal signal sequence, a unique and conserved 150 aa Wif-domain, five epidermal growth factor (EGF)-like repeats, and a 45 aa long hydrophilic tail (Hsieh et al., 1999; **Figure 1**). Crystal structure analysis of the Wnt ligand binding *N*-terminal part of the Wif1 domain (Wif1WD) and its interaction with Wnt3a revealed that the EGF domains of Wif1 are required for Wnt ligand binding (Malinauskas et al., 2011). In addition, EGF domains II–V have heparan sulfate proteoglycan (HSPG) binding properties. HSPGs strengthen the interaction between Wif1 and Wnt ligands (Avanesov et al., 2012), but they are also known to modulate diffusion of morphogens with which they interact such as Wnt proteins (Panakova et al., 2005; Yan and Lin, 2009). Thus, in addition to Wnt ligand binding, Wif1 might well influence the generation of morphogen gradients and/or protect cells within such gradients from signal cascade activation. This feature might not be restricted to Wnt ligands. The Drosophila Wif1 ortholog "Shifted" promotes hedgehog signaling (Glise et al., 2005; Gorfinkiel et al., 2005), and this was hypothesized to potentially occur also in vertebrates (Avanesov et al., 2012).

Intriguingly, a pocket for phospholipids was identified in the Wif1WD domain (Malinauskas et al., 2011) binding 1,2-dipalmitoyl-phosphatidylcholine (DPPC). This suggested that Wif1 shares similarities with lipoprotein particles that can sequester Wnt3a-linked lipids (Willert et al., 2003; Panakova et al., 2005; Malinauskas et al., 2011). However, since the Wif1WD pocket already binds DPPC, the exchange with a Wnt ligand-bound palmitoleoyl moiety would require energy in the aqueous environment and would therefore rather be unlikely. It was hypothesized that the lipid containing pocket in the Wif-domain may provide conformational flexibility and thereby indirectly contribute to expose the appropriate surface for ligand binding (Malinauskas et al., 2011). Interestingly, also RYK-receptors involved in non-canonical Wnt signaling contain Wif-domains, raising the possibility that the lipid pocket could also be used directly for the binding of moieties attached to Wnt ligands (Kawano and Kypta, 2003; Malinauskas and Jones, 2014).

WIF1 EPIGENETICS AND CANCER

The Wnt pathway is well known for regulating cell stemness in many organs and tissues including bone, intestine and skin (Steinhart and Angers, 2018). In these and several other tissues, deregulation of various Wnt pathway components has been implicated in cancer occurrence and/or recurrence (Tai et al., 2015; Nusse and Clevers, 2017). One of these components is Wif1, which is downregulated in prostate, breast, lung and bladder cancers, as shown by RNA microarray analysis (Wissmann et al., 2003). Genomic studies identified a CpG island in the human Wif1 promoter 1,5 kb upstream of the Wif1 gene (Reguart et al., 2004). Methylation of CpG islands is one of the major modes of inactivating tumor suppressor genes in cancer (Herman and Baylin, 2003). Indeed, hypermethylation of the Wif1 promoter, leading to Wif1 silencing (and thus activation of Wnt/beta-catenin signaling), was shown to be associated with various types of cancers such as lung cancer, in particular non-small cell lung cancer (NSCLC) (Wissmann et al., 2003; Tan et al., 2013; Zheng et al., 2016; Guo et al., 2017), osteosarcoma formation (Kansara et al., 2009), colorectal cancer (Hu et al., 2018), cervical cancer (Ramachandran et al., 2012) and others (**Figure 1**). In NSCLC, Wif1 promoter hypermethylation can be counteracted by microRNAs, which negatively regulate DNA methyltransferases in a regulatory feedback loop (Tan et al., 2013). The epigenetic silencing of secreted Wnt pathway inhibitors related to cancer appears not restricted to Wif1 but holds true also for most other members such as Dkk1-3, Sost, Igfbp4 or sFRP1-5 (Mazieres et al., 2004; Aguilera et al., 2006; Roman-Gomez et al., 2007; Sato et al., 2007; Elston et al., 2008; Kongkham et al., 2010; Fellenberg et al., 2013; Gopal et al., 2013). In addition, Wif1 function is required to prevent metastasis of cancer cells. Prostate cancer (PCa) cells can invade bone tissue. In PCa cell lines, *Wif1* expression is spontaneously downregulated by promoter hypermethylation. Restoring *Wif1* expression, however, leads to a reduction in cell invasiveness and motility by upregulation of epithelial markers (Yee et al., 2010). These studies are

encouraging for the development of cancer therapies. For instance, targeted disruption or addition of CpG islands in the Wif1 promoter using genome editing techniques would be informative with respect to the resulting cell behaviors in established Wnt cancer models. In parallel, effects at the developmental level can be analyzed *in vivo* in the physiological environment of the zebrafish. Such complementing studies in zebrafish would not only be interesting regarding the epigenetic regulation of genes in general, but would at the same time give important insights into potential side-effects when developing therapies.

Expression profiling experiments have revealed that Wif1 is a downstream target of Wnt/beta-catenin signaling suggesting that Wif1 may act as a feedback inhibitor (Wissmann et al., 2003; Reguart et al., 2004; Vaes et al., 2005; Boerboom et al., 2006; Zirn et al., 2006; Kansara et al., 2009). Thus, Wif1 could be a central player in the dynamic control of Wnt signaling through a regulatory feedback mechanism. Wif1 also plays roles during embryonic development and some evidences collected mainly in mice and zebrafish implicate that Wif1 is similarly self-regulating its own expression during developmental processes (Diep et al., 2004; Yin et al., 2012). Such Wif1 regulatory feedback loops can involve hedgehog (Hh) such that Hh positively regulates *Wif1* expression to inhibit Wnt signaling. In turn, Wnt signaling maintains *Hh* expression. This mechanism is important for swim bladder development in the zebrafish (Yin et al., 2012; **Figure 1**). Wif1 morpholino knockdown reduces cell proliferation resulting in defective swim bladder development such that epithelium and mesenchyme growth are inhibited, smooth muscle differentiation is abolished and the organization of mesothelium is perturbed.

WIF1 EXPRESSION AND FUNCTION IN EMBRYONIC DEVELOPMENT

Zebrafish *Wif1* starts to be expressed in the presumptive paraxial mesoderm during late gastrulation (Thisse and Thisse, 2005). During subsequent neurulation stages *Wif1* expression appears largely similar in *Xenopus* and zebrafish in the notochord, visceral arches, nasal placodes, swim bladder/lung, otic vesicles, somites (*Xenopus*), lateral line and corneal epithelium (zebrafish) and discrete domains of the brain (Hsieh et al., 1999; Thisse and Thisse, 2005; Yin et al., 2012; Lush and Piotrowski, 2014). In zebrafish, the latter comprise the ventral midbrain and developing dorsal diencephalon (Thisse and Thisse, 2005).

Similarly, in mammals and birds *Wif1* expression initiates relatively late during development and is mainly restricted to the brain, lung, retina, and cartilage (Hsieh et al., 1999; Hunter et al., 2004; Hu et al., 2008; Surmann-Schmitt et al., 2009). In adult mice, *Wif1* expression is retained in the heart and lung and also in the brain and eye, albeit at lower levels (Hsieh et al., 1999). The rather late onset of *Wif1* expression might explain the subtle effects observed in *Wif1* knock out mice, which exhibit accelerated development of radiation-induced osteosarcomas but no recognizable morphological malformations (Kansara et al., 2009). Only in more recent years, mammalian Wif1 was additionally implicated in lung development (Xu et al., 2011), tooth morphogenesis (Lee et al., 2015) and anorectal development (Ng et al., 2014; **Figure 1**).

The subtle impact on embryonic development and/or maintenance of embryonic structures caused by loss of Wif1 may rather be counterintuitive given its direct interaction with at least six different canonical and non-canonical Wnt ligands and several other proteins (Nakaya et al., 2008; Surmann-Schmitt et al., 2009, 2012). Indeed, forced early ectopic expression of *Wif1* mRNA in the ventral blastomeres of the *Xenopus* embryo causes secondary axes typical for early inhibition of the canonical Wnt signaling cascade (Hsieh et al., 1999). However, Wif1 DNA overexpression leading to ectopic activation at later developmental stages after mid-blastula-transition induces a mild somite phenotype. Furthermore, so far no work has reported a role for Wif1 function in non-canonical Wnt signaling during embryonic development. Thus, Wif1 has the potential to regulate fundamental early processes during axis formation similar to, for instance, Dkk1 (Glinka et al., 1998). However, the onset of its expression mainly after gastrulation implicates that the Wif1/Wnt interaction may only fine-tune the spatial and temporal patterns of Wnt activity (Hsieh et al., 1999). *Wif1* is discretely expressed in cells of tissues, in which Wnt morphogen gradients are at work. For instance, developing neuronal cells in the zebrafish dorsal diencephalon show *Wif1* expression at developmental times, when Wnt3a is active in and around the adjacent mid-diencephalic organizing center to pattern the zebrafish thalamus (Thisse and Thisse, 2005; Mattes et al., 2012). In contrast, cells in the dorsal diencephalon anterior to the thalamus show Wnt activity only later during development (Hüsken et al., 2014). Thus, it is conceivable that particular cells in close vicinity to the Wnt source are protected from premature Wnt signaling – at least for a certain period of time. A Wif1/Wnt feedback regulation for the temporal control of Wnt signaling activity during neurogenesis would be a favorable mechanism to react to dynamic changes within a morphogen gradient.

WIF1 IN STEM CELL PLASTICITY AND AS THERAPEUTIC TARGET IN THE EYE

Enriched expression of *Wif1* is reported in the cornea of the zebrafish (Thisse and Thisse, 2005), mouse (Davis and Piatigorsky, 2011), monkey (Ding et al., 2008) and human (Nakatsu et al., 2011) eye. Notably, *Wif1* is predominantly expressed in the limbus of the cornea where limbal epithelial stem cells (LESCs) are located (Ding et al., 2008; Nakatsu et al., 2011). LESCs are important for the homeostasis and wound healing capability of the corneal epithelium and hence there is great interest in restoring LESC function in presently untreatable pathologic conditions where corneal healing is impaired (Yazdanpanah et al., 2017). The strong *Wif1* expression in the limbal niche suggests a function in controlling the quiescent state of LESCs under normal physiological conditions (Nakatsu et al., 2011). Removal of Wif1 would allow activation of Wnt signaling associated with the high proliferative behavior of LESCs observed during wound healing and corneal regeneration (Nakatsu et al., 2011). Despite the evolutionarily conserved

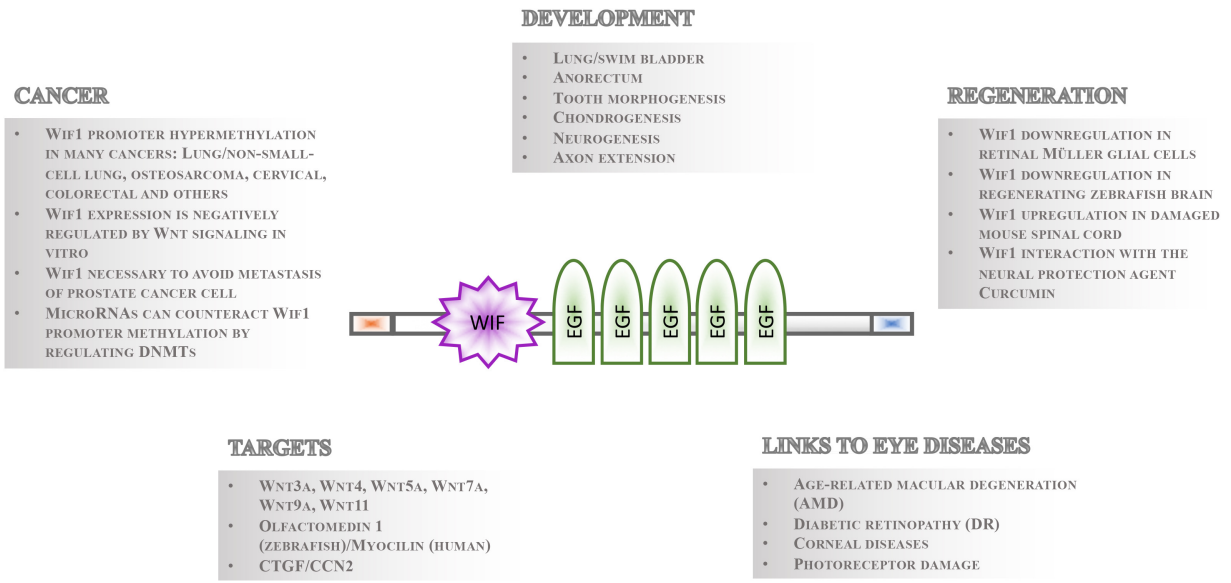

FIGURE 1 | Overview of Wif1 structure and function. The 379 aa Wif1 protein comprises an *N*-terminal signal sequence (orange box), a 150 aa Wif-domain, five epidermal growth factor (EGF)-like repeats, and a 45 aa long hydrophilic tail (blue box) at the *C*-terminus (Hsieh et al., 1999). Wif1 interacts with a number of proteins and has been linked to various processes in embryonic development, regeneration, cancer, and eye diseases as indicated.

Wif1 expression in the cornea and lens (Thisse and Thisse, 2005), surprisingly no studies on the role of zebrafish Wif1 have been reported. However, the combination of transgenes, genetic mutants and *in vivo* time-lapse imaging to visualize dynamic processes under physiological conditions would almost certainly help understanding more about the pathophysiology of LESCs/Wif1/Wnt related diseases. For instance, inducible systems to transiently activate Wif1 in injured wild type or *Wif1* mutant embryos, which carry additional transgenes like *anillin* and signaling reporter to simultaneously highlight cell proliferation (Paolini et al., 2015; Cepero Malo et al., 2017) and Wnt signaling activity (Moro et al., 2012) would be an excellent tool to study Wif1 dependent regeneration processes *in vivo*. The fact that cornea structure, development and maintenance appear comparable between zebrafish and mammals (Pan et al., 2013; Takamiya et al., 2015) should encourage to exploit the zebrafish as *in vivo* model to understand the role of Wif1 in corneal regeneration and homeostasis.

Wif1 is also expressed in early developing rod photoreceptors, and in the interphotoreceptor matrix of the mature retina (Hsieh et al., 1999; Hunter et al., 2004). Studies from Hsieh et al. suggest that Wif1 plays a role as opposing binding partner of Wnt4, providing a fine-tuning system for the regulation of rod photoreceptor production during development (Hsieh et al., 1999; Hunter et al., 2004). Dysregulated Wnt signaling has been linked to the pathophysiology of neovascular age-related macular degeneration (AMD) as well as diabetic retinopathy (DR) – leading causes of blindness in adults (Chen and Ma, 2017). Intriguingly, increased levels of *Wif1* were found in the aqueous humor of patients with AMD (Park et al., 2014) as well as DR (Kim et al., 2007), suggesting that Wif1 provides an attractive candidate drug target to treat these eye disorders (**Figure 1**). Furthermore, increasing levels of *Wif1* expression in the vitreous humor correlate with degrees of photoreceptor damage (Park et al., 2014). Alongside being a potential therapeutic target, these studies highlight Wif1 as candidate biomarker of retinal photoreceptor health and disease states.

Other Wif1 functions in the vertebrate eye involve its interaction with members of the Olfactomedin protein family. Within the zebrafish retina, the antagonistic interaction between Wif1 and Olfactomedin 1 in the extracellular space may fine tune retinal ganglion cell axon growth through modulation of Wnt signaling (Nakaya et al., 2008). In the human eye, Wif1 interaction with the Olfactomedin protein family member myocilin appears critical for the regulation of the intraocular pressure (IOP). High IOP in turn is a risk factor for glaucoma and myocilin has been linked to more than 10% of juvenile onset glaucoma cases, which result in the progressive degeneration of the optic nerve (Ortego et al., 1997; Kwon et al., 2009; Menaa et al., 2011).

WIF1 REGULATION DURING REGENERATION

Dynamic changes in *Wif1* expression appear tightly associated to regenerative events in the brain and retina (Gonzalez-Fernandez et al., 2014; Lambert et al., 2016; Yao et al., 2016; Lim et al., 2017). The capability to regenerate lost or damaged neurons in response to injury is a key feature of the fish central nervous system. In the retina, this is achieved through activation and transient asymmetric proliferation of retinal Müller glial cells - the potential stem cells of the retina (Nagashima et al., 2013;

Lust et al., 2016). Intriguingly, significant transcriptional down-regulation of *Wif1* and concomitant activation of canonical Wnt signaling was observed in transiently proliferating Müller glial cells (Yao et al., 2016) and also during early stages of zebrafish brain regeneration (Lim et al., 2017; **Figure 1**). In contrast, damaging the mouse spinal cord appears to induce up-regulation of *Wif1* and other Wnt antagonists (Gonzalez-Fernandez et al., 2014). These data suggest that Wnt signaling after injury might be the key for *de novo* neurogenesis, which is inhibited in the mammalian central nervous system with its limited regenerative capability (Gonzalez-Fernandez et al., 2014 and reviewed in Lambert et al., 2016). The different molecular responses to injury in zebrafish and mammals with respect to Wif1/Wnt signaling and regeneration open exciting possibilities for the development of novel therapeutic approaches to treat central nervous system injuries and possibly also neurodegenerative disorders. Recently, Wif1 has been implicated as potential molecular target of curcumin (Tiwari et al., 2016). Curcumin is a natural polyphenol product derived from the rhizome of the Indian spice turmeric (*Curcuma longa*) that appears to provide neuroprotection in cellular and animal models of neurodegenerative and neurological disorders (Pluta et al., 2015). The capability of curcumin to promote adult neurogenesis, neurite outgrowth and proliferation appears to occur through the interaction with Wif1 and concomitant activation of canonical Wnt signaling (Tiwari et al., 2015).

CONCLUDING REMARKS/OUTLOOK

In summary, the structure and mechanistic underlying Wif1 function is rather well described. Epigenetic silencing of the Wif1 promoter is a common feature, frequently resulting in cancer progression when uncontrolled. Wif1 appears to fine-tune cellular processes, perhaps also temporally controlling them, by dynamically regulating the delicate balance of Wnt signaling via feedback loop activation. Indeed, an increasing number of studies link Wif1/Wnt signaling to different comparably subtle, yet important processes in development and disease, particularly in the eye. Furthermore, Wif1 downregulation and concomitant upregulation of Wnt signaling has been connected to regeneration processes in the zebrafish CNS, while spinal cord lesions in mammals with limited regeneration potential has the opposing effect on *Wif1* expression. Combining the knowledge of the genetic and epigenetic feedback regulation of Wif1 with the regeneration capacities of the zebrafish nervous system has great potential to complement and aid research progress on neural regeneration in mammals.

AUTHOR CONTRIBUTIONS

LP and MC had the original idea and outlined the review. All authors contributed to writing the article.

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Heparan Sulfate as a Therapeutic Target in Tauopathies: Insights From Zebrafish

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Microtubule-associated protein tau (MAPT) hyperphosphorylation and aggregation, are two hallmarks of a family of neurodegenerative disorders collectively referred to as tauopathies. In many tauopathies, including Alzheimer's disease (AD), progressive supranuclear palsy (PSP) and Pick's disease, tau aggregates are found associated with highly sulfated polysaccharides known as heparan sulfates (HSs). In AD, amyloid beta (Aβ) peptide aggregates associated with HS are also characteristic of disease. Heparin, an HS analog, promotes misfolding, hyperphosphorylation and aggregation of tau protein *in vitro*. HS also provides cell surface receptors for attachment and uptake of tau seeds, enabling their propagation. These findings point to HS-tau interactions as potential therapeutic targets in tauopathies. The zebrafish genome contains genes paralogous to MAPT, genes orthologous to HS biosynthetic and chain modifier enzymes, and other genes implicated in AD. The nervous system in the zebrafish bears anatomical and chemical similarities to that in humans. These homologies, together with numerous technical advantages, make zebrafish a valuable model for investigating basic mechanisms in tauopathies and identifying therapeutic targets. Here, we comprehensively review current knowledge on the role of HSs in tau pathology and HS-targeting therapeutic approaches. We also discuss novel insights from zebrafish suggesting a role for HS 3-*O*-sulfated motifs in tau pathology and establishing HS antagonists as potential preventive agents or therapies for tauopathies.

Keywords: heparan sulfate, glycosaminoglycans, tauopathy, tau, Alzheimer's disease, zebrafish, drug discovery

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HEPARAN SULFATE PROTEOGLYCANS

Structure and Biosynthesis

Glycosaminoglycans (GAGs), formerly called mucopolysaccharides, are a major class of anionic polysaccharides, consisting of unbranched and often long polysaccharide chains made of disaccharide units. Most GAGs are bound to core proteins, forming proteoglycans (PGs), which are important components of extracellular matrices. All extracellular matrices contain PGs, but these glycoproteins are also found in membrane-bound secretory granules and in cell nuclei. PGs are evolutionarily ancient and are found in all Bilateria species (organisms with left-right symmetry). So far, five structurally different GAG species have been described: heparan

sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan or hyaluronic acid (HA). While heparin, a highly sulfated HS, is mainly produced by connective tissue-type mast cells and bipotential glial progenitor cells, HS is synthesized by all cell types (Couchman and Pataki, 2012; Lindahl et al., 2017). In the zebrafish (*Danio rerio*), HS is synthetized in developing embryos, larvae and adults (Zhang et al., 2009).

Glycosaminoglycans synthesis starts in the endoplasmic reticulum (ER), with the formation of a tetrasaccharide linker (Xyl-Gal-Gal-GlcA) bound to the core protein via a serine residue. During linker formation, xylose (Xyl) is first attached by a xylosyltransferase (XT); galactosyl-transferases I and II (GT I and II) then transfer two galactoses (Gal) from UDP-Gal to xylose, and finally, glucoronic acid (GlcA) is transferred from UDP-GlcA by glucuronosyl-transferase I (GlcAT). After the formation of the linker, GAG biosynthesis takes different routes for CS/DS or heparin/HS synthesis. Synthesis of CS/DS chains first involves the transfer of *N*-acetylgalactosamine (GalNAc) by GalNAc transferase to the terminal GlcA, while that of heparin/HS chains depends on the transfer of *N*-acetylglucosamine (GlcNAc) by GlcNAc transferase. The GAG chains are then elongated by sequential addition of repeated disaccharide units composed of an amino sugar, [*N*-acetylgalactosamine (GalNAc) or *N*-acetylgalactosamine (GalNAc)], and a uronic acid [glucoronic acid (GlcA) or iduronic acid (IdoA)]. In HS and heparin, the repeated disaccharide unit comprises *N*-acetylglucosamine and glucoronic acid residues. EXT enzymes mediate HS/heparin chain elongation (Kreuger and Kjellén, 2012).

After their synthesis, GAG chains are modified in the Golgi apparatus by different enzymes. All HS chain modifier enzymes have been identified in zebrafish (Bink et al., 2003; Chen et al., 2005; Ghiselli and Farber, 2005; Cadwallader and Yost, 2006a,b, 2007; Filipek-Górniok et al., 2015). Major modifications on the HS chains are sulfations, which are mediated by two classes of sulfotransferases in a hierarchical manner. The expression patterns of these enzymes are characterized in zebrafish through early developmental stages (0–48 hpf). First, the *N*-deacetylase/*N*-sulfotransferases (NDSTs) replace the acetyl group in GlcNAc by a sulfate group. Five NDST genes (*ndst1a, ndst1b, ndst2a, ndst2b, and ndst3*) have been identified in zebrafish. All isoforms are expressed in various brain regions during early zebrafish development, while a distinct isoform (*ndst3*) is expressed in the spinal cord (Filipek-Górniok et al., 2015). HS chains can be further modified through epimerization of GlcA into iduronic acid (IdoA) by C-5 epimerase. The zebrafish C-5 epimerases Glce-A and Glce-B are expressed in brain through early embryonic development (Cadwallader and Yost, 2007).

The second class of sulfotransferases comprises the HS *O*-sulfotransferases (OSTs) 2-OST, 6-OST, and 3-OST. Zebrafish 2-OST or HS2ST is expressed in all brain regions in early developmental stages. Of the four zebrafish 6-OSTs or HS6STs (6-OST-1a, 6-OST-1b, 6-OST-2, and 6-OST-3) all are expressed in the brain in early development, while only 6-OST-1a and 6-OST-1b isoforms are expressed in the spinal cord (Cadwallader and Yost, 2006b). Comparison of HS structure between adult zebrafish and porcine intestine revealed similar global structures. Differences were documented as higher 2-*O*-sulfated iduronic acid (IdoA2S) content and lower levels of GlcA (Zhang et al., 2009).

It has been suggested that the evolutionary origin of the Hs3sts goes back to a common ancestor of bilaterians and cnidarians in the early eumetazoan evolution. *Trichoplax*, a placozoan identified as a sister clade to bilaterians and cnidarians, has no Hs3st homologs, but expresses all the other HS sulfotransferases. Invertebrates express fewer Hs3st isozymes than vertebrates. *Hydra* expresses one Hs3st, whereas *Nematostella* (sea anemone), belonging to the Cnidarian phylum, expresses two Hs3sts. The Hs3st enzymes in *Hydra* and *Nematostella* share near 50% amino acid sequence identity with human Hs3sts. *Strongylocentrotus* (sea urchin) and *Planaria* (flatworm) express a single Hs3st, whereas *Drosophila* (fruit fly) and *Caenorhabditis* (nematode) have two Hs3st. Vertebrates express a greater number of Hs3sts. *Homo* and other mammals express 7 Hs3sts, while *Danio* (zebrafish) expresses 8 (Thacker et al., 2014). 3-*O*-Sulfated disaccharides and tetrasaccharides have been identified in HS from zebrafish embryos, which were diminished by morpholinos directed against specific *3-ost* genes. During early cleavage stages in the zebrafish, 3-OST enzymes are encoded by maternally deposited mRNAs, which are evenly distributed throughout the embryos. By contrast, during later stages, and particularly in the developing brain, the zebrafish Hs3st genes display a complex combinatorial expression profile with each gene showing a unique and cell-type specific expression pattern. The *3ost1* transcripts are ubiquitously accumulated to high levels during early cleavage stages prior to becoming restricted to head and anterior somites by 24 hpf. In 48 hpf embryos, *3ost1* expression is restricted to head, gut and pectoral fin. Expression of the *3ost2* gene is detected from 24 hpf onward in the brain and otic vesicle. At 48 hpf, *3ost2* is expressed in all major brain areas, as well as in the olfactory region. Zebrafish *3ost3X* and *-3Z* are the orthologs of the mammalian *3-OST-3a* and *-3b* genes, respectively. *3ost3X* transcripts are evenly distributed during early cleavage stages and are restricted to hindbrain and spinal cord from 36 hpf onward. By contrast, while expression of the *3ost3Z* gene is not detected during early cleavage stages, by 48 hpf it is transcribed in telencephalon, tectal regions, hindbrain, and spinal cord. *3ost4* RNAs are maternally expressed and ubiquitously accumulated in early cleavage stage embryos, before being specifically transcribed in midbrain, hindbrain, olfactory epithelium and otic vesicle by 48 hpf onward. Similarly, ubiquitous accumulation of maternal *3ost5* transcripts is detected in early cleavage and embryonic stages, while from mid-somitogenesis onward, these RNAs are specifically transcribed in forebrain, midbrain and spinal cord neurons. Distribution of *3ost6* transcripts is detected during early cleavage stages, while from 36 hpf onward these RNAs are expressed in the hindbrain. Finally, the *3ost7* gene is expressed during early cleavage stages and becomes restricted to brain neurons by 48 hpf (Cadwallader and Yost, 2006a). The zebrafish *3ost2* and *3ost3* generate receptors for herpes simplex virus 1 (HSV1) entry, highlighting the functionality and similarity of

zebrafish neuronal 3-*O*-sulfated motifs to those found in humans (Antoine et al., 2014).

Heparan sulfate PGs (HSPGs) comprise one or more HS-sulfated GAG chains covalently linked to a core protein. 17 distinct types of HSPGs have been identified, which are classified into three groups based on their locations: (i) syndecans and glypicans (membrane HSPGs), (ii) agrin, perlecan, and type-XVIII collagen (secreted extracellular matrix HSPGs), and (iii) serglycin (secretory vesicle HSPG) (Lindahl et al., 2017). Agrin also occurs as a transmembrane proteoglycan resulting from alternative splicing (Burgess et al., 2000; Neumann et al., 2001). The structure and function of zebrafish syndecan-4, required for neural crest migration, closely resembles that found in higher vertebrates (Whiteford et al., 2008). Using brains of BACE-1 deficient zebrafish, glypican-1 is identified as a substrate for the enzyme. BACE-1 is implicated in pathogenesis of AD by contributing to generation of Aβ peptide (Hogl et al., 2013).

Heparan sulfate chains of HSPGs can also be modified post-assembly by several enzymes. In particular, the shortening and degradation of HS chains involve heparanase (HPSE), and the removal of sulfate motifs by SULFs (a family of plasma membrane endosulfatases) entails finer modifications. A single gene orthologous to human heparanase is found in zebrafish (Wei and Liu, 2014). Orthologs to mammalian SULFs have been identified in zebrafish; they consist of three genes, all expressed in the central nervous system (CNS) (Gorsi et al., 2010). PG degradation is mediated by their internalization, which induces endolytic cleavage of the core protein by proteases and degradation of heparan sulfate chains by heparanase and endo-β-glucuronidase. Complete degradation of the remaining small HS chains takes place in lysosomes and involves a series of exoglycosidases and sulfatases (Lamanna et al., 2008; Lindahl et al., 2017). The ortholog of the human iduronate-2-sulfatase in zebrafish, whose defects are responsible for mucopolysaccharidosis type II (MPS II), has been identified (Moro et al., 2010). The identification of HSs in zebrafish, together with an array of orthologs for HS biosynthetic and chain modifier enzymes in the nervous system, makes zebrafish a model organism well-suited for the study of HS contribution to tauopathies.

HS-Protein Interactions

Heparan sulfates can bind non-covalently to a variety of proteins. An array of heparin-binding proteins have been identified, many of which interact with HSs under physiological conditions and modulate their biological activities (Bishop et al., 2007; Ori, 2008; Xu and Esko, 2014). Protein-HS interactions promote protein presentation, protection and stabilization, as well as conformational changes and oligomerization (Thacker et al., 2014). The conformation of GAG chains and the negative charges provided by the sulfate groups and uronic acid epimers constitute the binding site for positively charged amino acids of heparin-binding proteins (Lindahl and Li, 2009; Sarrazin et al., 2011). The ligand-binding sites of HSPGs rely on the orientation of carboxyl groups and organization of sulfate groups (Sarrazin et al., 2011). In particular, the sulfation pattern of HSPGs, which is more diverse than other GAG chain modifications, plays an essential role in the specificity of the binding sites of HSPGs to heparin-binding proteins (Aviezer et al., 1994; Sanderson et al., 1994; Herndon et al., 1999). Moreover, it has been shown that different patterns of sulfation in HSPGs with similar core proteins lend them different binding specificities (Kato et al., 1994; Sanderson et al., 1994). Hence the concerted action of enzymes confers highly variable patterns of sulfation to HS chains, thus driving their complexity and micro-heterogeneity (Shriver et al., 2012; Thacker et al., 2014). Importantly, data suggest that GAG chain sulfation does not go to completion on sulfated polysaccharide chains, yielding alternate highly sulfated domains (NS domains) and regions showing low or no sulfation (NA domains) (Sarrazin et al., 2011).

The sulfation pattern of most HSPGs changes during development, thus modifying their binding specificities (Nurcombe et al., 1993; Brickman et al., 1998; Yabe et al., 2005), a process playing a critical role in nervous system development (Irie et al., 2002; Bülow and Hobert, 2004). HS modifications dynamically evolve during zebrafish development (Cadwallader and Yost, 2006a,b, 2007; Zhang et al., 2009). Studies in zebrafish have demonstrated a critical requirement for HS in axon pathfinding during development (Lee et al., 2004; Kim et al., 2007; Kastenhuber et al., 2009; Wang et al., 2012; Poulain and Chien, 2013). Chemical disruption of HS and CS biosynthesis in early zebrafish development leads to brain disorganization (Beahm et al., 2014). However, the specific binding motifs for many heparin-binding proteins remain to be defined (Thacker et al., 2014). Full knowledge of HS-protein interactions has important therapeutic implications. Surfen, a non-specific heparan sulfate antagonist interferes with numerous biological processes, showing potential as a therapeutic agent. The properties of surfen ranges from anti-inflammatory (Warford et al., 2018) and immuomodulatory (Warford et al., 2014) to suppression of stem cell differentiation (Huang et al., 2018), inhibition of HIV type 1 infection (Roan et al., 2010) and rescue of tauopathy in a zebrafish model (Alavi Naini et al., 2018). However, non-specificity limits the beneficial effects of surfen. Surfen is reported to decrease neuroinflammation through HSPGs while increasing both the beneficial and harmful CS proteoglycans involved in remyelination (Warford et al., 2018). Thus complete knowledge of the PG interactome promotes development of specific therapies targeting harmful intractions and/or enhancing beneficial ones.

TAU AND TAUOPATHIES

Tauopathies are neurodegenerative disorders characterized by microtubule-associated protein tau (MAPT) hyperphosphorylation and aggregation into paired helical filaments (PHFs) or straight filaments (SFs), forming neurofibrillary tangles (NFTs) in brain. Unlike amyloid-beta (Aβ) aggregates, which are specifically detected in AD patients, tau tangles are found in many neurodegenerative disorders such as corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), Pick's disease, dementia pugilistica, frontotemporal dementia with parkinsonism linked to chromosome 17

(FTDP-17), and many others including AD (Alavi Naini and Soussi-Yanicostas, 2015). *MAPT* gene mutations are identified in a number of tauopathies. The mutations alter the amino acid sequence of tau, disrupt splicing or both (Alavi Naini and Soussi-Yanicostas, 2015). AD and other dementias are currently major challenges for health care systems, and major public health priorities worldwide, for which there is an unmet need for disease-modifying treatments (Frankish and Horton, 2017).

The tau protein is identified as a microtubule-associated protein (MAP) with a wide range of potential functions. In the adult human CNS six different tau isoforms are expressed (Goedert et al., 1989b). They are generated *via* alternative splicing of a single *MAPT* gene located on chromosome 17q21.31 and comprising 16 exons (Neve et al., 1986). Tau isoforms range in length from 352 to 441 amino acids, with a molecular weight of 45–65 kDa. The exons E1, E4, E5, E7, E9, E11, E12 and E13, are included in all tau isoforms. Tau isoforms are differentiated by three (3R) or four (4R) carboxy-terminal tandem repeats of 31 amino acids, which are designated as microtubule-binding domains (MBDs). These repeated domains, which show strong sequence conservation, are encoded by exons E9, E10, E11, and E12, the exclusion or inclusion of exon 10 generates the 3R or 4R tau isoforms, respectively (Goedert et al., 1989a,b; Andreadis et al., 1992).

The main functions recognized for tau are microtubule stabilization and polymerization. Microtubules are part of the eukaryotic cytoskeletal framework and are primarily composed of α- and β-tubulin, forming tubular polymers. Microtubules are essential for cytoskeletal maintenance and intracellular transport (Nogales, 2001). Tau mutations alter their affinity for interaction and binding with microtubules (Alavi Naini and Soussi-Yanicostas, 2015). Tau is able to bind to the outside and probably to the inside of microtubules, while the N- and C-terminal regions project outward (Kar et al., 2003; Santarella et al., 2004). The tandem repeat sequences in the (MBD) provide a net positive charge that interacts directly with the negatively charged residues of tubulin (Kar et al., 2003; Jho et al., 2010). The 4R-tau isoforms have higher affinity for binding to microtubules than 3R-tau and are more efficient at promoting microtubule assembly, likely due to the presence of the inter-repeat sequences between the first and second microtubule-binding repeats, which possess over twice the binding affinity of any individual microtubule-binding repeat (Goedert and Jakes, 1990; Goode and Feinstein, 1994).

Tau alterations as biomarkers for dementia have mainly been studied in AD. The amount of total tau (T-tau) in CSF correlates with the intensity of neurodegeneration (Gao et al., 2018) and hyperphosphorylated tau (P-tau) levels in CSF correlate with hippocampal atrophy in prodromal AD (De Leon et al., 2006; Fagan and Holtzman, 2010). Interestingly, Aβ peptide accumulation does not correlate with neurodegeneration in prodromal AD (Iaccarino et al., 2018). Moreover the mean content of abnormally phosphorylated tau from several brain regions in individual AD patients closely correlates with disease severity (Holzer et al., 1994). These observations along with tau mutations responsible for a number of tauopathies, highlight the importance of tau alterations as therapeutic targets in tauopathies.

ROLE OF HEPARAN SULFATE (HS) IN TAUOPATHIES

In 1855 Virchow reported the presence of polysaccharides in amyloid deposits in brain (Virchow, 1855). In 1942 Hass characterized the polysaccharides in amyloid lesions as sulfated amino-sugar based polysaccharides, later called heparin-like glycosaminoglycans (GAGs), and then heparan sulfate proteoglycans (HSPGs) (Hass, 1942). Tauopathies are amyloid disorders (Iadanza et al., 2018). Amyloid disorders are a diverse group of protein-misfolding disorders (PMDs) characterized by disease-specific protein aggregates containing heparan sulfate proteoglycans (HSPGs) in most cases (Ancsin, 2003; Nishitsuji and Uchimura, 2017).

The association of GAGs with protein aggregates in Alzheimer's disease (AD) and other tauopathies was described decades ago in the literature (Kato et al., 1991; Su et al., 1992; DeWitt et al., 1993, 1994). Further investigations demonstrated that sulfated GAGs played a critical role in tau hyperphosphorylation and aggregation, but also amyloid precursor protein (APP) cleavage and the resulting Aβ peptide fibrillization (Fraser et al., 1992; Brunden et al., 1993; Goedert et al., 1996; Hasegawa et al., 1997; Scholefield et al., 2003; Beckman et al., 2006). However, the nature of the sulfated GAGs involved in these processes remained unclear. In particular, GAG-tau interactions have received little attention as potential therapeutic targets.

Heparan sulfate proteoglycans are associated with both amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) (Snow et al., 1990; Kato et al., 1991; Perlmutter et al., 1991; Perry et al., 1991; Su et al., 1992), the most frequent form of tauopathy (Hernández et al., 2018). While the principal HSPGs accumulating in the AD-associated aggregates are membrane-associated HSPGs, the presence of perlecan (a non-membrane-associated HSPG) in Aβ deposits is debated (Van Gool et al., 1993; Snow et al., 1994; Van Horssen et al., 2002). Specifically, the initially reported association of perlecan with Aβ plaques and neurofibrillary tangles in AD was later challenged because of the cross-reactivity of the antibodies used (Verbeek et al., 1999). Perlecan is increased in AD brains (Liu et al., 2016). However, a study of the perlecan mRNA levels in the hippocampus of AD patients and age-matched controls has shown similar expression levels (Maresh et al., 1996), and mice overexpressing the perlecan core protein did not develop plaques or tangles (Hart et al., 2001). Agrin is strongly expressed in the hippocampus and amygdala (Bowe and Fallon, 1995; Donahue et al., 1999), two areas strongly affected in AD (Braak and Braak, 1991). Agrin is the major HSPG associated with both Aβ plaques and tau-containing NFTs in AD (Verbeek et al., 1999; Cole and Liu, 2006). Last, heparanase (HPSE) is overexpressed in brain areas showing degeneration in AD as measured by RT-PCR (García et al., 2017), suggested be a protective mechanim. Thus membrane-associated HSPGs provide a link between the two

major hallmarks of AD, Aβ plaques and NFTs. This suggests that the membrane-associated HSPGs might be involved in processes upstream of AD lesions.

In many neuropathies, abnormal HSPG accumulations are often observed at early stages of the disease. HSPGs accumulate in neuronal cell bodies in Down syndrome patients years before the first clinical symptoms of dementia occur. HSPGs are also associated with Aβ deposits in the early stages of AD. Specifically, HSPGs are associated with both immature diffuse Aβ plaques (Snow et al., 1990, 1994; Su et al., 1992; Cotman et al., 2000) and hyperphosphorylated tau aggregates, during the earliest stages of neurofibrillary pathology (Spillantini et al., 1999). Interestingly, in this situation HSPG staining is more intense than that of tau, suggesting that HSPGs play a role in initiation of disease.

In 1996 *in vitro* biochemical investigations demonstrated that interactions between heparin and tau protein led to the assembly of tau into NFT-like filaments (Goedert et al., 1996). Moreover, sulfated motifs on the GAG chain are critical for tau aggregation, as the non-sulfated hyaluronic acid does not enhance tau polymerization (Arrasate et al., 1997). Aside from structural changes, heparin was also shown to markedly stimulate the phosphorylation of tau by different protein kinases, leading to tau hyperphosphorylation, a key feature of tauopathies. In particular, tau phosphorylation by cdc28, cAMP-dependent protein kinase, GSK3b and several stress-activated protein kinases (SAP kinases) are markedly stimulated *in vitro* by heparin (Mawal-Dewan et al., 1992; Brandt et al., 1994; Yang et al., 1994; Goedert et al., 1997; Hasegawa et al., 1997). Heparin also prevents tau binding to taxol-stabilized microtubules and promotes disassembly of microtubules formed by tau and tubulin, suggesting that highly sulfated GAGs compete with microtubules for tau binding (Goedert et al., 1996). However, further research showed that the effect of heparin on tau was mimicked by several GAGs and depended on their degree of sulfation: highly sulfated dextran sulfate, pentosan polysulfate and heparin exerted a marked effect, while a number of intermediately sulfated GAGs such as HS, CS and DS had a moderate effect, and non-sulfated dextran and hyaloronic acid had no effect (Hasegawa et al., 1997). Interestingly, the concentration of sulfated GAGs required to stimulate tau phosphorylation was lower than that required for tau assembly and aggregation (Hasegawa et al., 1997), suggesting that tau phosphorylation may precede its assembly into NFTs. The increased phosphorylation and aggregation of tau observed in tauopathies may involve a substrate modulator effect of GAGs on tau conformation, as previously shown in other biological contexts (Faucher et al., 1988; Abdel Ghany et al., 1990).

Association of HSPGs with proteins can lead to conformational changes in the bound proteins, as demonstrated by the allosteric effect of heparin on anti-thrombin. Heparin induces a conformational change in tau (Paudel and Li, 1999; Sibille et al., 2006; Elbaum-Garfinkle and Rhoades, 2012), and heparin-induced conformational changes have been shown to expose previously masked tau phosphorylation sites (Zheng-Fischhöfer et al., 1998; Paudel and Li, 1999; Yoshida and Goedert, 2012). Electron microscopy data has provided evidence that sulfated GAGs present in tau aggregates affect paired helical filament (PHF) conformation: treatment of PHF-tau extracted from AD patients with heparinase or chondroitinase resulted in untwisting of PHF filaments (Arrasate et al., 1997). Furthermore, treatment of PHF-tau tangles with heparinase alters their immunodecoration properties, suggesting conformational changes upon heparinase treatment (Hernández et al., 2002). Specifically, heparin interacts with the second (R2) and third (R3) repeat regions of the tau microtubule-binding domain (Sibille et al., 2006). More recently, a small domain located in the N-terminal region of the R2 repeat was identified as critical for binding (Mukrasch et al., 2005; Zhao et al., 2017). Interestingly, Hasegawa et al. (1997), have suggested that sequence differences among moderately sulfated GAGs likely play a role in their interaction with tau (Hasegawa et al., 1997), as the overall level of sulfation cannot account for the observed effects. This further suggests that specific sulfated sequences on GAGs are required for interaction with tau.

Most importantly, cell surface HSs also act as receptors for Aβ (Kanekiyo et al., 2011) and extracellular tau aggregates, likely mediating a prion-like propagation of the pathology (Holmes et al., 2013), as reflected by the stages defined by Braak and Braak (1991). Membrane-associated heparan sulfate is involved in cell surface binding, uptake and internalization of tau seeds (Holmes et al., 2013) suggesting that propagation of tau seeds can also be blocked by pharmacological targeting of cell surface HSPGs. This approach may slow the progression of tauopathies (Holmes and Diamond, 2014). Alteration of heparan sulfate structure with aging may also be implicated in neurodegeneration. In particular, HS is important for adult neurogenesis by modulating FGF signaling; age-related alteration of HS binding properties in the hippocampus (Huynh et al., 2012), significantly compromises local regenerative capacities (Yamada et al., 2017). Membrane-bound HS, such as syndecans and glypicans are critical for regulation of neurogenesis (Yu et al., 2017).

Heparan sulfate has emerged as a potential multiplayer in tau pathology. HS is potentially implicated in the initiation and propagation of tau pathology and may play a role in limiting regenerative potential in dementia-susceptible regions. Further studies are needed to elucidate the role of different HSPGs in tauopathies. Memebrane-bound HS, mostly syndecan-3, syndecan-4 and glypican-3, are increased in AD brain (Liu et al., 2016). Particular attention should be paid to processes defining regional susceptibility to tauopathy.

ZEBRAFISH FOR DRUG DISCOVERY IN TAUOPATHIES

The zebrafish (*Danio rerio*), is a well-established model for the study of developmental biology, gene function and human diseases. As a vertebrate, it is closer to humans than invertebrate models, such as nematodes and fruit flies, and offers many advantages over invertebrate and other alternative vertebrate models.

The zebrafish is particularly well-suited for forward and reverse genetic and high-throughput functional studies. Major advantages of zebrafish include a precisely characterized genome with genes showing 50–80% sequence similarities with their

human orthologs. Orthologous counterparts of many genes relevant to tauopathies or specifically to Alzheimer's disease are identified in zebrafish. The zebrafish has undergone an ancient genome duplication followed by loss of many duplicated genes. Two paralogs of the human *MAPT* gene, *mapta* and *maptb*, have been identified in zebrafish. Interestingly, in early developmental stages *mapta* transcripts contain predominantly 4-6 Rs, while most *maptb* transcripts contain 3Rs (Chen et al., 2009). In embryonic stages, the expression of *mapta* and *maptb* show a positive correlation with sex-linked ubiquitin-specific peptidase 9 (USP9) (Köglsberger et al., 2017). In the adult zebrafish brain, hypoxia is reported to shift splicing of *mapta* and *maptb* transcripts toward 4-6 R isoforms (Moussavi Nik et al., 2014). Orthologs to human *APOE* (*apoea* and *apoeb*), *APP* (*appa* and *appb*) and the APP cleaving complex are also identified in zebrafish (Newman et al., 2014).

Further advantages of the zebrafish include low cost, high fecundity, short generation time, external fertilization, external embryonic development and embryo transparency allowing easy manipulation and visualization. This small fish is particularly advantageous for the study of the nervous system as its small brain size facilitates imaging. Despite differences in CNS development, anatomy and chemistry (Panula et al., 2010), overall brain organization (Rupp et al., 1996) with neuroanatomical (Rink and Wullimann, 2004; Mueller and Wullimann, 2009) and neurochemical (Mueller et al., 2004) pathways show striking similarities in zebrafish and humans. The adult zebrafish also displays high-order behaviors such as memory, social contacts and conditioned responses (Guo, 2004). In addition, the transparency and small size of its brain allow recording of neuronal activity by the recently developed molecular sensors such as genetically encoded calcium indicators or voltage sensors (Lin and Schnitzer, 2016).

Microinjection of antisense morpholino oligonucleotides (MOs) into one-cell stage zebrafish embryos allows rapid, simple and specific reverse genetic studies by inhibition of mRNA splicing or translation (Nasevicius and Ekker, 2000; Eisen and Smith, 2008). Efficient transgenesis methods and large-scale mutagenesis screens, mRNA injection, and genomic editing tools such as the CRISPR/cas9 approach are developed in zebrafish (Prykhozhij et al., 2018). Zebrafish embryos are also suitable for large-scale chemical screening to identify novel therapeutic targets and compounds. Whole cell, tissue or organism assays are twice as often successful in drug discovery efforts than target-based assays, and the ideal assay settings are whole organism assays. Phenotypic screens, particularly in CNS drug discovery, have been markedly more efficient in identifying novel therapeutic entities than target-based assays in recent pre-clinical studies (1999–2008) (Swinney and Anthony, 2011). Zebrafish embryos have also been used to study the potential toxicity of an imaging probe for NFTs (Anumala et al., 2013) and a neuroprotective agent for AD (Torres et al., 2014).

Recent successes in drug discovery in zebrafish rely on conserved physiological pathways and drug metabolism. Here we cite two examples of such discoveries. Clemizole, approved by the FDA, has been found to inhibit seizures in a zebrafish phenotypic screen for antiepileptic drugs (Baraban et al., 2013). Zebrafish larval assays have also revealed an FDA-approved substance that protects against the ototoxicity of aminoglycoside, which enters a phase I clinical trial this year (Smuga-Otto, 2018).

The analogy of brain structure and neuronal subtypes between zebrafish and humans, together with expression of key tau phosphorylating kinases, makes the zebrafish an attractive model organism for investigating tau pathology (Santana et al., 2012). Several transgenic zebrafish models of tauopathy expressing either the wild-type or mutant human tau protein are generated. These models represent the proof of concept that zebrafish models of tauopathy recapitulate key aspects of tau pathology. First, a zebrafish model transiently expressing a mutant human tau protein fused to GFP reproduced the cytoskeletal pathology in the brain including the accumulation of tau in NFT-like aggregates and the presence of hyperphosphorylated tau (Tomasiewicz et al., 2002). The hybrid protein was under the control of the pan-neuronal *gata2* promoter, which is expressed in the brain, retina and spinal cord and shows mosaic expression. This study demonstrated the conservation of pathways involved in tauopathy in zebrafish, making it possible to robustly model the pathology in this species.

More recently, a stable zebrafish transgenic line was produced that expresses the human 4-repeat tau under the control of the zebrafish *enolase-2* (*eno2*) promoter. The utility of the *eno2* promoter lies in its expression, which starts in differentiated neurons and persists into adulthood. Tau deposits resembling NFTs were found within neuronal cell bodies and proximal axons in brain regions relevant to PSP (Bai et al., 2007; Bai and Burton, 2011). Later, a stable zebrafish transgenic line expressing the human tau protein carrying the tau^{P301L} mutation linked to frontotemporal dementia (FTD) under the control of the zebrafish pan-neuronal HuC promoter was generated (Paquet et al., 2009). In this model, the Tg[HuC::$hTau^{P301L}$/DsRed], the vector system allows expression of the mutated human tau P301L along with simultaneous expression of DsRed fluorescent reporter, facilitating the identification of the mutated tau expressing neurons. Tg[HuC::$hTau^{P301L}$/DsRed] develops spinal motoneuron axon extention and elongation defects and neurodegeneration in early developmental stages and as a consequence deficits in the escape response reflex. Similar defects were also observed in zebrafish larvae expressing the mutant A152T tau protein (Lopez et al., 2017). The chronic expression of the P301L mutant tau into adulthood has also been recently examined in zebrafish (Cosacak et al., 2017). Focusing on the adult zebrafish brain, Cosacak et al reported tau hyperphosphorylation in the telencephalon. However, the authors did not find tauopathy-related symptoms in the telencephalon, such as formation of neurofibrillary tangles. This is in contrasts with another report (Bai and Burton, 2011) on tau mislocalization and tau-laden neurofibrillary like structures in tau transgenic adult zebrafish brain.

The observed discrepancies may be due to the specific brain regions studied, telencephalon (Cosacak et al) vs. optic tectum and brainstem (Bai and Burton), or different transgenic systems or labeling methologies used. However, the possible resistance of adult zebrafish telencephalon to tauopathy is an interesting avenue of investigation that may reveal potential protective

mechanisms. Moreover, HS plays a role in nervous system regeneration (Fuller-Carter et al., 2015; Yamada et al., 2017). High regenerative capacities in zebrafish (Gemberling et al., 2013) makes the organism a suitable model to study the implication of HS in regenerative processes.

A transient expression system is also established that expresses either full-length or truncated zebrafish 3R or human 4R tau, fused to GFP, under the control of HuC promoter (Wu et al., 2016). The expression of all constructs was found to be toxic, causing significant neurodegeneration. Using this experimental system enhancing an anti-apoptotic, anti-oxidative or neurotrophic mechanisms, was found to be protective against neurodegeneration.

The zebrafish offers numerous, diverse advantages as an animal model for the study of nervous system pathology. Numerous genes orthologous to those involved in tauopathies have been identified in the zebrafish. Experiments on zebrafish have confirmed the conservation of essential pathways between humans and the zebrafish that enable it to model tau pathology. Specific brain regions in the zebrafish are reported to bear resistance to NFT formation, possibly due to high regenerative capacities. Elucidation of these mechanisms, in particular the role of HS in the pathological vs. protective or regenerative processes in zebrafish will help identify potential pathways and targets to prevent or treat tau pathology.

THERAPEUTIC APPROACHES TARGETING HSPGS IN TAUOPATHIES

A better knowledge of HS-protein interactions has huge therapeutic implications for human pathologies (Capila and Linhardt, 2002; Lindahl, 2007). The primary therapeutic approach based on targeting PGs/GAGs in tauopathies consists of treatment with exogenous GAGs or GAG mimetics. Administration of exogenous GAGs is believed to competitively inhibit the harmful processes mediated by endogenous GAGs (Wang and Ding, 2014). A summary of therapeutic approaches involving exogenous GAGs and GAG mimetics is presented in **Table 1**. Indeed, LMW heparin oligomers efficiently inhibit the stimulatory effect of heparin on APP secretion, and inhibit the binding of heparin to $A\beta_{1-28}$ peptide *in vitro* (Leveugle et al., 1998). However, large sulfated GAGs such as heparin and DS inhibit the binding of HSPGs to APP *in vitro* (Narindrasorasak et al., 1991). Several polysulfated GAGs and synthetic sulfate-containing compounds such as the Aβ-binding sulfonated dye Congo Red, along with several other sulfonated dyes, have been found to attenuate the neurotoxic effects of $A\beta_{25-35}$ and $A\beta_{1-40}$ *in vitro* (Pollack et al., 1995a,b). Moreover, several small anionic substances with sulfonate and sulfate residues efficiently decrease Aβ deposition (Kisilevsky et al., 1995). Accordingly, arrays of LMW GAGs are developed as competitive inhibitors of PG interactions (Gervais et al., 2001). Neuroparin (C3), an LMW GAG derivative of heparin, has been shown to have neuroprotective and neuroreparative properties in several animal models of AD (Dudas et al., 2008). Interestingly, neuroparin is reported to attenuate the abnormal tau immunoreactivity (representative of an AD-related conformational alteration of tau) in rat hippocampus following unilateral injection of $A\beta_{25-35}$ into the amygdala (Dudas et al., 2002). In addition, this abnormal $A\beta_{25-35}$ induced tau immunoreactivity was fully prevented following chronic subcutaneous injections of the LMW GAG ceroparin (Walzer et al., 2002). Heparin-like oligosaccharides reduce uptake of tau oligomers limiting their infectivity (Wang et al., 2018).

Another ionic compound, tramiprosate (3-amino-1-propanesulfonic acid; 3APS; Alzhemed^{TM}), has been shown to bind preferentially the soluble Aβ peptide, maintain Aβ peptide in a soluble conformation, and reduce Aβ burden in TgCRND8 mice, a transgenic AD model that develops early-onset and aggressive brain Aβ amyloidosis (Gervais et al., 2007). However, in November 2007, tramiprosate was rejected for further pharmaceutical development because of a lack of proven efficacy in a phase III human clinical trial (Carter et al., 2010). However, while clinical trials of tramiprosate in mild-to-moderate AD have not shown therapeutic efficacy, *post-hoc* analyses have shown some significantly positive outcomes on secondary endpoints and in some particular subgroups of patients (Aisen et al., 2011; Caltagirone et al., 2012). In particular, the disease-modifying potential is highlighted in APOE4 allele carrier patients by the observed cognitive benefits (Caltagirone et al., 2012; Abushakra et al., 2017).

The classic strategies aimed at HSPGs are mostly tested in AD where benefits are observed in distinct patient subgroups. In these strategies, APP and Aβ (not tau) are mostly studied as targets. An alternative approach consists in targeting HS sulfation. Recently, overexpression of several HS biosynthetic enzymes such as 3-*O*-sulfotransferase-2 (3ost2 or Hs3st2) was demonstrated in the hippocampus of AD patients. Inactivation of the zebrafish ortholog of the HS chain modifier enzyme Hs3st2 significantly decreased tau hyperphosphorylation and tau-related neuropathology *in vitro* and *in vivo* in the Tg[HuC::hTau^{P301L}/DsRed] zebrafish model of tauopathy (Sepulveda Diaz et al., 2015). An additional approach consists of treatment with heparan sulfate antagonist molecules. In a recent proof of concept study a rescue of neuronal abnormalities upon treatment with surfen and oxalyl surfen was observed in the Tg[HuC::hTau^{P301L}/DsRed] zebrafish model (Alavi Naini et al., 2018). **Figure 1** summarizes the two approaches in Tg[HuC::hTau^{P301L}/DsRed] zebrafish.

3-*O*-Sulfation in HS chains forms binding sites for HSPG binding proteins. However, biochemical characterization of this rare modification among an array of other sulfated groups requires large sample quantities, and shortage of material for studying 3-*O*-sulfation has so far hampered work on these sulfated motifs. So far six HSPG-binding proteins have been shown to require 3-*O*-sulfation for their association with GAG chains (Thacker et al., 2014).

The addition of a 3-*O*-sulfate group is one of the last chain modifications in HS biosynthesis (Zhang et al., 2001a,b). This modification is a rare and discrete modification confined to a limited number of chains when it occurs at all, and its prevalence among naturally occurring HSs is largely unknown (Thacker et al., 2014). The 3-*O*-sulfate group was initially identified while

TABLE 1 | Classic strategies targeting HSPGs in tauopathies.

Category		Names	Targets	Settings/Organisms	Outcomes	References
Sulfated glycosaminoglycans (GAGs)	Conventional GAGs	Heparin, dextran sulfate and other GAGs	APP/Aβ	*In vitro*, PC12 cells	Inhibition of HSPG binding to APP	Narindrasorasak et al., 1991; Leveugle et al., 1994
		Heparin analogs	BACE1	*In vitro*	BACE1 inhibition	Patey et al., 2006, 2008
	Low molecular weight (LMW) heparins	Heparin oligosaccharides	APP/Aβ	*In vitro*, Human	Inhibition of the stimulatory effect of heparin on APP secretion and heparin binding to $Aβ_{1-28}$	Leveugle et al., 1998
			Tau	Neuroblastoma culture	Reduction in cellular uptake and cytotoxicity of tau oligomers	Wang et al., 2018
		Enoxaparin/Dalteparin	Aβ	*In vitro*	Disassembley of $Aβ_{40}$ fibrils	Zhu et al., 2001
		Certoparin/Neuroparin (C3)	Aβ/Tau	Rat	Reduction in tau abnormal conformation in hippocampus, stimulated by $Aβ_{25-35}$ intra-amygdaloid injection	Walzer et al., 2002/ Dudas et al., 2002, 2008
		Enoxaparin	Aβ	APP23 mice	Decrease in Aβ brain concentration, deposition and reactive astrocytosis	Bergamaschini et al., 2004
				APPswe/PS1dE9 mice	Improvement of spatial memory, decrease in Aβ deposition if treated in early stages of Aβ accumulation	Timmer et al., 2010
Low molecular weight anionic sulfonate or sulfate compounds		Congo red and analogs	Aβ	PC12/Hela cells	Attenuation of $Aβ_{25-35}$ and $Aβ_{40}$ toxicity	Pollack et al., 1995b,a
		Sodium 1,3-propanediol disulphate	Aβ	*In vitro*	Disassembly of $Aβ_{40}$ fibrils	Kisilevsky et al., 1995
		Tramiprosate (3-amino-1-propanesulfonic acid; 3APS; homotaurine; Alzhemed™)	Aβ	TgCRND8 mice	Significant reduction of Aβ in brain and plasma, reduction of Aβ plaques in brain	Gervais et al., 2007
				Humans: Phase II trial in probable mild to moderate AD	Satisfactory safety and tolerability, significant decrease of CSF $Aβ_{42}$ levels, no cognitive benefits	
				Phase III trial in mild to moderate AD	No cognitive benefits, *post-hoc* analyses: decrease in loss of hippocampal volume	Aisen et al., 2006
					Cognitive benefits in APOE4/4 patients	Abushakra et al., 2017

searching for sulfate group removing enzymes on heparin (Leder, 1980), and later confirmed with chemical, NMR and mass spectrometry analyses (Meyer et al., 1981; Yamada et al., 1993).

The best-studied case of 3-*O*-sulfate group-dependent binding is the association of anti-thrombin (AT) to heparin and HS. Binding of heparin to AT induces conformational changes in the protein that radically increase its catalytic activity (Rosenberg and Damus, 1973; Huntington et al., 1996). The activated AT then inactivates several proteases, including thrombin, factor IXa, and factor Xa, which are involved in blood coagulation. This is why heparin is used as a routine anticoagulant agent in clinical practice. The characterization of AT binding sites on the sulfated sugar chains is facilitated by their enrichment in heparin, which is readily available commercially (Yamada et al., 1993). The minimum AT binding site on heparin is a pentasaccharide sequence with a critical 3-*O*-sulfated motif on the middle *N*-sulfo-glucosamine residue (Thunberg et al., 1982; Choay et al., 1983). In the absence of this 3-*O*-sulfate group, the

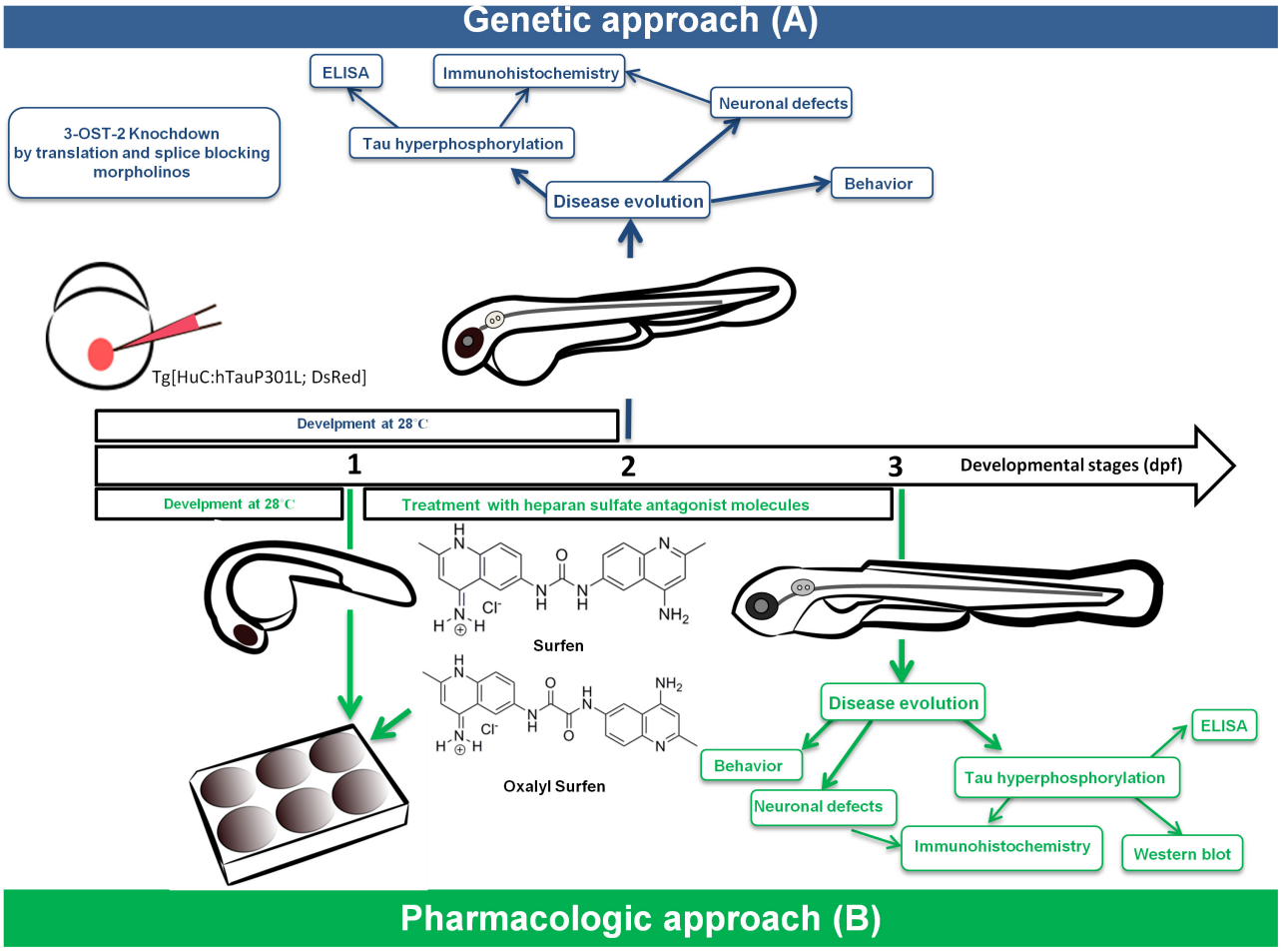

FIGURE 1 | Combination of genetic **(A)** and pharmacological **(B)** approaches identified novel therapeutics for tauopathies using a zebrafish transgenic model of tau pathology.

affinity of heparin to AT was markedly decreased, along with its inhibitory effect on factor Xa (Atha et al., 1985, 1987).

3-*O*-Sulfated HSs at the cell surface also serve as entry receptors for the herpes simplex envelope glycoprotein, glycoprotein D (gD) (Shukla et al., 1999), and the binding domain has been identified as an octosaccharide containing a 3-*O*-sulfated motif (Liu et al., 2002). In a similar manner, fibroblast growth factor 7 (FGF7) and fibroblast growth factor receptors (FGFR) are thought to rely on 3-*O*-sulfated motifs for binding to HS and heparin (McKeehan et al., 1999; Ye et al., 2001; Luo et al., 2006). Moreover, 3-*O*-sulfation on heparin/HS is suggested to mediate the binding of "cyclophilin B" and "stabilin" (Vanpouille et al., 2007; Pempe et al., 2012). Cyclophilin B promotes lymphocyte adhesion and migration following binding to HS located at the cell surface (Allain et al., 2002). Stabilins (1, 2) mediate heparin clearance in hepatic sinusoidal endothelial cells (Hansen et al., 2005; Harris et al., 2008).

Combination of genetic (A) (morpholino gene-KO) (Sepulveda-Diaz et al., 2015) and pharmacological (B) (small molecule) (Alavi Naini et al., 2018) approaches in the zebrafish Tg[HuC::hTau^{P301L}; DsRed] tauopathy model identified 3-*OS*-sulfated HS as a therapeutic target for tauopathies and the small molecules surfen and oxalyl surfen as novel therapeutic candidates.

Alterations in the fine structure of GAGs have previously been described in several pathological situations. The sulfate moieties on GAG chains have been shown to contribute to amylin fibrillization in islet amyloidosis (Castillo et al., 1998). Furthermore, alteration of the *O*-sulfation pattern and specific disaccharide compositions has been observed in amyloid-laden liver and spleen tissues (Lindahl and Lindahl, 1997). An analysis of the HSs in the cerebral cortex in AD and control subjects demonstrated an alteration in *N*-sulfated residue distribution in AD brain, with an increase in GlcNSO3 residues (Lindahl et al., 1995). However, in studies of cerebral tissue the abnormal HSs may have been diluted with normal HSs, as analyses were performed on whole brains. In particular, alterations in specific HSPG populations or modifications of small domains in HSPG chains may have been hidden. Moreover, in AD, analysis of skin

fibroblasts from patients revealed alterations in HS sulfation. Zebrower and Kieras reported that cultured skin fibroblasts from AD patients produced 30% more GlcA-GlcNSO3, and 40% less GlcA-GlcN-6-OSO3 compared to samples from age-matched healthy individuals (Zebrower et al., 1992). Authors suggested that an alteration in HS-sulfotransferase activity was responsible for their observations (Zebrower and Kieras, 1993). Importantly, a critical requirement for the presence of 6-*O*-sulfated disaccharide-containing HS in internalization and spreading of infectious tau species has recently been demonstrated (Rauch et al., 2018; Stopschinski et al., 2018). 6-*O*-Sulfation was also identified as critical for heparin-tau interaction by surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) spectroscopy (Zhao et al., 2017), while the requirement for 3-*O*-sulfation was not investigated in these recent studies. These studies together with studies on 3-*O*-sulfation (Sepulveda-Diaz et al., 2015) support the hypothesis that specific GAG chain sulfation mediated by a combination of 2-, 3- and 6-*O*-sulfotransferases play a key role in the physiopathology of tauopathies. Interestingly, hierarchical sulfation of disaccharide residues allows 3-*O*-sulfated motifs to be generated only on 6-*O*-sulfated domains of GAG chains.

Aside from hyperphosphorylation and aggregation of the tau protein, the sulfated motifs on GAG chains have also been shown to play a role in Aβ fibrillization and aggregation. This was first suggested by Fraser and coworkers, based on X-ray diffraction studies (Fraser et al., 1992). A later *in vitro* study showed that the sulfated motifs on GAG chains directly bind fibrillar Aβ (Gupta-Bansal et al., 1995). In addition, while removal of *N*-sulfated motifs on heparin GAG chains slightly reduced the heparin-induced Aβ fibril formation (Castillo et al., 1999), removal of *O*-sulfates led to a significant loss of heparin-enhanced Aβ fibrillization. Heparin enhancement of Aβ fibrillization was significantly greater than that induced by HS, consistent with the lower sulfate content in HS. By contrast, inorganic sulfate was found to have no effect (Castillo et al., 1999).

In turn, Aβ peptides have been suggested to influence GAG composition and localization (Miller et al., 1997; Timmer et al., 2009). In particular, $A\beta_{42}$ carrying the Dutch mutation has been reported to induce cellular relocalization and production of agrin and glypican-1 *in vitro*, together with altered glycosylation and increased sulfate incorporation (Timmer et al., 2009). Interestingly, the treatment of human brain pericytes (HBPs) with $A\beta_{42}^{Dutch}$ leads to the association of Aβ fibrils to cell surface glypican-1 prior to their internalization (Timmer et al., 2009). TGF-β enhancement of Aβ fibril formation is also suggested to involve an increase in HSPG synthesis or chain modification (Castillo et al., 1999). Aβ degradation takes place in the lysosomal compartment (Li et al., 2012), the intracellular compartment where HSPG degradation also occurs (Brauker and Wang, 1987; Yanagishita and Hascall, 1992; Bame, 1993). Interestingly, Aβ has been shown to inhibit the heparanase-mediated degradation of GAGs and HSPGs *in vitro* (Bame et al., 1997), suggesting that a similar process may hamper HSPGs degradation *in vivo* in tauopathies, creating a scenario similar to that observed in patients with mucopolysaccharidoses.

Apolipoprotein E (whose E4 allele is a major risk factor in sporadic AD) promotes GAG sulfation in cultured neuroblastoma cells, and this effect is greater for ApoE4 than ApoE3 (Bonay and Avila, 2001). The interaction between HSPGs and apolipoprotein is necessary for the uptake of apolipoprotein E-containing lipoprotein by low-density lipoprotein (LDL) receptor related protein (LRP) by hepatocytes *in vitro*. Lipoprotein uptake in neurons is also mediated by the HSPG/LRP pathway (Mahley, 1996). Interestingly, apoE has been shown to interact with HSPGs through a binding site, which is structurally complementary to HS rich in *N*- and *O*-sulfate groups (Libeu et al., 2001). Moreover, the inhibitory effect of ApoE4 (not observed for ApoE3) on neurite outgrowth *in vitro* is likely mediated by the HSPG/LRP pathway (Bellosta et al., 1995). Moreover, a synthetic peptide that contain the amino acid residues 141-155, together with a 22 kDa N-terminal thrombin-cleavage fragment of apoE display significant toxicity to neurites from embryonic chick neurons *in vitro*, with apoE4 fragments exhibiting greater toxicity than apoE3. The toxic effect is likely mediated via the HSPG/LRP pathway, and treatments antagonizing HS prevent neurite degeneration (Crutcher et al., 1994; Marques et al., 1996). HSPG acts jointly with LRP1 for cell surface binding and uptake of Aβ (Kanekiyo et al., 2011). Finally, ApoE (Li et al., 2012) and LRP (Fuentealba et al., 2010) promote lysosomal trafficking of Aβ. ApoE acts in an isoform-dependent manner, with apoE3 enhancing Aβ trafficking and degradation more efficiently than apoE4 (Li et al., 2012). These observations support the hypothesis that a defect in the metabolism of HS orchestrated by Aβ and apoE may play a role in the physiopathology of tauopathies.

The internalization of HS in the intracellular space has been described in several pathological situations (Cole and Liu, 2006). The intracellular accumulation of HS long before detection of tau pathology in neurons in AD and in Down syndrome was described decades ago (Snow et al., 1990; Goedert et al., 1996). However, their involvement in the series of events leading to the abnormal tau phosphorylation and neurodegeneration has been largely disregarded, possibly because of the paradigm confining the biological roles of HSPGs to the extracellular space (Shriver et al., 2012).

The association of sulfated GAGs with tau in neurons has long been puzzling and was seen to result from leakage of newly synthesized sulfated GAGs from intracellular organelles such as Golgi apparatus, endosomes and lysosomes (Díaz-Nido et al., 2002). Interestingly, under two distinct pathological situations in neuroblastoma cultures, expression of human tau^{P301L} protein and exposure to oxidative stress, HS was localized in intracellular space. An intense intracellular uptake of membrane-associated HS and the concomitant hyperphosphorylation of the tau protein were observed (Sepulveda Diaz et al., 2015). These findings point to a possible altered metabolism and distribution of sulfated HS in tau-related neuropathologies. Regarding the molecular mechanisms linking 3-*O*-sulfated HSs and tau abnormal phosphorylation, *in vitro* data suggest

that the sulfated GAGs bind to tau and not to the GSK3β tau-kinase (Sepulveda Diaz et al., 2015). Accordingly, in the presence of heparin (a highly sulfated HS), tau rapidly undergoes conformational changes that allow kinase access to epitopes otherwise inaccessible for phosphorylation (Hasegawa et al., 1997; Zheng-Fischhöfer et al., 1998; Paudel and Li, 1999; Sibille et al., 2006; Yoshida and Goedert, 2012). The substrate modulator effect of 3-*O*-sulfated HS motifs further supports the hypothesis that these sulfated polysaccharides act as molecular chaperones able to induce key conformational changes on tau protein and triggering its phosphorylation at newly unmasked tau residues. The hypothesis is further supported by the observation of an increase in hippocampal 3-*O*-sulfation with aging (Huynh et al., 2012). Recently, a GAG sulfotransferase inhibitor has been identified (Cheung et al., 2017) that sets a basic scaffold for the future development of highly specific and potent sulfotransferase inhibitors, which are promising candidates as potential therapeutics for tauopathies.

CONCLUSION

Heparan sulfate-protein interactions are emerging as potential therapeutic targets in tauopathies. Previous work suggest a multifactorial role played by GAGs in the pathogenesis of tauopathies. Increased biosynthesis, internalization and/or decreased degradation of HS GAGs, or alterations in HS chain modifications likely contribute to tauopathies. These observations raise the question of whether specific motifs on HS chains are involved in binding to tau and induction of misfolded states. The zebrafish has recently emerged as an attractive animal model for Alzheimer's disease research and provides an ideal tool for drug screening prior to clinical testing in mammals. Thanks to this small fish, HS is targeted in tau pathology by two different approaches. A critical requirement for 3-*O*-sulfated HS in tau pathology was discovered and HS antagonists (surfen and oxalyl surfen) were identified as efficient therapeutic candidates. 6-*O*-sulfated HS motifs have recently been identified as critical for tau seed internalization. 3-*O*-sulfation, a fine chain modification, is only generated in 6-*O*-sulfated regions. Together, these recent findings strengthen the hypothesis of distinct HS sequences implicated in tau misfolding, hyperphosphorylation, aggregation and propagation. These findings are steps toward defining of specific HS targets in tauopathies.

AUTHOR CONTRIBUTIONS

Both authors contributed to writing the manuscript.

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Hematopoietic Cytokine Gene Duplication in Zebrafish Erythroid and Myeloid Lineages

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Hematopoiesis is a precisely orchestrated process regulated by the activity of hematopoietic cytokines and their respective receptors. Due to an extra round of whole genome duplication during vertebrate evolution in teleost fish, zebrafish have two paralogs of many important genes, including genes involved in hematopoiesis. Importantly, these duplication events brought increased level of complexity in such cases, where both ligands and receptors have been duplicated in parallel. Therefore, precise understanding of binding specificities between duplicated ligand-receptor signalosomes as well as understanding of their differential expression provide an important basis for future studies to better understand the role of duplication of these genes. However, although many recent studies in the field have partly addressed functional redundancy or sub-specialization of some of those duplicated paralogs, this information remains to be scattered over many publications and unpublished data. Therefore, the focus of this review is to provide an overview of recent findings in the zebrafish hematopoietic field regarding activity, role and specificity of some of the hematopoietic cytokines with emphasis on crucial regulators of the erythro-myeloid lineages.

Keywords: zebrafish, hematopoiesis, cytokine, genome duplication, myelopoiesis, erythropoiesis

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INTRODUCTION

Hematopoiesis, the multistep process of formation and turnover of blood cells, is precisely regulated by an array of extrinsic and intrinsic factors (Kaushansky, 2006). Extrinsic factors include cytokines and growth factors that bind to their corresponding receptors and in turn activate intracellular signaling molecules that further modulate cellular responses, mainly by controlling activity of different transcriptional activators or repressors. The process of hematopoiesis begins already in early development, where red blood cells and macrophages are formed in a primitive wave to provide necessary support for the developing embryo. Later in development, the whole system is largely driven by proliferation, self-renewal, and differentiation of lineage restricted progenitors as well as hematopoietic stem cells (HSCs) and hematopoietic multipotent progenitor cells (MPPs) with reduced self-renewal capabilities. All erythroid and myeloid cells derive from HSCs with the exception of some tissue resident macrophages which are independent on HSC input (Ginhoux et al., 2010).

According to the classical hierarchical model of definitive vertebrate hematopoiesis (Akashi et al., 2000), the HSCs asymmetrically lose their long term self-renewing capabilities to form MPPs that further give rise to common lymphoid and common myeloid progenitors. The common myeloid progenitors afterward differentiate into bipotent megakaryocyte-erythrocyte progenitors (MEPs) and restricted common myelo-monocytic progenitors (CMPs). In this review, we will focus on cytokines that regulate formation and maintenance of these erythro-myeloid hematopoietic lineages.

Hematopoiesis is well conserved throughout the vertebrates, with all the major blood lineages – myeloid, erythroid and lymphoid – conserved from fish to men. Importantly, also the sequential waves of developing blood cells during ontogenesis are present during the development of the zebrafish embryo, leading finally to a fully fledged adult hematopoietic system, as has been described in other vertebrates. However, some differences between mammalian and non-mammalian hematopoiesis do exist (Svoboda and Bartunek, 2015), particularly in erythro-megakaryocytic lineages. In mammals, bi-potent cells termed megakaryocyte-erythrocyte progenitors (MEPs) give rise to either endoreduplicated megakaryocytes (Svoboda et al., 2014) that serve as a precursor for platelet biogenesis, or to enucleated erythrocytes (Muir and Kerr, 1958; Simpson, 1967). On the other hand, non-mammalian vertebrates possess bi-potent thrombocyte-erythrocyte progenitors (TEPs) instead that differentiate into functional homologs of platelets, termed thrombocytes or to nucleated erythrocytes (**Figure 1**; Ratnoff, 1987; Schneider and Gattermann, 1994; Svoboda et al., 2014). Besides these differences, the rest of the hematopoietic differentiation tree is well conserved in all vertebrate animals.

Hematopoietic cytokines signal via their cognate receptors to drive target cell proliferation and/or differentiation. In general, cytokines are pleiotropic in their function and for this reason also many factors regulating erythro-thrombocytic differentiation have broader effect on all hematopoietic lineages (Nicola, 1994).

In vertebrates, the major cytokines regulating red blood cell development from bipotent TEPs or MEPs through committed burst forming units-erythroid (BFU-E), colony forming units-erythroid (CFU-E) and erythroblasts, are erythropoietin (EPO) and stem cell factor (SCF, or KIT ligand, KITLG). On the other hand, thrombopoietin (TPO) is the key mediator of thrombocyte or platelet formation from TEPs/MEPs and is also responsible for platelet formation from polyploid megakaryocytes (Kaushansky et al., 1995; Kato et al., 1998). Other important erythro/thrombocytic regulators that promote self-renewal of erythroid progenitors or their differentiation include insulin (INS) and insulin-like growth factor (IGF1) (Miyagawa et al., 2000). Moreover, transforming growth factor α (TGFα) and TGFβ family members (Krystal, 1994; Huber et al., 1998; Gandrillon et al., 1999; Fuchs et al., 2002; Harandi et al., 2010), interleukin 3 (IL3), and fibroblast growth factor 2 (FGF2) (Bartunek et al., 2002) also play a crucial role in this process.

In thrombocytic differentiation, TPO interacts with and activates its cognate receptor, TPOR (c-MPL) (de Sauvage et al., 1994; Alexander, 1999b) and this signaling has been shown to be necessary for proper thrombopoiesis (Alexander, 1999a). This signaling is complemented by IL12 and SDF1, necessary for proper megakaryocytic maturation and proper platelet formation (Gordon and Hoffman, 1992). Function of these lineage-restricted factors is complemented by other regulators, especially interleukins (IL3, IL6, IL11), G-CSF, GM-CSF (McNiece et al., 1991; Gordon and Hoffman, 1992) and SCF (Steinlein et al., 1995; Broudy, 1997) that can enhance both erythroid and thrombocytic differentiation.

The CMPs also give rise to other substantial myelo-monocytic cell types – granulocytes, monocytes/macrophages and dendritic cells (Akashi et al., 2000), whose proliferation and differentiation from hematopoietic stem and progenitor cells (HSPCs) is regulated by macrophage colony-stimulating factor (M-CSF, or CSF1), granulocyte-macrophage CSF (GM-CSF, or CSF2), granulocyte CSF (G-CSF, or CSF3) and interleukin 3 (IL3) (Metcalf and Nicola, 1983; Metcalf, 1985; Migliaccio et al., 1991; Lieschke et al., 1994; Liu et al., 1996). These cytokines act via their cognate receptors – M-CSF receptor (M-CSFR, or CSF1R), G-CSF receptor (G-CSFR, or CSF3R), GM-CSF receptor (GM-CSFR, or CSF2RA) and interleukin 3 receptor (IL3RA), respectively.

Due to poor sequence homology between teleost and mammalian cytokines, the mammalian cytokines generally do not cross-react with zebrafish hematopoietic cells (Stachura et al., 2009, 2011, 2013; Svoboda et al., 2014) and for the same reason, the identification of zebrafish cytokine orthologs has been challenging. However, many successful attempts of identifying, generating and using recombinant zebrafish cytokines have been reported in recent years (Stachura et al., 2009, 2011, 2013; Svoboda et al., 2014).

Due to an extra round of whole genome duplication (WGD) during the evolution of teleost fish, which occurred 320–350 million years ago (Hoegg et al., 2004; Amores et al., 2011), zebrafish possess multiple paralogs of many important genes. After WGD, duplicated paralogs are often lost through a process of pseudogenization, where detrimental mutations accumulate in the duplicated gene (Nei and Roychoudhury, 1973; Takahata and Maruyama, 1979; Watterson, 1983). Alternatively, the two paralogs of the ancestral gene can be retained and either acquire new functions (i.e., neofunctionalization) or split the original function between the two paralogs (i.e., subfunctionalization) (Force et al., 1999).

Importantly, the event of duplication brought an increased level of complexity in such cases when both ligands and receptors have been duplicated (and retained) in parallel. Therefore, precise understanding of binding specificities between duplicated ligand-receptor signalosomes, as well as an understanding of their differential expression, will provide an important basis for future studies to better understand evolution of the vertebrate genome. Although many recent studies in the field have partly addressed functional redundancy or sub-specialization of some of these duplicated paralogs (Hultman et al., 2007; Wang et al., 2008; Stachura et al., 2013; Butko et al., 2015), there are still many unknowns.

Understanding the precise relationship between mammalian and non-mammalian hematopoiesis may have an important

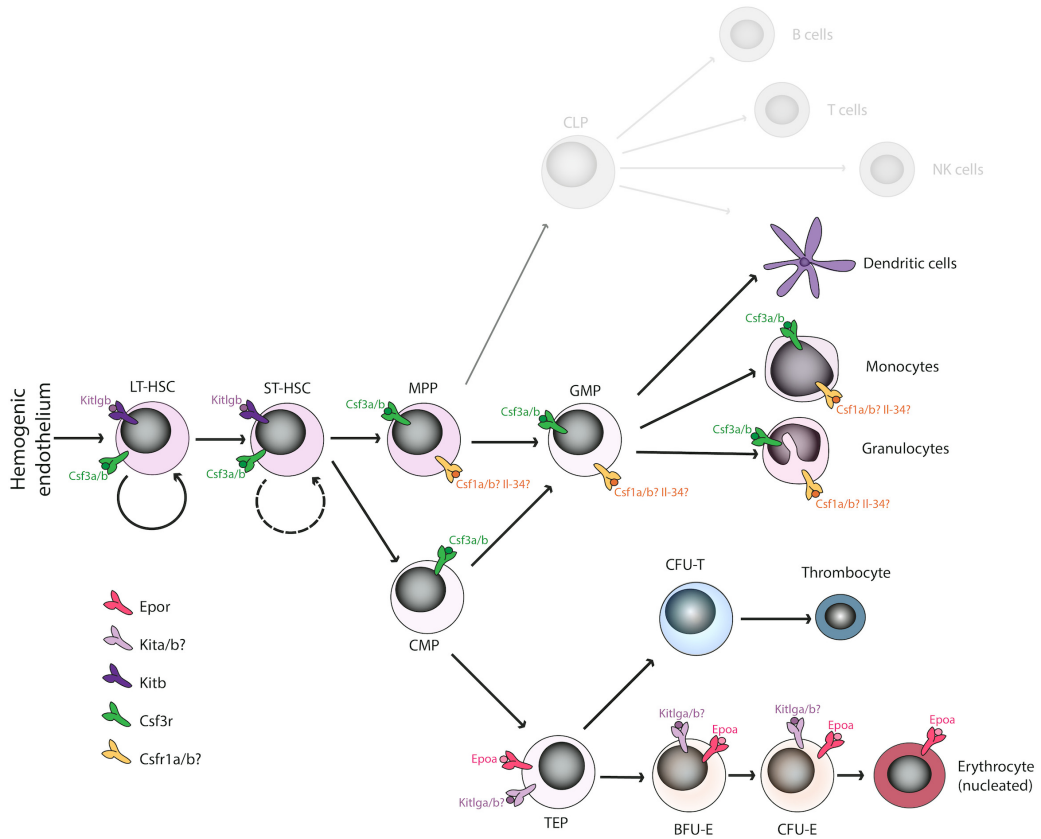

FIGURE 1 | Overview of duplicated hematopoietic cytokines and receptors regulating the erythro-myeloid lineage in *Danio rerio*. Various cytokine receptors have been proposed to act at the level of hematopoietic stem cells (Kitb, Csf3r), the myeloid lineage (Csf1ra/b, Csf3r), or the erythroid lineage (Epor, Kita/b) in *D. rerio* binding the corresponding ligands. The function of missing cytokines (Il-3, Il-5, GM-CSF, Fli3) is most likely compensated by other factors. Some duplicated paralogs have undergone sub-specification (e.g., Kitlga/b), whereas some other seem to have lost their function in hematopoiesis (e.g., Epob). LT-HSC, long-term hematopoietic stem cells; ST-HSC, short-term hematopoietic stem cells; MPP, multipotent progenitor cells; GMP, granulocyte-macrophage progenitors; CMP, common myeloid progenitors; TEP, thrombo-erythroid progenitors; BFU-E, burst forming units-erythroid; CFU-E, colony forming units-erythroid.

impact on general hematopoiesis research. Due to the interference with sophisticated mammalian megakaryocytic and erythroid enhancements, the employment of mammalian model organisms brings only partial success in the quest of identifying novel key regulators of cell fate determination. Once we have detailed understanding of cytokines and the receptors driving hematopoiesis in zebrafish, we can overcome these obstacles and efficiently utilize this non-mammalian model organism, instead.

DUPLICATED ERYTHRO-MYELOID CYTOKINE GENES IN ZEBRAFISH

Epoa/b

Erythropoietin (EPO) is the major regulator of erythropoiesis, mediating self-renewal, survival and differentiation via the Epo receptor (EPOR) (Krantz, 1991). EPOR homodimerization mediated by EPO binding leads to auto-phosphorylation of JAK2 that is bound to Box1/2 and that in turn phosphorylates the receptor itself as well as other signaling molecules such as STAT5 (signal transducers and activators of transcription) transcription factor, PI-3K (phosphatidylinositol 3-kinase) and mitogen-activated protein kinase (MAPK) (Drachman and Kaushansky, 1997; Constantinescu et al., 1999).

We previously identified two copies of the epo gene in zebrafish, termed epoa and epob. Although zebrafish epoa probably plays a similar role as its mammalian ortholog, the epob has not been studied so far and it is likely that it formed a pseudo-gene without having any biological role. Zebrafish erythropoietin encoded by the epoa gene is a crucial cytokine for erythroid cell development and maintenance (Krystal, 1994) and it has been shown to stimulate proliferation, and differentiation of erythroid cells (Paffett-Lugassy et al., 2007; Stachura et al., 2011). Moreover, recombinant zebrafish Epoa has been shown to expand and differentiate erythroid cells *ex vivo* (Stachura et al., 2009) and in combination with other factors such as Gcsfa or Tpo, and it promotes multilineage erythro-myeloid hematopoiesis in semisolid media (Stachura et al., 2011; Svoboda et al., 2014). To date, a single erythropoietin receptor has been identified in zebrafish (Paffett-Lugassy et al., 2007).

Kitlga/b

KIT ligand (KITLG) is a classic example of a cytokine acting on many different levels. KIT signaling plays an important role in a variety of tissues and cells, such as HSCs and germ stem cells. Moreover, KIT also plays a significant role during erythro-myelopoiesis (Hayman et al., 1993), neurogenesis and pigmentation, and mutations in this gene have been reported in many types of cancers including erythroleukemia (Kosmider et al., 2005; Abbaspour Babaei et al., 2016). Binding of KITLG to its cognate receptor (KIT) – member of the receptor tyrosine kinase type III family (RTKIII) – promotes various signaling cascades. Ligand-based activation of Kit triggers auto-phosphorylation and activation of PI-3K, MAPK, SRC, but also JAK kinase pathways (Ashman, 1999; Abbaspour Babaei et al., 2016). Notably, KITLG exists in two forms *in vivo* – transmembrane, which seems to be important to regulate stem cells in their niches, and soluble, that affects distant tissues (Ashman, 1999).

Interestingly, both Kit and Kitlg have been duplicated in teleost to form two receptors (Kita and Kitb) and two ligands (Kitlga and Kitlgb). This duplication of the whole ligand-receptor signalosome is relatively uncommon and raises many questions about diversification of both ligands and receptors as well as their binding specificities for each other. These questions have been poorly studied so far and very little is known about Kit involvement during zebrafish hematopoiesis. So far, it has been shown that both kitlg paralogs might have subspecialized during teleost phylogenesis. Kita is expressed in neural crest, lateral line or in the notochord. Similar to their mammalian counterparts, both Kitlga as well as Kita are involved in melanogenesis, since overexpression of kitlga results in a hyper pigmentation phenotype (Hultman et al., 2007), whereas kita receptor mutants (sparse) show severe pigmentation defects (Parichy et al., 1999). On the contrary, the second zebrafish Kit paralog, Kitb, does not seem to play a role during melanogenesis. It has been shown to be expressed in neural tube and otic vesicles (Mellgren and Johnson, 2005).

As mentioned above, Kit signaling has been poorly studied in the hematopoietic context and there are only two studies that present any possible Kit involvement in these processes. It has been shown that even though the kita is expressed in hematopoietic tissues, surprisingly and in contradiction to the other vertebrate models, hematopoiesis does not seem to be affected in the kita receptor (sparse) mutants (Parichy et al., 1999). So far, the only studies demonstrating any potential importance of Kit signaling during hematopoiesis in zebrafish at present are a mild increase of HSCs upon overexpression of kitlgb (Mahony et al., 2016) and decrease of HSCs upon downregulation of kitb (Mahony et al., 2018).

Csf3a/b

Colony stimulating factor 3, CSF3, also known as granulocyte colony stimulating factor (GCSF), is a cytokine crucial for proliferation, differentiation and survival of monocytes, macrophages and neutrophilic granulocytes (Metcalf and Nicola, 1983; Nicola et al., 1983, 1985; Lieschke et al., 1994; Liu et al., 1996). CSF3 binds to its cognate receptor, CSF3R, activating signaling cascades including JAK2/STAT5 and MAPK pathway, important for neutrophil production during both steady state and emergency hematopoiesis (Touw and van de Geijn, 2007).

Two paralogs of Csf3, the major regulator of granulocytic, monocytic and megakaryocytic differentiation, have been reported in zebrafish with a slightly diverged function. Based on extensive synteny analysis, it has been suggested that chromosomal regions harboring Csf3a/b share common ancestral origin and probably emerged from a chromosome/genome duplication event (Stachura et al., 2013). Although like in mammals, both zebrafish Csf3 paralogs stimulate granulocytic differentiation (Nicola et al., 1983, 1985) and monocyte/macrophage differentiation (Migliaccio et al., 1988), they appear to play a broader role in hematopoiesis, including HSCs specification and expansion. Both paralogs differ in the levels of spatio-temporal expression during development and in the adult animals. Csf3a expression is low in early development, rising gradually over time, whereas Csf3b is highly expressed starting from 6 hpf but its levels decrease over time. Despite these differences, overexpression of both ligands during development indicates redundant functions. Slight differences in binding kinetics to Csf3r, indicate another possible mechanism that controls spatio-temporal activity of both zebrafish Csf3 paralogs (Stachura et al., 2013).

High csf3b expression has been detected in the kidney, the main site of hematopoiesis in zebrafish, as well as testes, skin and gills. Csf3a has been detected at lower levels in these tissues, with high expression in heart and spleen. Although the differences in tissue expression might indicate that the major player could be csf3b, both of these ligands retain the ability to differentiate myeloid progenitors. As in mammals, Csf3 is important for both primitive and definitive waves of generation of myelomonocytic cells (Stachura et al., 2013).

These findings suggest that during vertebrate evolution, Csf3 was involved in many levels of hematopoiesis, but after the radiation of mammals other specialized cytokines evolved and have likely taken over the function of Csf3 (Avery et al., 2004; Huising et al., 2006). Csf3 probably represents an ancient cytokine whose functions were diversified in evolution following duplication events (Stachura et al., 2013).

Csf1a/b and IL34

In mammals, CSF1 is the major regulator of many myeloid cells, such as monocytes, macrophages, dendritic cells, microglia, osteoclasts, or Langerhans and Kupffer cells, and it also plays an important role in disease development (Hamilton, 2008; Hamilton and Achuthan, 2013). The ligand binds to its specific receptor (CSF1R), which is another member of RTKIII family, and activated CSF1R further promotes JAK2/STAT5, PI3K, and MEK signaling (Pixley and Stanley, 2004; Martinez and Gordon, 2014). It has been shown that CSF1 is not the only ligand to bind and activate this receptor. Alternatively, it can be activated also by IL34 in certain tissues – Langerhans cells and microglia inside mouse brain (Greter et al., 2012; Wang et al., 2012).

Similarly to other cytokines described, the CSF1 has been identified in the form of two paralogs in zebrafish (csf1a/b) (Wang et al., 2008). Along with two csf1 receptors (csf1ra/b) found in fish, this provides another relatively unique example of duplication of the whole receptor-ligand signalosome (Braasch et al., 2006). Regarding the IL34 that has been similarly reported to exist in two copies in salmon (Pagan et al., 2015), only a single il34 gene has been identified in zebrafish so far.

Zebrafish Csf1a and Il34 signaling has been shown to play a role in microglia development in the retina (Huang et al., 2012) as well as in pigment pattern formation during development (Patterson et al., 2014) (Csf1a only). Supporting this, the csf1ra mutant fish (panther) have decreased numbers of microglia and macrophages (Herbomel et al., 1999; Pagan et al., 2015). Il34 has recently been shown to regulate distribution of yolk sac macrophages and microglial precursors and seeding of the brain in zebrafish embryos (van Ham et al., 2018; Wu et al., 2018). On the other hand, any information about the functional role of csf1rb are missing.

Examples of Potentially Missing Cytokine Genes

Genome evolution in the teleost fish did not only bring another rounds of duplication, but it also brought many losses of individual genes or clusters of genes including some of the class I cytokine family members (Liongue and Ward, 2007). This includes a missing cluster of il3 family genes with the disappearance of ligands and receptors for il3, il5, and gmcsf. In mammals and birds, these factors are responsible for the maintenance of myelo-monocytic lineages and their loss in teleost indicates that they were possibly substituted by other newly duplicated genes (Stachura et al., 2013).

Another example of a cytokine potentially missing in the zebrafish genome is flt3l, an important regulator of HSCs and myeloid cells (Guermonprez et al., 2013; Jacobsen et al., 2016; Tornack et al., 2017). However, the possibility exists that it has not yet been identified due to sequence divergence. This hypothesis is supported by the fact that flt3l is present both in mammals and even some lower vertebrates including *Latimeria* and elephant shark (Tan et al., 2012) and its cognate receptor, Flt3, is expressed in the developing zebrafish embryo and adult HSCs and monocytes (He et al., 2014; Macaulay et al., 2016; Tang et al., 2017).

CONCLUSION

The whole-genome duplication in teleost fish raises interesting questions regarding hematopoietic cytokine sub-specialization, redundancy and gain/loss of function. In this review, we have reviewed particular examples of all of these events, discussing epob loss of function, sub-specialization of kitlga/b, or the redundancy between csf3a/b (**Figure 1**). We hypothesize that some specific gene duplications might even have enabled or compensated for the loss of some specific genes in the zebrafish genome (GM-CSF/IL3 cluster).

Although several reports have addressed the question of duplicated cytokines in zebrafish, many functional links are still missing, especially the ligand receptor specificities in cases, when the whole ligand-receptor signalosome has been duplicated (e.g., Kitlg/Kit, Csf1/Csf1r). Moreover, functions of many hematopoietic cytokines have yet not been elucidated. One example is IL11, a crucial regulator of megakaryocyte maturation (Paul et al., 1990) that binds to IL11Rα and gp130, activating the JAK/STAT pathway (Heinrich et al., 2003). Two paralogs of il11 have been identified in teleost (Huising et al., 2005); however, data indicating respective functions of each of the paralogs are still missing.

Zebrafish is a powerful model organism for studies of hematopoietic cell maintenance and differentiation both in the course of development and in the adult animal using the wide range of available *in vivo* and *ex vivo* tools. Therefore, it is very important to understand the precise function of each of the paralogs and elucidate the functions of the yet uncharacterized cytokines that would enable more complex experiments elucidating the fine details of hematopoietic regulatory mechanisms.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PERSPECTIVE
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The Zebrafish as an Emerging Model to Study DNA Damage in Aging, Cancer and Other Diseases

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Cancer is a disease of the elderly, and old age is its largest risk factor. With age, DNA damage accumulates continuously, increasing the chance of malignant transformation. The zebrafish has emerged as an important vertebrate model to study these processes. Key mechanisms such as DNA damage responses and cellular senescence can be studied in zebrafish throughout its life course. In addition, the zebrafish is becoming an important resource to study telomere biology in aging, regeneration and cancer. Here we review some of the tools and resources that zebrafish researchers have developed and discuss their potential use in the study of DNA damage, cancer and aging related diseases.

Keywords: zebrafish, DDR, aging, telomeres, cancer, genome maintenance, disease model, p53

INTRODUCTION

Dysfunctional DNA damage repair (DDR) underlies multiple diseases, including age-associated diseases and cancer. The zebrafish, a model organism exploited mostly in developmental studies, is now emerging as a powerful tool to study adult diseases, and scientists are exploring the potentials of this organism for studying how DNA damage dysfunctions impact human health and life span.

Scientists gathering at the 5th European Zebrafish PI meeting held in Trento on 20–23 March 2018 had a dedicated workshop to present their latest research in Aging, DDR and Cancer. This gave them the opportunity to plan the writing of an overview article that summarizes the state of the art in this field. This perspective article conveys our view on the advantages of zebrafish as a model vertebrate organism to study aging, DDR and cancer and sets up the stage for investigating the complex interplay of metabolic, immune and pathophysiological changes associated with these conditions.

In this perspective article, we summarize the recent advances using the zebrafish model for studying diseases with a major DNA damage component. We survey the tools to investigate the impact of DDR dysfunctions on diseases and review the contribution of the zebrafish telomerase mutant to the understanding of aging and its relation with cancer. Moreover, we report how technical advances in zebrafish disease modeling impacted our field, not only by

providing more technical resources, but also by furthering our understanding of the mechanisms of gene compensation following mRNA depletion and DNA repair following CRISPR-Cas9 genetic manipulations.

PERSPECTIVES IN STUDYING AGING AND CONGENITAL DISEASES LINKED TO AGING AND DDR IN ZEBRAFISH

Until now, aging research has mainly focused on single diseases. However, this approach does not address the fact that approximately 60% of people over 65 suffer from multiple diseases at the same time, termed multimorbidity (Tinetti et al., 2012; Fabbri et al., 2015). There is, therefore, the urgent need to understand the hallmark mechanisms that go awry with aging and promote and underlie multiple chronic diseases of the elderly.

One important hallmark of aging in humans is telomere dysfunction. Critically short telomeres cause cells to stop dividing, and either die by apoptosis or enter a state of "dormancy" termed senescence. Importantly, senescent cells accumulate aberrantly with aging in multiple organisms, including humans (Dimri et al., 1995), mice (Krishnamurthy et al., 2004), and zebrafish (Carneiro et al., 2016b) and there is strong evidence implicating telomere dysfunction and senescence in age-associated pathologies, such as atherosclerosis (Minamino and Komuro, 2007), arthritis (Price et al., 2002), liver cirrhosis (Wiemann et al., 2002), chronic obstructive pulmonary disease (Amsellem et al., 2011), and cancer (Campisi, 2013).

The zebrafish has recently emerged as a powerful complementary model to investigate the fundamental mechanisms of aging underlying disease. Like humans, and unlike most lab-mice, both naturally aged (Carneiro et al., 2016b) and telomerase mutant zebrafish, accumulate telomere-dependent replicative senescent cells in aged tissues (Anchelin et al., 2013; Henriques et al., 2013; Carneiro et al., 2016b), making the zebrafish telomerase mutant an excellent model of premature aging (see "Telomerase and TR" section below for a detailed discussion of the telomerase mutant).

Zebrafish age-dependent tissue degeneration occurs in a time- and tissue-dependent manner, and, for most tissues, it is anticipated and exacerbated in the absence of telomerase. There are critical tissues that age before others, such as the intestine and muscle, and this is tightly linked to telomere shortening and DNA damage in natural, wild type (WT) aging fish (Carneiro et al., 2016b). Importantly, this is reminiscent of the human scenario, where telomerase loss-of-function mutations, or mutations affecting telomere stability, lead to premature aging syndromes (termed progeria, Hofer et al., 2005; Alter et al., 2012).

In the past decades, the study of human monogenic accelerated aging disorders, such as ataxia-telangiectasia (A-T), Bloom's syndrome (BS), Cockayne syndrome (CS), Dyskeratosis congenita (DC), Fanconi anemia (FA), Rothmund-Thomson syndrome (RTS), and Werner syndrome (WS), has uncovered some commonalities in their etiology. One recurring theme appears to be impairment of one or more pathways related to DNA repair: RecQ class DNA helicases in BS, RTS and WS (de Renty and Ellis, 2017; Lu et al., 2017; Oshima et al., 2017), the homeostatic protein kinase ATM in A-T (Shiloh and Lederman, 2017) and at least some CS and FA gene products (Brosh et al., 2017; Karikkineth et al., 2017) have all been implicated in the maintenance of genomic stability.

Given the relatively large number of genes involved in monogenic aging disorders it is striking that up to date only a handful of *bona fide* mutations have been characterized in zebrafish: *brca2/fancd1*, *fancl* and *rad51/fancr* (Rodríguez-Marí et al., 2010, 2011; Botthof et al., 2017). Synthetic antisense morpholino (MO) knockdown of *ercc6* (Wei et al., 2015) and *fancd2* (Liu et al., 2003) have been also described, and TALEN and CRISPR mutants for *atm* (Thomas et al., 2014) and *ercc5/xpg* (China Zebrafish Resource Center [CZRC], 2017) have been reported, but not characterized in detail. As for almost all other genes, insertional and ENU mutants already exist at ZIRC and EZRC. Given how straightforward it is to generate novel alleles with CRISPR, it is likely that this apparent dearth of zebrafish aging disorder models will change soon.

A peculiar characteristic of zebrafish as a model for DDR-related conditions is that the impairment of this pathway often results in biased sex ratios. The first documented cases were described in *brca2/fancd1* and *fancl* mutants, both models for FA, where homozygous mutants all developed as males (Rodríguez-Marí et al., 2010, 2011). The sex bias was restored when *tp53* was also blocked, demonstrating that p53-dependent apoptosis has a role in the development of sex bias.

Recently, mutations in 19 genes related to the Fanconi anemia (FA) pathway were created (*fanca, fancb, fancc, fancd1/brca2, fancd2, fance, fancf, fancg, fanci, fancj/brip1, fancl, fancm, fancn/palb2, fanco/rad51c, fancp/slx4, fancq/ercc4, fanct/ube2t, faap100, and faap24*). Knockouts for 12 of these genes showed complete female-to-male sex reversal, and partial sex-reversal was seen in KOs of five more genes. Sex reversal in the case of fancp was Tp53-dependent, just as with the previously reported fancd1, fancl, and fancr mutants. And while mutant males and females were mostly fertile, fancd1, and fancj mutants showed partial and complete sterility, respectively (Ramanagoudr-Bhojappa et al., 2018). These results substantiate the role of the FA-pathway in the PGC-dependent sex determination process of zebrafish and provide further proof for the role of some DDR genes in germ-cell specification.

The germ line has an active role in zebrafish sex-determination (Siegfried and Nüsslein-Volhard, 2008). More precisely, primordial germ cells (PGCs) in the developing juvenile ovary have a key and most likely inductive role in gonad differentiation and their number is a deciding factor in sex-determination. Individuals with lower PGC number become male, whereas high PGC count generally results in females (Tzung et al., 2015). Therefore, the survival and expansion of the PGC population during early phases of development adds another layer of control to the complex polygenic sex determination system, characteristic for zebrafish (Liew et al., 2012).

With this foresight, the sex biased phenotype of *brca2/fancd1* and *fancl* mutants suggests that genes involved in DDR might have an important role for the survival and proliferation of PGCs

in the developing gonads. Defects in meiotic recombination do not automatically lead to PGC death, however, for instance *mlh1* mutant females and males are capable of producing eggs and sperm but resulting embryos are aneuploid (Leal et al., 2008). Also in the telomerase mutant, PGCs proliferation is impaired (Anchelin et al., 2013; Henriques et al., 2013) and telomerase has been reported to respond to mild DNA damage with an increased activation (Akiyama et al., 2013).

RECENT TECHNICAL ADVANCES IN DISEASE MODELING AND IMPACT ON DDR STUDIES

Previous large-scale mutagenesis approaches, such as the Zebrafish Mutation Project (ZMP) have created mutations in most of the relevant zebrafish genes, and these lines are available from major zebrafish stock centers ([1]Zirc and EZRC). Furthermore, with the adoption of TALEN-based (Sander et al., 2011; Bedell et al., 2012; Reyon et al., 2012) and CRISPR/Cas9-based genome editing technologies (Hruscha et al., 2013; Hwang et al., 2013a,b; Jao et al., 2013; Gagnon et al., 2014; Talbot and Amacher, 2014) for zebrafish, practically any research group can create loss-of-function alleles for the gene(s) of interest.

With regards to DNA repair, it is also noteworthy that over the past couple of years, novel genetic and immunohistochemistry-based tools have been developed that can help to understand the prevalence of different DNA double-strand break (DSB) repair pathways during the repair process, for instance GFP based constructs are available that can report NHEJ, MMEJ, SSA, and HR-based repair (Liu et al., 2012; He et al., 2015).

While during the previous decades of zebrafish research, MOs have been the tool of choice for creating loss-of-function phenotypes (Nasevicius and Ekker, 2000), recently, several studies have highlighted the limitations of this approach (Schulte-Merker and Stainier, 2014; Kok et al., 2015; Stainier et al., 2017). MOs are extremely stable and can be easily delivered into embryos at early stages, where they interfere with translation or splicing. However, the effect of MOs is only transient, therefore they are usually injected in molar excess to have a longer lasting effect. The injection of large amounts of synthetic molecules could be the reason why MOs often elicit strong, specific p53-dependent effects (Robu et al., 2007) and the activation of an innate immune response (Gentsch et al., 2018). Given that DNA damage repair is inseparably linked to p53 activation, it is not surprising that MOs have not been widely adopted in areas of research where DNA damage responses (DDR) play a central role, such as cancer and aging.

Bona fide mutants offer a more promising avenue for research modeling diseases related to DDR. One caveat of the genetic engineering approach is that, in zebrafish, mutations caused by genetic engineering manipulations often result in altered mRNA processing (Anderson et al., 2017) or trigger genomic compensation effects through non-sense mediated decay (NMD, Rossi et al., 2015; El-Brolosy and Stainier, 2017) ultimately failing to induce a strong phenotype. New research suggests, however, that these problems can be circumvented with the efficient targeting of the promoter region (El-Brolosy et al., 2018). These data are likely to be particularly useful in the functional analysis of aging-related genes.

[1]Zirc: http://zebrafish.org/fish/lineAll.php, EZRC: https://www.ezrc.kit.edu

TELOMERASE: THE STATE OF THE ART

The telomerase zebrafish mutant has revealed a role for telomeres and telomerase in aging and disease in zebrafish (Carneiro et al., 2016a). Compared to common laboratory mice that possess very long telomeres (40–150 kb), zebrafish telomere length is similar to human telomeres (5–15 kb). Also like humans, telomere length and telomerase expression decrease over time in zebrafish. Telomerase deficient zebrafish ($tert^{hu3430/hu3430}$ *or* $tert^{-/-}$) have shorter telomeres, premature aging phenotype and reduced lifespan. These defects do not occur all at the same time. Strikingly, the majority of tissue dysfunction phenocopies the events occurring during natural zebrafish aging (Anchelin et al., 2013; Henriques et al., 2013; Carneiro et al., 2016b). Over natural aging, the zebrafish intestinal epithelium is one of the first tissues to show gradual DNA damage response activation (53BP1 and γH2AX foci associated with telomeres), increased onset of apoptosis and senescence, and functional defects. Remarkably, telomere shortening in $tert^{-/-}$ mutants anticipates these alterations in this tissue. However, other proliferative tissues, such as testis or kidney marrow showed altered phenotypes independently of significant telomere shortening. Nevertheless, absence of telomerase has a clear deleterious effect in the adult zebrafish tissues. Thus, in absence of visible telomere shortening in some tissues, lack of telomerase does have a clear impact on their functions, resembling degeneration observed in old age (Carneiro et al., 2016b). This leaves open the hypothesis that, rather than just elongating short telomeres, presence of telomerase may be required for the regenerative capacity of adult organs. In agreement with this idea, telomerase was shown to have a non-catalytic role in hematopoietic cell differentiation (Imamura et al., 2008) and in DDR (Akiyama et al., 2013).

Similarly, also the RNA component (*TERC/TR*) of the telomerase holoenzyme has a non-canonical role in hematopoiesis. DC is a hereditary disease caused by defects in telomere maintenance, and is due to mutations in telomerase components or in telomere-stabilizing components. DC is characterized, in 85% of cases, by cutaneous defects and premature death due to failure in hematopoiesis and immunodeficiency. Mutations in *TERT* (catalytic subunit) and *TERC/TR* (RNA component) are responsible for the dominant autosomic form of DC. Although 90% of patients with DC have problems with the production of three types of blood cells, and all have telomere shortening, the incidence of aplastic anemia (AA), myelodysplastic syndromes (MDS) and cancer is greater in patients with mutations in *TERC/TR*. However, the variability and severity of the symptoms due to the different mutations cannot be accounted for by the sole influence on telomere length (Vulliamy et al., 2011). Indeed, genetic inhibition of *terc* in zebrafish results in neutropenia and monocytopenia

(Alcaraz-Pérez et al., 2014), similarly to DC patients. This defect is fully independent of both telomerase activity and telomere length. *Terc/tr* is expressed at very high levels in isolated neutrophils, whereas *tert* is undetectable, suggesting that *terc/tr* has a non-canonical function in these cells. Human *TERC* physically interacts with 2,198 sites throughout the human genome by recognizing the target sequence CCACCACCCC (Chu et al., 2011). These findings suggest that *TERC/TR* has an additional role to that as a telomerase component, and acts as a long non-coding RNA which regulates myelopoietic gene expression, revealing a new target for therapeutic intervention in DC patients.

During aging, short telomeres are deprotected and recognized as DNA damage. As a consequence, both WT and $tert^{-/-}$ mutants zebrafish accumulate γH2AX foci at telomeres with aging, mainly in gut and muscle (Carneiro et al., 2016b). The formation of γH2AX telomeric foci correlates with telomere shortening, supporting the idea that short telomeres are sensed as DNA damage and activate the DDR. Damaged DNA is recognized by the MRN complex, which recruits the kinases ATM and ATR mediating H2AX phosphorylation. In addition to γH2AX foci, gut and muscles of both aged WT and $tert^{-/-}$ animals showed a significant reduction of proliferating cell nuclear antigen (PCNA) staining and an increase of senescence markers (e.g., senescence-associated β-galactosidase staining), indicating that telomere shortening leads also to reduction of proliferation and induction of senescence (Anchelin et al., 2013; Henriques et al., 2013; Carneiro et al., 2016b). Decrease of proliferation and accumulation of senescent cells cooperate to the disruption of tissues homeostasis with aging. These effects are mediated by the activation of p53, as the combination of $tert^{-/-}$ and $tp53^{-/-}$ mutations rescues the replication rate and partially abolish senescence in the gut (Anchelin et al., 2013; Henriques et al., 2013).

Cells with critically short telomeres rely on either of two mechanisms to avoid cell proliferation. They can either engage a cell death program, through a p53-dependent expression of pro-apoptotic proteins, such as PUMA (Wang et al., 2007), or they can irreversibly arrest the cell cycle, upregulate a second CDKi, *p16Ink4a*, and become senescent (Stein et al., 1999). To date, it remains largely unclear what determines if a cell with short telomeres undergoes senescence or apoptosis. Evidence suggests that most cells are capable of both (Campisi, 2007). Given that both outcomes are initiated by the same type of stimulus and involve the same type of molecular players, this raises the question of how do damaged cells, in an organism, decide whether to continue living in a dysfunctional state or die.

Cancer may result from the occurrence of DNA damage that generates oncogenic events, bypass senescence and apoptosis and activates telomere maintenance mechanisms (TMMs), to allow proliferation and overcome telomere attrition (Reddel, 2014). The study of TMMs in zebrafish cancer models could shed light on the development of this important cancer hallmark. Defining which TMM is adopted by different cancer types, whether overexpression of telomerase (usually linked to mutations or positive regulation at the level of the promoter region) or Alternative Lengthening of Telomeres (ALT), can support diagnosis (mesenchymal and pediatric cancer being more prone to ALT, Apte and Cooper, 2017), prognosis (i.e., Alt+ glioblastomas in adults have a better prognosis, Hakin-Smith et al., 2003) and responses to anti-telomerase therapy (Agrawal et al., 2012). A zebrafish model of brain cancer (Mayrhofer et al., 2017) shows progressive induction of ALT (Idilli et al., unpublished) and recalls the prevalence of this TMM in pediatric brain tumors (Abedalthagafi et al., 2013). One of the hallmarks of ALT is the accumulation of DDR markers at telomeres, which suggests that the repair machinery may have a role in the homologous recombination and chromatid exchange occurring during ALT (Koschmann et al., 2016). Here, the cooperation between the activation of telomere maintenance mechanisms and DNA damage signaling leads to a predominance of surviving and proliferative signals, ensuring the survival and expansion of cancer cells.

HOW TO STUDY DDR IN ZEBRAFISH?

With the mechanisms and signals of DDR conserved from yeast to mammals, it is not surprising that the reports so far indicate a strict conservation of the molecular players in DDR between zebrafish and humans (see first section). Zebrafish are members of the Teleostei infraclass, and its ancestors underwent an additional round of whole-genome duplication (WGD) called the teleost-specific genome duplication (TSD) (Meyer and Schartl, 1999). Comparison to the human reference genome shows that approximately 71.4% of human genes have at least one obvious zebrafish ortholog and reciprocally, 69% of zebrafish genes have at least one human ortholog (Howe et al., 2013). Interestingly, of the 648 DDR-associated genes, only 70 were found to be duplicated (**Supplementary Table S1**). Although the zebrafish genome has no *BRCA1* ortholog, it has an ortholog of the *BRCA1-associated BARD1* gene, which encodes an associated and functionally similar protein and a *brca2* gene. Several studies found that zebrafish is a suitable animal model to study DDR.

The DDR response can be studied from within minutes upon DNA damage (recruitment of proteins, foci formation) to hours (residual DNA damage, apoptosis) and days (morphological changes) up to months later (tumor formation). When evaluating the expression of genes involved in the DNA repair system, time kinetics experiments are important – this parameter has to be taken into account and may vary depending on the DNA damage reagent exposed to, or the type of damage to be restored (e.g., Sandrini et al., 2009). We mainly focus here on DNA DSB repair.

Within the first minutes after induction of DNA damage, a wide range of proteins are recruited to the damaged site. This damage can be visualized through immunohistochemistry. Subsequently, after damage recognition, repair proteins are recruited. For example, dividing cells will repair DNA DSB in part through Homologous Recombination (HR), which can be visualized by Rad51 immunostaining. Rad51 foci formation is an important event in HR and defects in genes upstream in this pathway may lead to decrease or even absence of Rad51 foci (Vierstraete et al., 2017).

Unfortunately, there is still a lack of antibodies that can be used reliably in zebrafish, and good DNA repair protein antibodies are rare. Phosphorylated H2AX has been exploited by numerous groups and can be used to visualize and quantify DNA damage (Santoriello et al., 2009; Pereira et al., 2011, 2014; Francia et al., 2012; Drummond and Wingert, 2018), but does not show the repair pathway(s) that are used. There are only sporadic reports on other important DNA repair proteins; Fernández-Díez et al. (2018) published an immunostaining for p53BP1, which promotes non-homologous end-joining-mediated DSB repair while preventing homologous recombination, but signals appeared mainly cytoplasmic, however, Henriques and Ferreira (2012) reported nuclear staining. Here, higher resolution analysis and costaining with Rad51 to confirm the occurrence of HR would be beneficial.

Furthermore pAtm has been detected on tissue sections, but costaining with other DDR proteins has not been shown (Santoriello et al., 2009). Besides immunohistochemical stainings, qPCR, RNAseq and Western blotting provide quantitative information about up- or downregulation of expression and proteins. Although these techniques are (semi-) quantitative, they do not allow visualization of the localization of the damage (e.g., Zheng et al., 2018).

Other assays are "in development," the van Eeden group fortuitously generated a GFP loss of heterozygosity (LOH) reporter system based on a strong inhibition of Hypoxia Inducible Factor (HIF) by the presence of the Vhl protein (Santhakumar et al., 2012); inactivation of the remaining functional copy of *vhl* in heterozygous (*vhl*/+) cells leads to activation of HIF which in turn activates a sensitive downstream HIF reporter: *phd3:eGFP*. In this system the *vh*l gene is therefore used as a detector of gene damage, where it is expected to be representative of the entire genome, every cell in the embryos that have lost VHL function after a particular treatment will express GFP. This system detects all forms of damage capable of inactivating the gene; chromosome loss, base changes, indels. Thus, it cannot distinguish between different forms of genome stability defects.

Comet assays using larvae have also been reported (Jarvis and Knowles, 2003) as they are popular in toxicology studies. At least one group has used a comet assay to show effects of genetic mutations on levels of DNA damage (Lim et al., 2009). Detailed protocols for several techniques were published by the Amatruda Lab (Verduzco and Amatruda, 2011).

After protein recruitment, repair is performed through either non-homologous end-joining (NHEJ), homologous recombination (HR) and single strand annealing (SSA). Repair through these pathways in zebrafish embryos can be analyzed by utilizing specialized vectors, such as those developed by Liu et al. (2012). Most genetic tools for DSB analysis rely on fluorescence-based systems, where efficient repair following a nuclease-triggered DSB break in the GFP coding sequence leads to functional fluorescent protein. Visualizing efficient repair with such tools is easiest during early stages of development, when essentially all cells can be imaged at once. However, it is important to keep in mind that during these earliest stages of embryogenesis alternative end joining (alt-EJ) is the favored repair mechanism as demonstrated recently by the characterization of *polq* mutants (Thyme and Schier, 2016). Furthermore, although the experiments with these reporter constructs appear technically easy to perform (DNA micro-injection in freshly fertilized eggs) the resulting uneven distribution of the DNA often leads to high variance in the response, and high concentrations of DNA will lead to strong developmental abnormalities.

The lack of good reporter systems for DDR could be alleviated by the use of CRISPR mediated genome modification to tag endogenous proteins involved in DDR, thus circumventing the scarcity of verified antibodies in zebrafish. Unfortunately such precise genome editing has so far been difficult in fish, but novel methods like tethering the repair template to the Cas9 enzyme, using Biotin-Avidin systems (Gu et al., 2018) or Cas9 related enzymes that do not destroy their own binding site (Moreno-Mateos et al., 2017) hold a lot of promise.

Faulty repair of DSBs can lead to the presence of micronuclei in dividing cells, which can be assessed with the micronucleus assay (Pereira et al., 2011; Moreno-Mateos et al., 2017). More micronuclei indicate higher doses of specific DNA damage agents, or higher sensitivity to a specific agent (Oliveira et al., 2009) due to one or more defects in the DDR pathway.

Accumulation of DNA damage might lead to cell death by apoptosis. For this, the TUNEL assay can be applied, on both whole mount zebrafish embryos, as well as sections of embryos or adults. Alternatively, the less sensitive acridine orange assay can be used (Reimers et al., 2006; Usenko et al., 2007).

Defects in the DDR pathway can lead to morphological aberrations in developing embryos. For example, exposure to amifostine will radiosensitize embryos, leading to morphological perturbations during development (Geiger et al., 2006). Interestingly, *tp53* deficiency may revert the effect of irradiation:

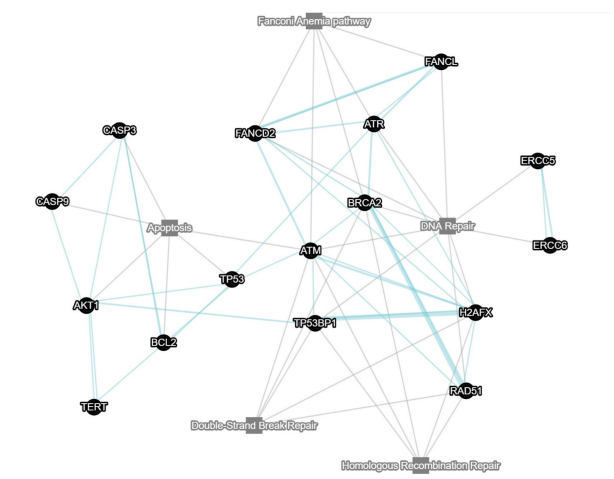

FIGURE 1 | Network displaying key genes involved in apoptosis, DSB repair, Fanconi anemia pathway and homologous recombination. Each node is clickable and linked to a pathway in ZFIN (write to the corresponding author for a hyperlinked version of the figure). Gray lines indicate which pathway a certain gene belongs to. Blue lines indicate pathway associations between genes. Multiple blue lines indicate data from multiple sources. Network was constructed using GeneMANIA.

for example, in irradiated zebrafish embryos *tp53* deficiency does not lead to altered morphological features and actually increases survival, when compared to irradiated wild type controls (Duffy and Wickstrom, 2007).

Accumulation of mutations caused by faulty DDR can lead to genomic instability and ultimately tumorigenesis. For example, *brca2* mutant fish form neoplasia at later stages in life (Rodríguez-Marí et al., 2010). In combination with *p53* deficiency, an accelerated tumorigenesis process can be observed, proving that Brca2 conserved its tumor suppressor role in zebrafish (Shive et al., 2010).

In conclusion, the DDR is a complex process that starts seconds after DNA damage, with effects potentially visible up to months after initial damage. All of the different aspects of DNA damage and (faulty) repair can be observed through a number of different methods in zebrafish. This demonstrates that zebrafish is a powerful system to investigate the DDR response.

CONCLUSION AND OUTLOOK

Research on DNA damage repair dysfunctions and their impact on aging and cancer is progressing fast and the zebrafish offers an excellent toolbox for these studies, particularly for *in vivo* observation of whole organism effects. Advantages over the mouse system include the similarities in telomere length and maintenance mechanisms (Anchelin et al., 2013; Henriques et al., 2013; Carneiro et al., 2016a), the toolbox for genetic manipulation (reviewed in Mayrhofer and Mione, 2016), the *in vivo* assays, including fluorescent reporters and high throughput chemical screens for drugs affecting DDR.

The number of models (including mutant and reporter lines) is growing (see **Figure 1** for a network of DDR-related zebrafish tools, linked to ZFIN pathway database). This is mostly due to the recent addition of somatic transgenics (reviewed in Idilli et al., 2017; Callahan et al., 2018) and somatic knock outs (Ablain et al., 2015; Di Donato et al., 2016). Reporters of DDR and different DNA repair systems are being perfected, while more tools for detection (antibodies, techniques, etc.) are being tested and protocols developed that can benefit our field. Our understanding of the complex relationships between DNA damaged cells and the microenvironment, including the important role of the immune system and damage signals outside of the affected cells is developing and so is our ability to detect and interpret them.

We are confident that zebrafish research will soon be able to provide unique and important contributions to the wider and distinguished community of DDR scholars, with a perspective that embraces molecular events at a tissue level throughout the life-time of a vertebrate.

AUTHOR CONTRIBUTIONS

All authors participated in conceptualization and writing of this perspective articles. JV prepared the figure and the table. MM drafted the article and assembled the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00178/full#supplementary-material

TABLE S1 | List of human and zebrafish genes involved in DDR, and the corresponding tools (mutant, antibody, etc.) available for zebrafish research. In the second page of the excel file, gene ontology classification of the same gene.

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Comparative Transcriptomics Between Zebrafish and Mammals: A Roadmap for Discovery of Conserved and Unique Signaling Pathways in Physiology and Disease

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Zebrafish is a small fresh-water species widely used as a vertebrate model in human physiology and pathologies. In addition, the zebrafish genome has recently been sequenced providing a plethora of opportunities for comparison with the genomes of other animals, such as rodents and humans (Howe et al., 2013). However, there is an emerging need for a concise synthesis of the literature findings and approaches used in comparing transcriptomes of mammals with that of zebrafish. In this opinion article, we first focus on features which make zebrafish genome and transcriptome unique and invaluable for comparative studies. Exploring research findings under multiple topics such as comparative physiology, toxicology, and cancer reveal important aspects of functional conservation among vertebrates and effective research pipelines for comparative transcriptomics studies.

FEATURES OF ZEBRAFISH GENOME/TRANSCRIPTOME

The zebrafish genome is similar to the human genome with respect to the number of chromosomes (25 vs. 23 pairs, respectively) (Postlethwait et al., 2000) and of protein coding genes (Howe et al., 2013). On the other hand, a distinguishing aspect of the zebrafish genome, as that of most fish genomes, is the extra whole-genome duplication (WGD) event that has been estimated to occur at least 320 million years ago (Hoegg et al., 2007). As a result, the zebrafish genome contains paralogous pairs of genes, exhibiting divergent or complementary functions, for a set of mammalian orthologous genes (Laprairie et al., 2016). The divergence of zebrafish paralogous genes with respect to their methylation pattern in correspondence to expression levels in gametes and early embryos has been studied (Zhong et al., 2016). Two thousand four hundred and forty paralogous pairs of genes have been identified and more than 75% of these genes are found to be under purifying selection. In addition, around 600 duplicate pairs in zebrafish exhibit divergent promoter methylation as well as a negative correlation between levels of promoter DNA methylation and mRNA expression (Zhong et al., 2016). Future studies are still needed to better understand the expression patterns of zebrafish paralogs in comparison to human orthologous genes. In this context, we are developing a web-based tool called *CompariZome* for comparative statistical examination of public human and zebrafish expression datasets extracted from GEO repository (Edgar et al., 2002; Lopes et al., 2016).

IDENTIFICATION OF CONSERVED FUNCTIONAL PROCESSES BY COMPARATIVE TRANSCRIPTOMICS

Based on previous studies, two effective and complementary approaches exist for comparative transcriptomics between different species: comparison of significantly modulated (a) genes; and/or (b) pathways (**Figure 1**). We briefly explain them below with selected examples from the literature.

For any comparative study dealing with zebrafish and mammalian expression, multiple data sources (Edgar et al., 2002; Kolesnikov et al., 2015) need to be searched for a specified biological question (**Figure 1**). Upon obtaining differentially expressed genes (DEGs), the next crucial step is to identify the pathways enriched in the gene lists of each species, separately. A commonly used web-based software is DAVID (Huang da et al., 2009a,b; Schartl et al., 2012; Sucularli et al., 2016), used to identify the enriched functional terms, e.g., KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways or gene ontology (GO) (**Figure 1**). Gene Set Enrichment Analysis (GSEA), which was developed to rank significantly enriched biological/cellular processes based on differentially expressed genes (Subramanian et al., 2005), is also widely used (Zheng et al., 2011; Yildiz et al., 2013). One of the most important steps in comparative transcriptomics is orthology mapping (**Figure 1**). This can be performed using different resources that include Ensembl Biomart (Saraiva et al., 2015; Shih et al., 2015); Unigene clusters (Lam et al., 2006; Zheng et al., 2011, 2014); and Homologene (Driessen et al., 2015). Statistics based on Fisher's Exact test (Sucularli et al., 2016) or GSEA (Zheng et al., 2011) are essential elements to reveal significant associations between different species in terms of the selected genes, pathways or enrichment terms (**Figure 1**). Moreover, the visualization of such associations between different species requires the use of multivariate exploratory techniques among which Principal Component Analysis (PCA) and heatmaps (Zheng et al., 2014; Saraiva et al., 2015; Tarifeño-Saldivia et al., 2017), and/or coinertia (COA) plots (Kaya et al., 2011) are commonly preferred (**Figure 1**). Representation by Venn diagrams is another way to show the intersection of gene lists/pathways between species (Lam et al., 2006; Tarifeño-Saldivia et al., 2017). The abundance of one-to-many orthology relationships between zebrafish and mammals, however, complicates orthology mapping and hence may restrict downstream analyses to the use of only one-to-one orthologous pairs that exhibit moderate-high sequence similarity (Saraiva et al., 2015; Davison et al., 2017; Tarifeño-Saldivia et al., 2017). Finally, the development of online tools and use of meta-analysis methodologies can lead to easier access to data/analyses and more robust conclusions on the degree of functional conservation between zebrafish and mammals (Kaya et al., 2011; Sucularli et al., 2016; Davison et al., 2017).

To demonstrate different aspects of the pipeline described in **Figure 1**, we provide the below examples of comparative transcriptomics studies focusing on physiology, toxicology, and cancer.

PHYSIOLOGICAL PROCESSES AND DISEASE RELEVANCE

Inter-species transcriptome analysis is highly useful to discover common, as well as unique, signatures in a diverse set of physiological and relevant pathological conditions. For example, RNA-sequencing of zebrafish heart has led to the identification of 96 chamber-specific genes, 68 of which possess orthologs in humans. An OMIM database search reveals the 25 of these 68 genes are disease-associated, and five of them are specifically involved in cardiac diseases (Singh et al., 2016). Similarly, 49 orthologs of 51 known human dilated cardiomyopathy-associated genes are found to be conserved in zebrafish, further promoting the use of the zebrafish model for human cardiac disease research (Shih et al., 2015). However, 19 of these genes have two or more paralogs in zebrafish, pointing to one of the complexities that needs to be resolved for studying disease association between zebrafish and humans. Currently, the selection of a gene that is enriched within the specified tissue emerges as a potential strategy to prioritize one or more of the paralogous genes for future studies (Shih et al., 2015).

Maternal to zygotic transition (MZT), during which maternally provided RNA and proteins are replaced by those produced by the zygote alone, is a shared phenomenon among animals (Bensaude et al., 1983; Harvey et al., 2013). The comparison of expression changes in MZT of *Lymnaea stagnalis* with the published data from several arthropod, nematode, urochordate, and chordate species including zebrafish, has indeed led to the extraction of a shared expression signature. An analysis of orthologs common to all species (identified using tBLASTx) reveals that maternal transcripts are often enriched with housekeeping functions and are involved in nucleotide binding, cell cycle progression, and protein degradation (Liu et al., 2014). This extensive comparative transcriptomics study of embryogenesis shows the power of such an approach and contributes to our understanding of the patterns of embryonic conservation among vertebrates and invertebrates. Future comparative transcriptomics studies between zebrafish and mammals can help reveal the functional divergence of paralogous genes in zebrafish MZT.

Other areas receiving attention in comparative transcriptomics include regulation of the circadian clock between zebrafish and mouse (Boyle et al., 2017) and the degree of conservation of ovulation in humans, mice, and zebrafish (Liu et al., 2017). Zebrafish has also become a useful model for revealing the resemblance between zebrafish swim bladder and mammalian lung (Zheng et al., 2011), renal distal convoluted tubules of zebrafish and mouse (Sugano et al., 2017), pancreatic cell populations of zebrafish, mouse, and human (Tarifeño-Saldivia et al., 2017), and olfactory systems of mouse and zebrafish (Saraiva et al., 2015). In addition, the conservation of immune response between zebrafish and mammals has been shown using meta-genomics of gut microbiota (Davison et al., 2017) and with respect to IFN gamma signaling (Filiano et al., 2016).

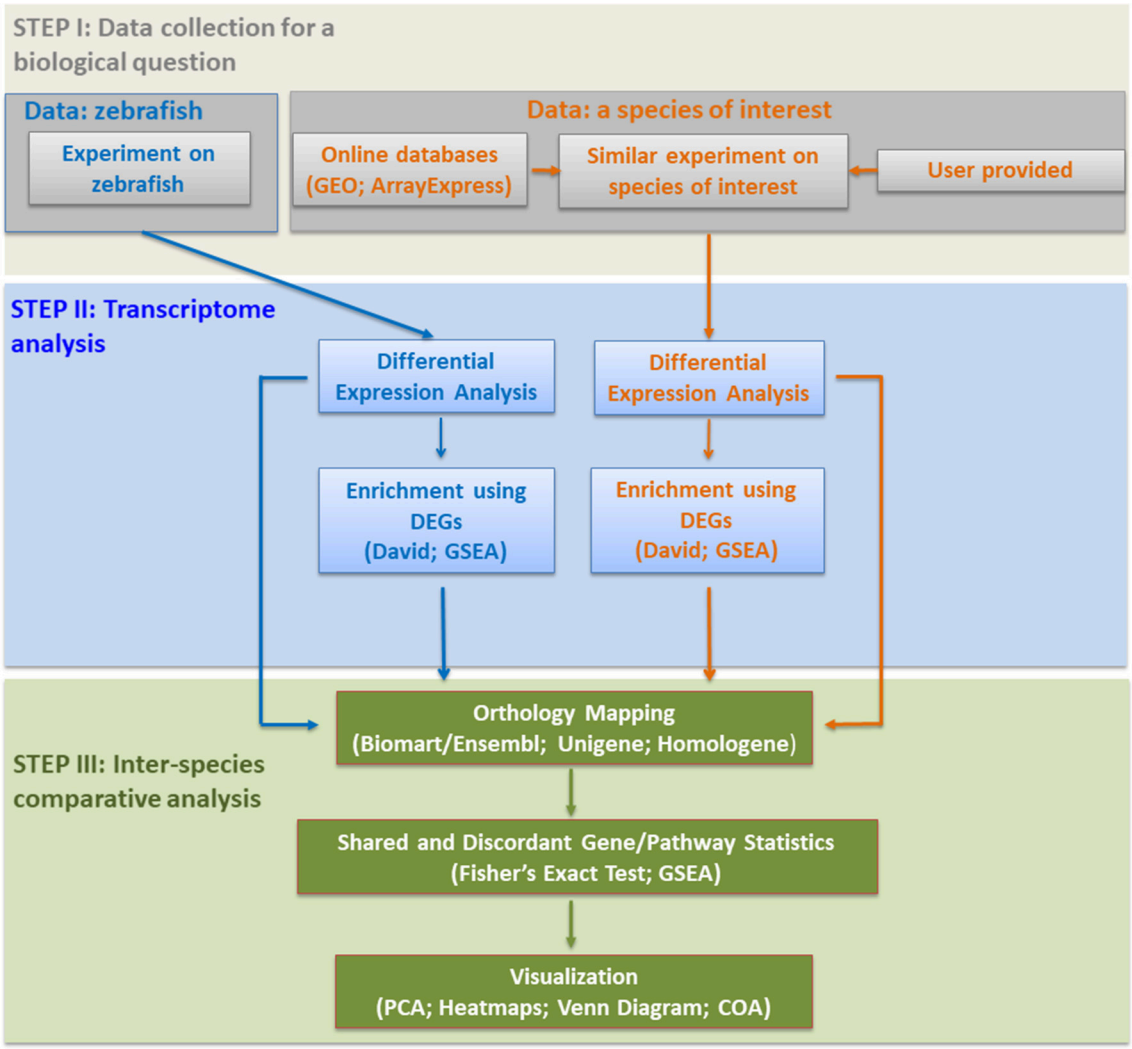

FIGURE 1 | A research pipeline for comparative transcriptomics between zebrafish and mammals.

CANCER

Cancer, one of the complex human diseases threatening large numbers of people across different age groups involves the deregulation of expression of genes relevant to cell proliferation, apoptosis, and senescence (Jones and Baylin, 2007). Hence, understanding how similarly genes and signaling pathways are modulated between zebrafish and humans can help identify novel therapeutic gene/protein targets and signaling pathways in cancer.

For example, comparisons between medaka, zebrafish, and human melanoma transcriptomes using RNA sequencing have identified more downregulated than upregulated genes that are commonly modulated between fish and human samples (Schartl et al., 2012). Another study focusing on the comparison of genetic alterations in zebrafish and human melanoma (exhibiting congruent BRAF mutation and P53 deletions) points to lower rates of UV-induced mutations in zebrafish while providing novel candidate genes to pursue in drug-resistant human melanomas (Kansler et al., 2017). Similarly, a study performed with an inducible zebrafish embryonal rhabdomyosarcoma model has identified the shared upregulation of *MYF5* mRNA in zebrafish and human expression datasets based on GSEA comparisons (Langenau et al., 2007). Further transcriptomics and mechanistic studies in zebrafish and human rhabdomyosarcomas have demonstrated the potent role of the transcription factors MYF5 and MYOD in the development and growth of muscle tumors (Tenente et al., 2017).

Using statistical approaches, such as binomial tests and GSEA has shown that zebrafish liver cancer most significantly resembles human liver cancer in terms of changes in gene expression (Lam et al., 2006). Further studies demonstrate that deregulations in several cancer-related transcription factors, MYC, E2F1, YY1, and STAT, are commonly observed in zebrafish and human

liver cancers (Ung et al., 2009). It has also been shown that different human liver cancer subtypes can be paired successfully with *xmrk*-, *kras*-, and *myc*-induced zebrafish tumors using comparative transcriptomics at the level of gene as well as at the level of cellular pathways (Zheng et al., 2014). The authors have found that the human orthologs of the genes upregulated in zebrafish liver tumors are also upregulated in different stages of hepatocellular carcinomas (HCC); and altogether, three types of zebrafish models represent almost half of the human HCC samples (Zheng et al., 2014).

TOXICOLOGY AND PHARMACOLOGY

Toxicology and pharmacology are the two relevant fields in which the comparison of zebrafish and mammalian transcriptomes provide invaluable information for translational biology. Liver cells, mainly due to their ability for detoxification, are frequently expression-profiled in human toxicology studies where comparisons with zebrafish liver or embryos/larvae exposed to drugs are made (Driessen et al., 2015). For example, mercury can induce early and late response genes *in vivo* in zebrafish liver and *in vitro* in human liver carcinoma cells. In addition to the identification of concordant upregulated (proteasome and DNA damage response) and downregulated (mitochondrial fatty acid beta oxidation, electron transport chain, nuclear receptor signaling) pathways, this study also reveals discrepancies between transcriptomes of zebrafish and humans in response to mercury, signifying caution in such comparative studies (Ung et al., 2010). Gene-centric analyses in toxicology also exist; for example, H2O2 exposure in zebrafish larvae and in human keratinocytes shows that the expressions of 41 genes are modulated in common between the two species (Lisse et al., 2016). Scatterplots of logarithmically transformed fold expression changes (LogFC) from different expression datasets help test similarity in expression profiles between experiments and have been used in recent comparative transcriptomics literature (Yildiz et al., 2013; Lisse et al., 2016).

Zebrafish is a highly preferred model for testing drugs commonly used in treating human diseases. For example, a study performed with the immunosuppressant drug, cyclosporine, at the levels of gene, pathway and transcription factors, strengthen the confidence on the effective use of zebrafish embryos for comparative toxicogenomic studies (Driessen et al., 2013). Pathway-focused comparative transcriptomics analysis of rapamycin exposure, *in vitro*, between a zebrafish embryonic fibroblast cell line and five mouse cell lines also demonstrate the presence of a strong and positive inter-species association between expression profiles (Sucularli et al., 2016). Furthermore, the comparison of pathways/gene ontology terms via meta-analysis pipelines, as well as the stringent use of association tests, can be listed among the strategies to increase the robustness of findings in comparative transcriptomics (Sucularli et al., 2016). Using *in vivo* models in addition to *in vitro* comparisons may further lead to more comprehensive findings since a larger number of shared genes and stronger enrichment scores can be uncovered with *in vivo* studies (Driessen et al., 2015).

CONCLUSION

Herein, we have provided a brief pipeline from hypothesis testing to the visualization of results for comparative transcriptomics studies between zebrafish and other animals based on the existing literature (**Figure 1**). We also reiterate the need to focus on the employment of effective strategies to deal with paralogous gene expression in comparative studies. Moreover, the use of meta-analysis in comparative transcriptomics is likely to increase the robustness of conclusions in regard to the functional conservation of genes and pathways between zebrafish and mammals. Similarly, the development of web-based analysis tools will enhance accessibility to comparative transcriptomics data and will allow researchers to make better decisions while selecting genes/pathways to target for further mechanistic studies or translational research.

AUTHOR CONTRIBUTIONS

HS and OK contributed to the ideas and the literature search of the manuscript, wrote the manuscript, and revised it.

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Resident Immunity in Tissue Repair and Maintenance: The Zebrafish Model Coming of Age

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The zebrafish has emerged as an exciting vertebrate model to study different aspects of immune system development, particularly due to its transparent embryonic development, the availability of multiple fluorescent reporter lines, efficient genetic tools and live imaging capabilities. However, the study of immunity in zebrafish has largely been limited to early larval stages due to an incomplete knowledge of the full repertoire of immune cells and their specific markers, in particular, a lack of cell surface antibodies to detect and isolate such cells in living tissues. Here we focus on tissue resident or associated immunity beyond development, in the adult zebrafish. It is our view that, with our increasing knowledge and the development of improved tools and protocols, the adult zebrafish will be increasingly appreciated for offering valuable insights into the role of immunity in tissue repair and maintenance, in both health and disease throughout the lifecourse.

Keywords: adult zebrafish (Danio Rerio), tissue resident immunity, tissue repair and regeneration, ageing, gut, heart, retina, brain

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INTRODUCTION

It is becoming increasingly clear that the innate and adaptive immune systems play crucial roles in tissue maintenance and repair during health and disease. Studies in animal models are crucial to identifying complex functions of immunity in sometimes surprising aspects of biology. For example, it was discovered relatively recently that macrophages, previously thought of as purely cell debris-eating machines, promote fibrosis and scarring in mammals after an injury. Further, they have been identified as being crucial for tissue regeneration, directly communicating with epithelial cells in a variety of vertebrate models, reviewed elsewhere (Pott and Hornef, 2012; Ginhoux and Guilliams, 2016). Zebrafish are well placed as a model to decipher the complex functions of immune cells in tissue regeneration and other disease related processes due to their genetic tractability and the ease of live imaging. However, the majority of studies are largely limited to embryonic and larval stages due to their rapid, external development, genetic tractability, and transparent embryonic development. However, to best study tissue regeneration and human disease, fully differentiated tissues and organs are required. Here we put forward the adult zebrafish as a relevant and valid model for studying tissue immunity in health and disease throughout the whole animal's lifecourse. We highlight the recent advances in our knowledge of tissue immunity in adult zebrafish and the best tools currently available to study it. It is our view that our increasing knowledge and the ongoing development of tools and protocols are already making the adult zebrafish a valuable model

offering insights into the role of immunity in tissue health throughout the lifecourse, and this model is likely to become more and more eminent in the future of the field, if we push forward for the continuous development of tools.

Ontogeny of Tissue Immunity in Zebrafish

It wouldn't make sense to delve into adult zebrafish tissue immunity before addressing their ontogeny. Unfortunately, though, this is where the problem starts. In the mouse, the most commonly used vertebrate immunology model, the origin of tissue resident or associated immune cells is generally well described, exemplified in (Bain et al., 2014; Ginhoux and Guilliams, 2016; Ferrero et al., 2018), whereas in the zebrafish, our knowledge is still largely incomplete.

In mice, extensive work over decades has shown that most tissues have resident immune cells, both innate (mainly macrophages and NK cells, depending on the tissue) and adaptive (T- and B-cells). The different flavors within these immune cells vary depending on tissue and disease status (Mowat et al., 2017; White et al., 2017). Amongst these, we know the most about macrophages. In mice, tissue-resident macrophages seem to derive from embryonic precursors that populate most tissues during embryogenesis, becoming a specialized, tissue-resident, self-renewing population in the adult (Hoeffel et al., 2012; Hashimoto et al., 2013; Yona et al., 2013; Hoeffel and Ginhoux, 2015). A well-known exception, at least in mice, is the gut. Recent work has shown that the macrophage population in the adult mouse gut is constantly re-populated by circulating monocytes, which then differentiate into mature macrophages and are maintained *in situ* (Bain et al., 2014; Bain and Mowat, 2014a,b). In zebrafish, our knowledge is more limited. Nevertheless, recent work by Alemany et al. has identified distinct signatures in resident immune cells in the adult zebrafish, using sophisticated single-cell sequencing and tracking analysis (Alemany et al., 2018). Their work shows that haematopoietic cells in the kidney marrow derive from a small set of multipotent embryonic progenitors. Surprisingly, the authors indicate that resident immune cells in the fin do not originate from haematopoietic stem cells and instead seem to arise either from epidermal and mesenchymal transdifferentiation, or from ectodermal ancestors similarly to mesenchymal cells. The origin and maintenance of resident immune cells remains to be fully elucidated in other organs such as the gut. Notwithstanding, the zebrafish model is also making key contributions to the understanding of tissue immunity in vertebrates, thanks to an impressive availability of transgenic reporter lines for different immune cells/inflammatory markers (see **Table 1** for details). Seminal work has shown that, like in other vertebrates, zebrafish have a fully functional tissue-associated immunity, including T-cells, B-cells, macrophages, neutrophils, eosinophils, and mast cells (Moss et al., 2009; Renshaw and Trede, 2012; Nguyen-Chi et al., 2015; Pereiro et al., 2015; Dee et al., 2016), even if it is not yet determined whether they are resident in all tissues or not. Emerging data, however, is shedding light on the ontogeny of tissue immunity in zebrafish.

Recent work has shown that microglia, the specialized macrophages in the Central Nervous System (CNS); have different origins depending on the age of the animals. In the adult zebrafish, microglia derive from haematopoietic stem cells (HSCs) and not from primitive macrophages, which occurs only in early development (Ferrero et al., 2018). This has also been shown for adult zebrafish Langerhans cells in the skin and suggested to also be the case for liver, heart, gut and brain (He et al., 2018). Together, these elegant recent studies suggest that most zebrafish adult resident or associated immunity derives from the second wave of hematopoiesis, mainly from the ventral wall of the dorsal aorta (VDA region), and not from erythro-myeloid progenitors (EMPs) as previously thought. This is also emerging as the current model in mammalian systems (Sheng et al., 2015) although there are still uncertainties and some controversy in the field (Perdiguero et al., 2015).

Crucially, recent work is showing that, more than ontogeny, tissue immunity seems to be particularly dictated by the tissue in which it resides. There are key tissues in adult zebrafish that are being intensely investigated and multiple studies highlight that the role of immunity in tissue repair and maintenance is largely conserved in zebrafish. Key examples where this has been shown are in the heart, gut, brain, and retina.

Selected Examples of Tissue Immunity in Adult Zebrafish

Heart

Recent years have seen many studies identify crucial and perhaps surprising roles for immune cell populations in the heart in homeostasis and disease, although much remains to be discovered. A recent study in mice indicated a remarkable role for resident cardiac macrophages in the distal atrioventricular node where they make direct connections to cardiomyocytes via Connexin 43 and facilitate electrical conductance (Hulsmans et al., 2017). In zebrafish, we currently know very little about cardiac macrophages under homeostatic conditions although our own experience has revealed a population of immune cells, labeled with L-plastin and transgenic markers of macrophages (see **Table 1**), is present in the unwounded heart and recent work suggests these may be derived from HSCs (see above; **Figure 1**). Recently, many studies have shown important contributions of different immune cell lineages in response to cardiac injury and disease in mammalian models. In particular, vital roles have been suggested for macrophages in complete regeneration of the neonatal mouse heart (Aurora and Olson, 2014; Lavine et al., 2014). However, the inflammatory response in the adult zebrafish heart has been less well characterized. Recent studies revealed that immune cells are recruited to the heart following cryoinjury of the ventricle in adult zebrafish (Schnabel et al., 2011). Two recent reports have also shown that macrophages are required for cardiomyocyte proliferation and therefore regeneration in the heart of adult zebrafish (de Preux Charles et al., 2016; Lai et al., 2017). Our own experience suggests that all immune cell lineages that we were able to analyse are recruited to the heart after injury and whereas roles can be assigned for macrophages of the innate immune system, the precise roles for other cell types remain more

TABLE 1 | Selected list of key transgenic zebrafish lines and antibodies to detect immune cells and/or inflammation markers.

Promoter	Immune cell type	Commercial source	Reference	Technical notes: conditions that work	Conditions that don't work
pu.1	Primitive myeloid cells		Hsu et al., 2004		
mpx	Neutrophils		Renshaw et al., 2006; Mathias et al., 2006		
lyz	Neutrophils/some macrophages		Hall et al., 2007		
gata2a	Eosinophils		Traver et al., 2003		
mpeg1	Macrophages		Ellett et al., 2011		
c-fms	Macrophages		Dee et al., 2016		
mfap4	Macrophages		Walton et al., 2015		
tnfα	Pro-inflammatory, *tnfα*+ cells		Marjoram et al., 2015; Nguyen-Chi et al., 2015		
lck	T-cells		Langenau et al., 2004		
cd4	$CD4^+$ T-cells and macrophages		Dee et al., 2016		
foxp3a	Foxp3a+ T-cells		Hui et al., 2017		
rag2	Lymphoid cells		Langenau et al., 2003.		
mhc2dab, cd45	Allows distinction between B-cells, macrophages/dendritics and T-cells/neutrophils		Wittamer et al., 2011		
IgM	B-cells		Page et al., 2013.		
CD79	B-cells		Liu et al., 2017		
apoe	Microglia		Oosterhof et al., 2017		
Antibodies					
Rabbit anti L-plastin	Pan-leukocyte	Ref: GTX124420, Genetex	Redd et al., 2006.	1:300/1:500 on whole mount tissue, paraffin and cryosections with Citrate pH6 antigen retrival	cryosections without citrate pH6 Ag retrieval
Rabbit anti-Mpeg1 (C terminus)	Macrophages	Ref: ANA55917, AnaSpec Inc		1:50 on cyosections and (weakly) on paraffin sections, both after *in situ* hybridisation heating steps. Note that, at least in our hands, in adult zebrafish gut, this antibody does not dettect all of mpeg-mcherry transgenically labeled macrophages	
Rabbit anti-TCR-alpha (N terminus)	T-cells	Ref: AS-55868, AnaSpec Inc	Our unpublished data (see **Figure 1**)	1:200 on cryosections(In our hands, we noticed a significamnt decline in the quality of staining iof the cryosections were not fresh, i.e, frozen for more than a week or so) with citrate pH6 Ag retrieval	
Isolectin GS-IB4	vascular endothelial cells and microglia	Thermo Fisher I21411	Zou et al., 2013	Cryosections or paraffin sections witjh Citrate Ph6 antigen retrieval	

Continued

TABLE 1 | Continued

Promoter	Immune cell type	Commercial source	Reference	Technical notes: conditions that work	Conditions that don't work
Mouse anti-7.4C4	microglia (vascular-derived, resident macrophages)		Becker and Becker, 2001	Cryosections or paraffin sections witjh Citrate Ph6 antigen retrieval (1:100 dilution)	
Mouse anti-RFP		Ref: ABP-MAB-RT008, Insight biotechnology		1:500 on paraffin or cryosections with citrate pH6 Ag retrieval	
Chicken anti-GFP		Ref: AB13970, Abcam		1:500 on paraffin or cryosections with citrate pH6 Ag retrieval	
Mouse anti-Glutamine Synthetase	Muller Glia	MAB302 clone GS6	Gramage et al., 2015	Cryosections with citrate Ph6 antigen retrieval 1:200 dilution	
Rabbit Spi1 spleen focus forming virus (SFFV) (Pu.1)	progenitor myeloid cells	GTX128266-S Genetex			In our hands (CMH) we did not achieve clear results in either paraffin or cryosections with or without antigen retrieval
Goat anti-EPX (eosinophil specific peroxidase)	Eosinophils	SC-19148 Santa Cruz Biotechnology			In our hands (CMH) we did not achieve clear results in either paraffin or cryosections with or without antigen retrieval
Purified Rat Anti-Mouse CD11b Clone M1/70 (RUO)	Multiple leukocyes	557394 BD			In our hands (CMH) we did not achieve clear results in either paraffin or cryosections with or without antigen retrieval

of a mystery (RJR, unpublished). However, another recent report has shown important roles for a Treg subset of zebrafish T-cells in promoting regeneration in a number of different tissues including the heart (Hui et al., 2017), suggesting intriguing and important roles for different immune cell populations in varying aspects of regeneration and disease remain to be elucidated.

Gut

The gut can be considered the biggest compartment of the immune system, and it is constantly exposed to multiple foreign antigens, which it must distinguish from harmless dietary proteins and the resident microbiota (Mowat, 2018). When this goes wrong, the immune system can "misfire" and contribute to chronic inflammatory disorders such as Inflammatory Bowel Disease (IBD) (Bain and Mowat, 2014b; Mowat et al., 2017; Andrews et al., 2018; Corridoni et al., 2018; Liu et al., 2018) and age-associated gut degeneration (Man et al., 2014; Sato et al., 2015; Soenen et al., 2016). Additionally, gut immunity is essential for the steady-state epithelial renewal (Andrews et al., 2018) that, similarly to humans, occurs roughly every 3 days in adult zebrafish (Wallace et al., 2005; Crosnier et al., 2006). Recovery after DSS intestinal injury has also been shown to be dependent on Myd88 signaling in myeloid cells (Malvin et al., 2012). Adult zebrafish are also showing great promise as a model to study gut inflammation and repair in health and disease (Marjoram and Bagnat, 2015 Brugman, 2016), including ageing (Henriques et al., 2013; Carneiro et al., 2016, Martins et al., 2018). These works have shown that, similarly to humans, critical aspects of gut homeostasis become compromised with ageing in zebrafish, namely increased permeability, inflammation and telomere-dependent cellular senescence.

Thanks to the development of key transgenic reporter lines and antibodies (**Table 1**), adult zebrafish gut has been shown to be populated by abundant T-cells, B cells, mast cells, macrophages, and dendritic cells in the normal steady-state context (CMH unpublished data and others) (Wittamer et al., 2011; Lewis et al., 2014), similarly to mammalian vertebrates (**Figure 1**). Importantly, macrophage M1 and M2-like subsets have also been identified in zebrafish, thanks to the development of mpeg-mcherry-TNFα-GFP double transgenic line. Using this line, M1 macrophages were characterized by high TNFα-GFP expression (mpeg+TNFα+), as well as expression of TNFβ, IL-1β, and IL-6 (Nguyen-Chi et al., 2015), which are well-known markers of M1-like macrophages in mammals. Moreover, these subsets were shown to respond to injury similarly to human macrophage subsets. Additionally, Il-1β reporter lines have recently been developed allowing visualization of cells expressing this pro-inflammatory cytokine (Hasegawa et al., 2017; Ogryzko et al., 2018). What is very different in the zebrafish gut, however, is the absence, as far as reported, of defined intestinal crypts and Peyer's patches (Ng et al., 2005; Cheng et al., 2016). Despite the absence of Peyer's patches there is a clear distribution of leukocytes along the adult zebrafish gut, lining the enterocytes, which could be considered analogous to the mucosal associated lymphoid tissue (MALT). The apparent absence of secondary lymphoid structures though, means that we still do not understand fully how antigen presentation occurs in zebrafish

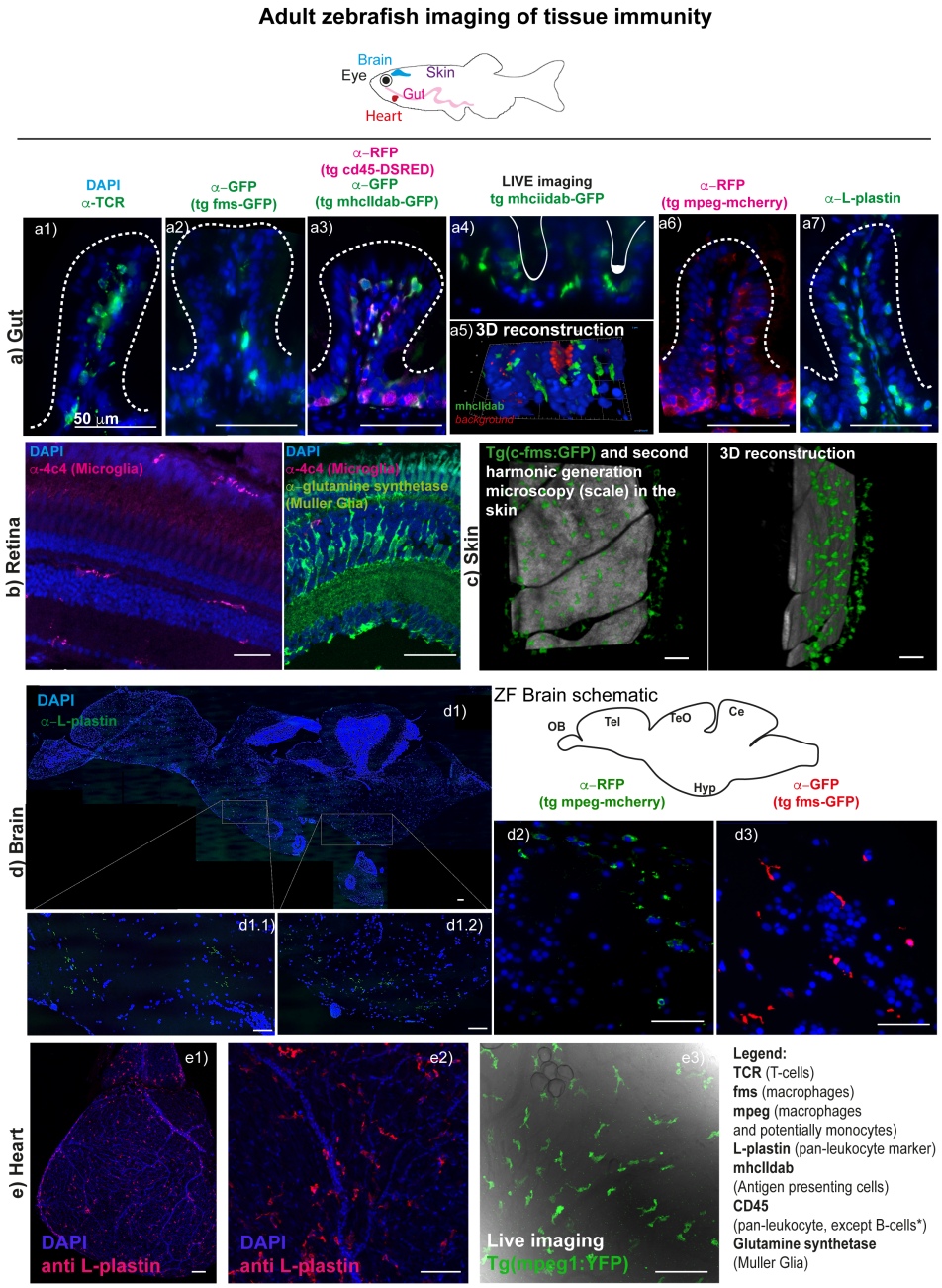

FIGURE 1 | Imaging tissue immunity in adult Zebrafish. **(a)** CMH unpublished images showing examples of cryo/paraffin sections of adult Zebrafish gut, followed by immune fluorescence of selected antigens (*see table 1 for antibody details*), all counterstained with DAPI for cell nuclei detection and imaged in a Deltavision light microscope. **(a1)** T-cells are stained with anti-TCR alpha antibody **(a2)** macrophages in Tg(fms-GFP) animals (Dee et al., 2016) are detected with an anti-GFP antibody; **(a3)** multiple leukocyte lineages can be detected using anti-RFP and anti-GFP antibodies in a double transgenic line for CD45-DSRED and mhcIIdab-GFP respectively (Wittamer et al., 2011). Single red cells are neutrophils/T-cells, double green and red cells are macrophages/dendritic cells, single green cells are B-cells, since, in this line, the CD45 promoter used is not active in B-cells; **(a4)** and **(a5)** Adult Zebrafish gut can be imaged in Light sheet microscopy for short durations, following dissection and gentle embedding in low percentage agarose in E3 media; **(a6)** macrophages are detected with an anti-RFP antibody in Tg(*mpeg1:mCherryCAAX*) (Ellett et al., 2011; Ogryzko et al., 2018) animals; **(a7)** leukocytes are stained with L-plastin (aka LCP-1) and these can n = be seen lining the gut epithelia cells (enterocytes). **(b)** RBM unpublished images showing selected cryosections of adult Zebrafish retina, imaged by confocal microscopy followed by immune fluorescence of selected antigens (*see table 1 for antibody details*), all counterstained with DAPI for cell nuclei detection. **(b1)** Microglia are detected with an anti-4c4 antibody and can be seen dispersed throughout the tissue, displaying a simple ramified state. Upon insult these microglia rapidly migrate and engulf damaged cells or debris. **(b2)** Muller Glia can also be detected in the retina using an anti-glutamine synthetase antibody. **(c)** RJR unpublished images of adult zebrafish skin where macrophages can be live-imaged, shown here using the Tg(*c-fms-GFP)* line and using second harmonic generation microscopy to identify

(Continued)

FIGURE 1 | Continued

the scale surface. **(d)** RRM unpublished images showing paraffin sections of adult Zebrafish brain, imaged using a Deltavision light microscope or by confocal microscopy; **(d1)** multiple-panel reconstitution of a whole adult brain, imaged by confocal microscopy, stained with anti-L-plastin, which marks all microglia in the brain. Microglia in the adult brain can also be detected for example using the **(d1)** Tg(*mpeg1:mCherryCAAX*) line (Ellett et al., 2011; Ogryzko et al., 2018) or **(d2)** the Tg(fms-GFP) line (Dee et al., 2016). Sections **(d1)** and **(d2)** show the diencephalon. **(e)** RJR unpublished images showing whole mount immunostaining of an entire heart **(e1)** or the surface of the ventricle **(e2)** with an anti-L plastin antibody and imaged with confocal microscopy. Live imaging using confocal microscopy of an unwounded adult zebrafish heart reveals cardiac macrophages using a Tg(*mpeg1:YFP*) line **(e3)**.

(Lewis et al., 2014). MHC class II-expressing, antigen presenting cells (APCs), namely macrophages, dendritics (DCs) and B-cells are, however, described and appear to function similarly to their mammalian counterparts (Lugo-Villarino et al., 2010; Wittamer et al., 2011, Lewis et al., 2014; Dee et al., 2016). See **Table 1** for further details and references.

Nevertheless, and importantly, the protective immune status of the gut has also been shown to occur in zebrafish, and this has been largely attributed to the secretion of IL-10 by CD41+foxp3+ Treg-like T-cells in the gut, once again showing that these sophisticated immune-regulatory elements are already evolved in teleost fish (Dee et al., 2016; Hui et al., 2017). It has also been shown that in adult zebrafish, the gut is rapidly populated by eosinophils upon parasitic infections (Balla et al., 2010), highlighting another conserved response to infection in the gut.

Brain

The neuro-immunology field has been growing in recent years, highlighting the complex crosstalk between the immune system and the central nervous system (CNS) and how this plays a key role in maintaining brain homeostasis, reviewed in (Oosterhof et al., 2015). Although mammalian models are more prominent at the moment, the zebrafish model is starting to gain traction, particularly due to its genetic and imaging amenity, small size and relatively low maintenance costs (de Abreu et al., 2018).

As in mammals (Cuadros and Navascues, 1998; Ginhoux et al., 2010), microglia are the tissue-resident immune cells in the zebrafish brain (Xu et al., 2015). In zebrafish, primitive microglia originate from yolk sac-derived macrophages that migrate to the cephalic mesenchyme and then invade the brain, being detectable from 60 h post-fertilization (hpf) (Herbomel et al., 2001). Only more recently it was recognized that these are not the definitive microglia observed in adulthood. As described above, an elegant recent study (Ferrero et al., 2018), showed the existence of a second wave of re-population of the brain's microglia, which originate from HSCs – a process that occurs between 2 weeks and 3 months of age. Microglia can be detected by L-plastin (or LCP-1), a pan-leukocyte marker (Redd et al., 2006; Cvejic et al., 2008; Mathias et al., 2010; Van Houcke et al., 2017), by macrophage-expressed gene 1 (*mpeg1*), labeling all mononuclear phagocytes (Ellett et al., 2011; Wittamer et al., 2011), by the 4c4 antibody (Becker and Becker, 2001)as well as Apolipoprotein E (ApoE) [Oosterhof et al. (2015), see **Table 1**; **Figure 1**]. Note that, although ApoE specifically labels microglia if compared with the other markers mentioned, it has also been shown to be expressed astrocytes (Boyles et al., 1985; Poirier et al., 1991; Xu et al., 2006), oligodendrocytes (Stoll et al., 1989), as well as by some neurons albeit at lower levels (Han et al., 1994; Achariyar et al., 2016). Additionally, cells in the choroid plexus as well as smooth muscle cells in blood vessels also seem to express ApoE (Xu et al., 2006). Thus, in order to ensure specific labeling of microglia, ApoE should be used in combination with another leukocyte or macrophage marker.

Microglia have been described as ramified cells that constantly sense the environment searching for physiological disturbances in the surroundings (Oosterhof et al., 2015). Like in mammals (Lucin and Wyss-Coray, 2009), adult zebrafish microglia proliferate and migrate to the injury or inflammation site ('gliosis'), upon activation in response to a stab lesion (Kroehne et al., 2011; Kyritsis et al., 2012), excitotoxin injection (Skaggs et al., 2014) or nitroreductase (NTR)-mediated neuronal ablation (Oosterhof et al., 2017). Also, there is an increased number of L-plastin^{+} cells in response to optic nerve injury in both young (5 months) and older (22–24 months) zebrafish, but this is decelerated in the old fish, suggesting age-related dysfunctional immune response in ageing (Van Houcke et al., 2017). Once activated, microglia change their appearance from a ramified to an amoeboid shape (Svahn et al., 2013). These immune cells have the important function of clearing cellular debris, such as dead or damaged neurons, by phagocytosis (Peri and Nusslein-Volhard, 2008); and, when activated can release anti- and pro- inflammatory cytokines, at least in mice primary microglia cultures (Cai et al., 2017). To our knowledge, microglia inflammatory cytokine release remains to described for zebrafish, despite extensive characterization of other aspects of zebrafish microglia (van Ham et al., 2014). Peripheral immune cells can infiltrate the CNS in cases of Blood Brain Barrier (BBB) alterations, such as those observed in Multiple Sclerosis (MS) or cerebral ischemia (Holtmaat and Caroni, 2016), in particular, infiltration of monocytes or perivascular macrophages has been described in mammals (Lucin and Wyss-Coray, 2009). Similarly, upon NTR-induced cell death, peripheral macrophage-like cells infiltrate the embryonic zebrafish brain, contributing to the first inflammatory response (van Ham et al., 2014). In opposition, it has been reported that no major infiltration of periphery macrophages occurs in the brain after neuronal ablation (Oosterhof et al., 2017). Thus, more studies are needed to address this question. More surprisingly, T cells were reported to infiltrate the brain in a mouse model of ALS (SOD1^{G93A}) during progression of the disease (Chiu et al., 2008) and to invade the human brain in Parkinson's Disease (PD) (Brochard et al., 2009). Also, CD4^{+} T cells and B cells have been detected in the brain of patients with MS, and this is thought to contribute to inflammation in the CNS (Jelcic et al., 2018). To our knowledge, so far, there are no studies reporting the presence of T cells or B

cells in zebrafish brain. Zebrafish Treg-like (zTreg) cells seem to move towards damaged sites in CNS, such as retina and spinal cord, contributing to regeneration; however, the brain was not explored in this study (Hui et al., 2017). Moreover, it remains unknown whether neutrophils invade the adult zebrafish brain in contexts of severe inflammation. Neutrophils were found in the brain of a nlrc3-like mutant model zebrafish embryo, where there is a systemic inflammation (Shiau et al., 2013). However, no recruitment of neutrophils was observed after injury either in the embryo brain (van Ham et al., 2014) or peripheral nervous system (Pope and Voigt, 2014). Additionally, Goldshmit et al. reported to rarely find neutrophils at the injury site after spinal cord transection in adult zebrafish (Goldshmit et al., 2012). Unfortunately, though, other studies have yet to be reported for adult zebrafish to help clarify this matter.

Retina

The retina is viewed as a unique "window" into the brain and is one of the most established systems to study neural development and disease processes in the CNS (London et al., 2013). The zebrafish retina is a true vertebrate retina as it has the same organisation and contains largely the same types of neurons and glial cells as the human eye. The innate immune system in the zebrafish retina is composed of two major types of glial cell, the Müller glia (MG) and the microglia (**Figure 1**). The mammalian retina also houses astrocytes that will contribute to immunity. However, their presence in the zebrafish CNS, including the retina, remains unclear (Lyons and Talbot, 2014). The MG and microglia will contribute to the maintenance of homeostasis, phagocytose debris and are critical for tissue repair (Reichenbach and Bringmann, 2013). MG are the most abundant glial cell in the tissue, have a fixed radial morphology which allows them to contact surrounding neurons (Jadhav et al., 2009) and can modulate innate retinal immunity (Kumar et al., 2013; Vecino et al., 2016). Retina microglia are migratory, as in the brain, and survey the tissue for damage and debris (Silverman and Wong, 2018). Crosstalk between these two glial cell types may mediate their response to damage and injury by coordinating inflammation (Wang et al., 2011, 2014). Activated MG and microglia are associated with almost every pathological condition in the retina (Bringmann et al., 2006; Silverman and Wong, 2018). This includes retinal degenerative conditions, such as age related macular degeneration and diabetic retinopathies (Ramirez et al., 2017). The zebrafish is an established model for studying cellular and molecular mechanisms underlying many ocular diseases (Gestri et al., 2012). However, linking immunity with confounding factors for disease, such as ageing, remain challenging in many models. A recent study in zebrafish has shown that there is progressive degeneration of photoreceptors with age when interfering with Crumbs, a gene family linked with human retinal degeneration (Fu et al., 2018). However, the contribution of the innate immune system to degeneration and pathologies of disease remains largely unknown.

After damage the innate immune systems plays a key role in the phagocytosis of debris and removal of dead or dying cells (Kumar et al., 2013). However, in the zebrafish retina after damage or disease the MG will generate neurons to restore vision (Hitchcock and Raymond, 2004). This is an area of intense study and the molecular mechanisms regulating it are beginning to be identified (Goldman, 2014), yet the role of microglia in these processes is not clear (Mitchell et al., 2018). By imaging the glial dynamics in real time *in vivo* in the zebrafish retina, microglia have been shown to change their morphology to the activated state and maintain this activation after regeneration is complete, potentially to ensure correct retinal function is re-established (Mitchell et al., 2018). Further, by pairing the imaging capacity of the zebrafish with the ease to which they can be treated with pharmacological inhibitors a recent study investigated roles of the innate immune system during rod photoreceptor regeneration (White et al., 2017). They show that the role of microglia is to regulate MG responsiveness to cell death, and thereby control neuronal regeneration kinetics. Further, immunosuppression can either inhibit or accelerate photoreceptor regeneration kinetics depending on the timing of treatment (White et al., 2017). Thus, utilizing the precise advantages of the zebrafish, paired with the well-characterized retina, makes this an exciting model to study the resident immune system in retinal disease and regeneration.

CONCLUDING REMARKS

Despite multiple advances in developing reporter transgenic lines marking different types of immune cell lineages in zebrafish, there are still multiple sub-types of immune cells we have no markers for or antibodies available e.g., mast cells. Nevertheless, advances in single-cell sequencing technology have already enabled the identification of specific immune subsets, such as different subtypes of NK cells (Carmona et al., 2017; Tang et al., 2017) and innate lymphoid cells (ILCs) (Hernandez et al., 2018), which have contributed to the understanding of the similarities and differences between zebrafish and human immune subsets. Despite the overall similarity between human and zebrafish immune subsets, highlighted here, there are key differences, which are important to keep in mind, reviewed elsewhere (Trede et al., 2004; Renshaw and Trede, 2012, Kanwal et al., 2014). The first obvious difference is that during the first week of zebrafish development, this organism relies entirely on an innate immune system (Lam et al., 2004), a difference which has been extensively used to understand the relative contribution of innate versus adaptive immunity in response to different bacterial, viral, and fungal pathogens (Meijer and Spaink, 2011; Meijer et al., 2014). Another key difference is the absence, at least not reported thus far, of secondary lymphoid organs in zebrafish. Moreover, the zebrafish does not have a bone marrow, and instead, T-, B- as well as myeloid cells are present in the spleen and head kidney, which act as the zebrafish equivalent of bone marrow. There are also key differences in zebrafish immune receptors and/or response to specific ligands reviewed in (Kanwal et al., 2014) and this is contributed to by the gene duplication detected in many of the zebrafish genes (Lu et al., 2012) An example are the novel immune-type receptors (NITRs), which appear to be homologues of mammalian NK-like receptors and seem to also have homologous functions (Yoder et al., 2010). Additionally, despite the fact that most of Toll Like receptors

have been described in zebrafish, there are key differences such as the fact that Tlr4 is not involved in sensing LPS. Indeed, in zebrafish, LPS signals via a Tlr4- and MyD88-independent manner (Sepulcre et al., 2009). Nevertheless, zebrafish still respond to lipopolysaccharide (LPS), and careful analysis has shown that the overall response to LPS stimulation at the level of gene transcription is highly conserved with that of mammals (Forn-Cuni et al., 2017).

We have highlighted in **Table 1** the working tools available as well as some antibodies that we have tested but have failed to get to work. We believe this will be a valuable starting point for future researchers wanting to use zebrafish to study tissue immunity.

In summary, we can clearly identify microglia, macrophages (including distinguishing a pro-inflammatory phenotype), T-cells, B-cells, and neutrophils in tissues using a combination of transgenic lines and antibodies. It will be particularly important to develop these techniques further if we are to improve our live imaging capability, but also the ability to detect multiple immune lineages in the same tissue without requiring crossing multiple transgenic lines, which dramatically increases the time and cost of experiments. Unfortunately, we are still missing transgenic reporters and/or antibodies for some sub-types of T-cells (e.g., Th1, Th2, cytotoxic, and NKT), NK-cells and mast cells.

We hope that the studies highlighted here show how zebrafish can offer an incredible tool to study immunity and its role in tissue repair and maintenance, across the lifecourse, in a time and cost-efficient manner, and how it can improve so much more with the continuous investment, not only of this scientific community, which is growing, but also of commercial companies, particularly in the development and validation of zebrafish-specific antibodies.

ETHICS STATEMENT

This study complied with the Animals (Scientific Procedures) Act 1986 using Home Office approved licenses [PPL numbers: 30/3318 (RJR), 70/8681 (CMH), and 40/3727 (RBM)]. The licenses and protocols were ethically reviewed and approved by each local Animal Welfare and Ethical Review Body (AWERB) (University of Sheffield and University of Bristol). Both Universities are signatories of the Understanding Animal Research Concordat on Openness and as Signatories to the Concordat have agreed to be more open about their use of animals in research, and to abide by the four commitments.

AUTHOR CONTRIBUTIONS

CMH, RRM, RBM, and RJR contributed equally to the writing of the manuscript, figure, and table. PSE contributed to the development of key techniques illustrated in **Figure 1** and contributed to **Table 1**.

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Nano-Sampling and Reporter Tools to Study Metabolic Regulation in Zebrafish

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In the past years, evidence has emerged that hallmarks of human metabolic disorders can be recapitulated in zebrafish using genetic, pharmacological or dietary interventions. An advantage of modeling metabolic diseases in zebrafish compared to other "lower organisms" is the presence of a vertebrate body plan providing the possibility to study the tissue-intrinsic processes preceding the loss of metabolic homeostasis. While the small size of zebrafish is advantageous in many aspects, it also has shortcomings such as the difficulty to obtain sufficient amounts for biochemical analyses in response to metabolic challenges. A workshop at the European Zebrafish Principal Investigator meeting in Trento, Italy, was dedicated to discuss the advantages and disadvantages of zebrafish to study metabolic disorders. This perspective article by the participants highlights strategies to achieve improved tissue-resolution for read-outs using "nano-sampling" approaches for metabolomics as well as live imaging of zebrafish expressing fluorescent reporter tools that inform on cellular or subcellular metabolic processes. We provide several examples, including the use of reporter tools to study the heterogeneity of pancreatic beta-cells within their tissue environment. While limitations exist, we believe that with the advent of new technologies and more labs developing methods that can be applied to minimal amounts of tissue or single cells, zebrafish will further increase their utility to study energy metabolism.

Keywords: zebrafish, metabolomics, fluorescent reporter, nano sampling, nano scaling, live imaging, beta-cell, diabetes

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INTRODUCTION

Zebrafish (*Danio rerio*) have evolved from being a model organism primarily used for studies of vertebrate development to a widely applied research tool, including its use in behavioral research, genetics, physiology, disease modeling, toxicology, and drug discovery (Lieschke and Currie, 2007; Kalueff et al., 2013; MacRae and Peterson, 2015; Gut et al., 2017). The growing use of zebrafish is based on the presence of a vertebrate body plan and observations that tissue-specific physiological

processes are surprisingly similar between zebrafish and humans (Schlegel and Gut, 2015; Gut et al., 2017). The conservation of the core mechanisms has led to the development of genetic, dietary and pharmacological models to study the principles of energy metabolism under physiological and disease conditions (Santoro, 2014; Gut et al., 2017).

Metabolic diseases are systemic disorders driven by the failure of single or multiple tissues to maintain homeostasis. Loss of metabolic homeostasis occurs when specialized tissues have lost their reserve capacity to react to daily metabolic challenges. For example, the diagnosis of diabetes is preceded by years of compensation during which a reduced number of functional beta cells maintains normoglycemia after meal intake. With further decline of beta-cell function during persistent metabolic stress, a minimum threshold of functional beta-cell mass is reached, causing elevated fasting and post-prandial glucose excursions and ultimately leading to diabetes (Chen C. et al., 2017). Challenging this reserve capacity to study the biochemical processes of cells that protect or accelerate functional responses to metabolic stressors are a cornerstone of metabolic research (Tschop et al., 2012). Due to their small body size, many of the challenges that are routinely done in larger laboratory animals can be difficult to implement in zebrafish. A workshop on metabolism at the 5th European Zebrafish Principal Investigator meeting (March 20–23, 2018 in Trento[1]) was dedicated to discuss novel solutions to remaining obstacles that prevent a wider and more impactful use of zebrafish for metabolic studies. As a result of this discussion, we summarize recent progress in "nano-sampling" approaches for metabolomics studies that provide possibilities to quantify metabolites with tissue-resolution in zebrafish larvae. Furthermore, we outline strategies using reporter tools and live imaging to study heterogeneity of cellular function within the tissue environment, an avenue that holds great promise to investigate the early cellular events leading to metabolic disease with clear advantages of using translucent zebrafish.

RECENT ADVANCES IN NANO-SAMPLING FOR METABOLOMICS

Metabolomics technologies have facilitated systematic studies of energy substrates and their intermediates in response to different metabolic states, as well as the contribution of pathologically elevated metabolites to disease onset and progression. Over the past years, metabolomics methods have also been increasingly applied to zebrafish. Typically, samples from pooled embryos/larvae and from adult tissues have been analyzed by nuclear magnetic resonance (NMR) spectroscopy as well as chromatographic or mass spectrometric (MS) methods (Ong et al., 2009; Papan and Chen, 2009; Soanes et al., 2011). [For recent examples by the workshop participants, see (Chatzopoulou et al., 2015; Martano et al., 2015; Weger et al., 2016) and below]. Targeted MS methods can reduce the amount of required material to as few as five embryos at 3 dpf (Kantae et al., 2016), but despite these efforts data interpretation remains challenging considering that metabolites are determined from pooled tissues. Efforts to increase tissue resolution have been made by applying manual microdissection, for example to determine the specific lipid composition of the embryo proper vs. the composition of yolk lipids at different stages, thereby separating two groups of tissues, but still using material from 15 embryos per sample (Fraher et al., 2016). To improve tissue resolution with minimal input material, microneedle sampling has been used to take yolk samples from single embryos for the quantification of drug uptake by targeted UPLC-MS (Ordas et al., 2015). Recently it also has become possible to draw blood from larvae: small drops could be obtained from the posterior cardinal vein at 5 dpf using a microneedle in conjunction with imaging to calculate the sample volume (van Wijk et al., 2018). Using this method the blood concentration, distribution volume and clearance of paracetamol was estimated in response to exposure from the water. Further optimization is required to compare blood sampling from different anatomical locations and to provide additional proof-of-concept examples beyond paracetamol. The determination of pharmacokinetic properties of a small molecule in a zebrafish larvae at nano-scale is a step forward in making zebrafish a suitable complementary model for drug discovery and development.

In another proof-of-concept study *Xenopus* embryos were applied for nano-sampling using a microprobe single cell CE-ESI-MS technique, which could determine about 70 metabolites from single blastomeres from the 32 cell stage (Onjiko et al., 2017). As the sample volumes used in these microneedle-based approaches are similar between zebrafish [20–200 nL; whole 3 dpf larva ∼290 nL (Kantae et al., 2016)] and *Xenopus* (10–15 nL), the capillary sampling method might also allow for untargeted metabolomics in the zebrafish.

Recent technical advances in single cell metabolomics with cultured cells demonstrate the feasibility of reaching cellular resolution also for cells smaller than early embryonic blastomeres with subcellular sampling on the horizon (Esaki and Masujima, 2015); reviewed in Armbrecht and Dittrich (2017), Yang et al. (2017), Qi et al. (2018). Although challenging, microdissections using microneedles or capillaries on tissues from zebrafish will be ideally suited for single cell metabolomics facilitated by the rich resource of reporter transgenic lines for the identification of embryonic and larval anatomical structures.

An alternative approach to nano-sampling is mass spectrometry imaging, which may achieve even higher spatial resolution and give snapshots of *in situ* metabolite distribution. For example, Dueñas et al. (2017) used MALDI imaging to map phospholipid distributions in early zebrafish embryos (up to 16 cell stage) at about 10 μm resolution. However, a downside of MALDI imaging is that it requires cryosectioning of the embryos and has an analytical bias for lipids (Baker et al., 2017; Emara et al., 2017). Secondary Ion Mass Spectrometry (SIMS) achieves higher resolutions than MALDI imaging, but is equally limited to fixed samples (Passarelli and Ewing, 2013; Armbrecht and Dittrich, 2017).

Toward a better understanding of metabolite dynamics, continuous recording of metabolome changes *in vivo* will be required. *In vivo* Nuclear Magnetic Resonance (NMR)

[1]http://events.unitn.it/en/ezpm2018

TABLE 1 | Nano-sampling approaches for metabolomics.

Method	Tissue	Description	Amount of material used per sample	References
Manual microdissection	Yolk and embryo proper	Separation of yolk and embryo body with forceps and fine scalpel	Pooled tissues from 15 embryos	Fraher et al., 2016
Microneedle sampling	Yolk, blood	Puncturing and suction of yolk or larval vasculature with glass capillary	Yolk: 50 nL (range 20–200 nL) from 1 embryo Blood: samples pooled from 15–35 individual larvae	Ordas et al., 2015
Mass spectrometry imaging	Early embryos (1–16 cell stage)	Matrix assisted laser desorption/ionization (MALDI) mass spectrometric imaging of phospholipids on cryosections	Cryosections of 1 embryo, spatial resolution 10 μm	Dueñas et al., 2017
In vivo magnetic resonance microscopy (MRM)/magnetic resonance spectroscopy (MRS)	Adult brain	Live MRM/MRS of adult fish in flowthrough chamber of microimaging probe	1 adult fish, voxel size 1.5 mm^3	Kabli et al., 2009

spectroscopy is a promising approach for such metabolic monitoring, and a few pioneering studies have followed metabolite changes during development and in response to hypoxia or herbicide exposure in medaka embryos (Viant et al., 2002, 2006; Pincetich et al., 2005). As spectroscopic analysis can be combined with magnetic resonance (MR) imaging, also spatial information on metabolite distribution is accessible to these techniques. For example, Kabli et al. (2009) recorded high resolution localized MR spectra from live adult zebrafish brains with a voxel size of 1.5 mm^3 and could detect several amino acids and other metabolites. Further improvements of the instruments are likely to increase both metabolite and spatial resolution as well as sensitivity of these methods.

Another strategy to examine metabolism beyond steady state levels is the application of tracer technologies to assess flux rates through different pathways. Mugoni et al. (2013a,b) used ^{13}C isotope labeling to study prenyl lipid metabolism in zebrafish embryos, showing reduced Coenzyme Q10 and Q9 synthesis based on HPLC analysis of extracts from 25 embryos mutant for *UbiA-domain containing protein 1* (*ubiad1*) and their wild-type siblings. Combining such tracer studies with the cellular and sub-cellular analysis methods currently being developed should provide unprecedented insight into metabolic pathways and their (patho-)physiological changes *in vivo*. **Table 1** summarizes methods that are relevant for nano-sampling strategies in zebrafish.

Looking forward, key applications for metabolomics studies in zebrafish include the investigation of cancer metabolism; metabolic reprogramming is a hallmark described as an intrinsic property of cancer and is based on the observation that proliferating cells require a large amount of nutrients, energy, and biosynthetic activity to produce the macromolecular components of the newly generated cells. The zebrafish, with its large collection of genetic models of cancers and the popular transplantation assays, represents the ideal model system for analysis of metabolism during cancer progression (White et al., 2013). While the tools for studying changes in metabolism *in vivo* are being developed, a number of studies have already revealed altered metabolism in a zebrafish model of glioma progression, including changes in glycolytic rate as well as lipid and nucleotide metabolism (Bräutigam et al., 2016; Tan et al., 2016; Zhang M. et al., 2018). Known oncogenes have been reported to rewire metabolic pathways in zebrafish. For example, Yap was found to reprogram glutamine metabolism to increase nucleotide biosynthesis in a zebrafish model of liver hyperplasia (Cox et al., 2016).

To provide a framework to compare metabolic changes in response to reprogrammed metabolic pathways between mammalian and fish metabolism, a metabolic network model (MetaFishNet[2]) has been generated (Li et al., 2010) and refined recently (Bekaert, 2012). Metabolic network models integrate genetic, epigenetic and metabolic information and allow predictions of cancer type-specific metabolic pathways, drug targets and therapeutic strategies, and have been constructed for a number of organisms and tissues [reviewed in Masoudi-Nejad and Asgari (2015)]. The MetaFishNet model was used to draw a comparison between human and fish metabolic pathways, showing a large overlap, and to analyze gene expression data in a zebrafish liver cancer model (Lam et al., 2006). Several metabolic pathways were predicted to be misregulated in zebrafish liver cancer (Li et al., 2010), and Wnt signaling was found to remodel lipid metabolism in tumors induced by overexpression of the oncogene Ras in hepatocarcinoma cells and zebrafish liver tumors (Yao et al., 2018). Advances in nano-sampling and metabolomics requiring low-input of materials will provide powerful technologies to investigate cancer-type specific metabolic changes in zebrafish cancer models.

TISSUE-RESOLUTION OF METABOLIC REGULATION USING REPORTER TOOLS

An alternative to the direct quantification of metabolites in tissues is the indirect visualization of physiological processes (Gut et al., 2013), metabolite ratios (Panieri et al., 2017), or signaling effects of metabolites (Niethammer et al., 2009) in zebrafish using fluorescent probes [Reviewed in Santoro (2014)]. The transparency of zebrafish larvae and amenability to genetic modification has enabled live imaging of these reporter tools in tissues and single cells (**Figure 1**). Further advantages of these strategies include the possibility to monitor processes continuously and in response to challenges, such as in the

[2]http://metafishnet.appspot.com

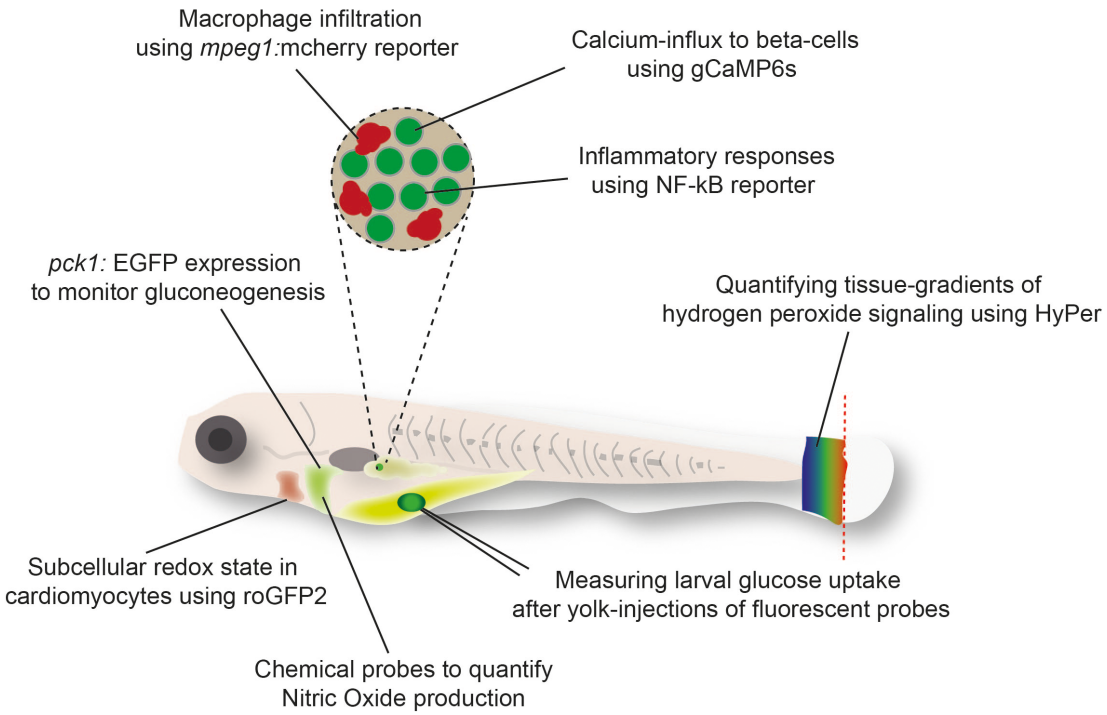

FIGURE 1 | Examples of fluorescent reporter tools used in zebrafish larvae to monitor metabolic responses in cells or tissues.

background of genetically modified zebrafish, following the treatment with drugs or toxins, or after exposure to tissue damage. For example, the use of genetically encoded H_2O_2 sensor HyPer has revealed the critical function of H_2O_2 as a chemoattractant released from the wound edge (Niethammer et al., 2009). However, HyPer is also sensitive to changes in pH and therefore requires careful control with pH sensors such as SypHer (Roma et al., 2012; Weller et al., 2014). Further pioneering work has been done using sensor probes for the cellular redox state that are less sensitive to pH, such as recently developed novel transgenic zebrafish lines expressing the metabolic redox biosensors roGFP2-Orp1 and Grx1-roGFP2 (Morgan et al., 2011) in endothelial and myocardial cells (Panieri and Santoro, 2017). These reporters rely on ratio-metric imaging of the sensor for real-time imaging of hydrogen peroxide (H_2O_2) levels and the redox potential of glutathione (E_{GSH}) in specific subcellular compartments (Panieri and Santoro, 2017). Specifically, imaging these sensors showed higher basal levels of H_2O_2 in the mitochondrial matrix than other sub-cellular compartments (Panieri et al., 2017). Similarly, the mitochondrial matrix was characterized by more oxidizing E_{GSH} compared to the cytosol and the nucleus (Panieri et al., 2017). Pharmacologic treatments suggest that the pentose phosphate and glutathione biosynthetic pathways exert a protective antioxidant role *in vivo* in endothelial cells and cardiomyocytes (Panieri et al., 2017).

Similarly, transgenic expression of other ratio-metric fluorescent biosensors for metabolites are on the way. Among those, Perceval HR (Berg et al., 2009) for measuring ATP/ADP ratio and Peredox for measuring NADH-NAD(+) ratio (Hung et al., 2011) are widely used biosensors in mammalian systems. Validation in zebrafish is lacking, but efforts to use them in live larvae are ongoing (unpublished data; YF). Förster Resonance Energy Transfer (FRET) based metabolite reporters are also promising in their application in zebrafish live imaging. Transgenic expression of the lactate FRET reporter. As more genetically encoded metabolite reporters are developed (Jensen et al., 2006; Gruenwald et al., 2012; Luddecke et al., 2017), we envisage that many of these reporters can also be used in zebrafish models to image and quantify metabolism at the cellular and subcellular levels.

In addition to genetically encoded metabolite probes, fluorescently labeled carbon sources such as glucose, lactate, and lipids analogs can be used to trace their uptake into cells *in vivo* in zebrafish embryos [(Marin-Juez et al., 2015; Anderson et al., 2016) and unpublished data; YF]. There is also increasing interest in developing novel fluorescent chemical probes for various metabolites, ions and redox species. Some of these tools have been successfully tested in zebrafish embryos such as a fluorescent sensor to detect Nitric Oxide in liver cells of zebrafish (Zhang et al., 2018a). Other probes include a polymer micelles-based ratio-metric fluorescent probe for hypochlorous acid (HClO) to monitor HClO generation during liver injury *in vivo* in zebrafish embryos (Zhang et al., 2018b).

However, most metabolite sensors are developed and optimized in mammalian tissue culture systems. Additional efforts are required to validate their sensitivity and accuracy in zebrafish embryos, which is particularly the case for FRET

TABLE 2 | Selection of reporter tools to quantify metabolites.

Tool	Method	Description	Validated in zebrafish	Reference
Perceval	Fluorescent biosensor	Genetically encoded ratiometric fluorescent reporter for ATP/ADP ratios	No	Berg et al., 2009
GCaMP6s	Fluorescent biosensor	Genetically encoded intensiometric fluorescent reporter for calcium	Yes	Chen J. et al., 2017; Singh et al., 2017; Janjuha et al., 2018a
RoGFP2-Orp1	Fluorescent sensor	Genetically encoded ratiometric fluorescent reporter for H_2O_2 detection	Yes	Panieri et al., 2017
Grx1-RoGFP2	Fluorescent sensor	Genetically encoded ratiometric fluorescent reporter for GSH:GSSG redox potential	Yes	Panieri et al., 2017
Cyto-roGFP	Fluorescent biosensor	Genetically encoded ratiometric fluorescent reporter for Redox state (Cytosol)	No	Waypa et al., 2010
Matrix-roGFP	Fluorescent biosensor	Genetically encoded ratiometric fluorescent reporter for Redox state (Mitochondrial Matrix)	No	Waypa et al., 2010
GPD-roGFP	Fluorescent biosensor	Genetically encoded ratiometric fluorescent reporter for Redox state (Mitochondrial Innermembrane space)	No	Waypa et al., 2010
Pyronic	FRET biosensor	Genetically encoded ratiometric fluorescent reporter for pyruvate	No	San Martin et al., 2014
Laconic	FRET biosensor	Genetically encoded ratiometric fluorescent reporter for lactate	No	San Martin et al., 2013
pHRed	Fluorescent biosensor	Genetically encoded intensiometric fluorescent reporter for pH	No	Tantama et al., 2011
Peredox-mCherry	FRET biosensor	Genetically encoded intensiometric fluorescent reporter for NADH/NAD ratio	No	Hung et al., 2011
iNap1	Fluorescent biosensor	Genetically encoded ratiometric fluorescent reporter for NADPH	Yes	Zou et al., 2018
SoNar	Fluorescent biosensor	Genetically encoded fluorescent reporter for NADH	Yes	Zhao et al., 2016; Zou et al., 2018
HyPerRed	Fluorescent biosensor	Genetically encoded intensiometric fluorescent reporter for H_2O_2	Yes	Zou et al., 2018

probes that often have been optimized to function at 37°C. Once a reliable imaging protocol is established, these sensors will be invaluable tools to monitor dynamic metabolic changes in specific tissues, cells and sub-cellular compartments in physiology and disease conditions. **Table 2** summarizes reporter tools that can be used to quantify metabolites and includes information whether these tools have been tested in zebrafish yet. Although these tools will not be able to replace traditional biochemical approaches on sampled tissues, the live observation of metabolites and signaling events *in vivo* can provide invaluable insights into metabolic regulation.

REPORTER TOOLS TO SHED LIGHT INTO CELLULAR HETEROGENEITY OF BETA-CELLS

A pertinent example for employing reporter tools to understand the function of individual cells within their tissue-context comes from studies of pancreatic beta-cells. Insulin-secreting beta-cells play a central role in glucose homeostasis, as their loss or malfunction can lead to the onset of diabetes. Beta-cells show a high plasticity in response to metabolic challenges or in pathological conditions, increasing interest in studying beta cell turnover and function at cellular resolution (Ninov et al., 2013; Chen C. et al., 2017). Studies on beta-cell biology in zebrafish so far have mainly used fluorescent reporter lines to study the processes of beta-cell differentiation and regeneration (Prince et al., 2017). These studies have defined the progenitor lineages for beta-cell formation during development and regeneration using genetic lineage-tracing techniques (Hesselson et al., 2009; Wang et al., 2011; Ninov et al., 2013; Delaspre et al., 2015). In addition, they have revealed novel signaling pathways that regulate beta-cell differentiation, proliferation and regeneration (Andersson et al., 2012; Tsuji et al., 2014; Wang et al., 2015) as well as the importance of inter-organ communication (Lu et al., 2016) and the gut microbiota for these processes (Hill et al., 2016).

However, several critical aspects of beta-cell biology that have taken a central stage in the mammalian pancreas field require monitoring of functional read-outs, and await to be examined in zebrafish. For example, the process of maturation of beta-cells toward glucose-stimulated insulin secretion has not been investigated extensively in the zebrafish pancreas. Addressing functionality is important as recent studies in mice have shown that beta-cell death might not be the primary reason for the loss of functional beta-cells in diabetes. Instead, beta-cells in diabetic conditions lose their identity and undergo a process of dedifferentiation, in which they stop expressing beta-cell markers (Bensellam et al., 2018). Thus, it will be necessary to establish models in zebrafish that recapitulate the dedifferentiation of beta-cells observed in mouse and human islets.

Toward this end, it was recently shown that beta-cells in zebrafish larvae show glucose-stimulated calcium influx and expression of markers of mature beta-cells, opening an avenue to use the zebrafish as a model to address the final step of beta-cell differentiation and maturation (Singh et al., 2017). Specifically, the genetically encoded calcium indicator, GCAMP6s, was expressed under the insulin promoter to quantify influx of calcium into beta-cells. Calcium binds to

GCaMP6s and activates a conformational change leading to the emission of green fluorescence. Since calcium influx in beta-cells correlates with insulin secretion (Bergsten et al., 1994), this system makes it possible to visualize the function of beta-cells with single-cell precision. When combined with lineage-tracing of different beta-cell populations, this approach revealed the presence of a functional heterogeneity and a trade-off between proliferative potential and maturity among beta-cells (Singh et al., 2017). Further efforts and new tools will be necessary, however, to visualize the actual release of insulin from zebrafish beta-cells, which remains an outstanding goal in the field.

Moreover, the interactions between the immune system and beta-cells play a critical role in diabetes pathogenesis, yet these processes have not been modeled in the zebrafish pancreas. Implementing models of beta-cell inflammation and auto-immunity would allow one to study how these interactions are controlled in response to metabolic stress and aging (Janjuha et al., 2018b). A recent study applied the zebrafish genetics and transgenic reporter for activated inflammation to reveal the presence of an inflammatory clock that marks the proliferative-decline of beta-cells with age. In this clock, beta-cells that activate inflammatory NF-kB signaling also prematurely upregulate *socs2*, an age-related gene that inhibits their proliferation (Janjuha et al., 2018b). This work suggests that certain aspects of beta-cell biology such as their capacity to proliferate depend on interactions with the islet-resident innate-immune cells. However, it will be necessary to further validate the zebrafish as a model to investigate the complex crosstalk of metabolism, immunity and organ function. In this regard, two recent papers showed that *foxp3* marks regulatory T-cells (Tregs) in zebrafish and that *foxp3* mutants display systemic inflammation, suggesting an involvement of these cells in the maintenance of immune tolerance (Hui et al., 2017; Kasheta et al., 2017). These results recapitulate in part the situation in humans where mutations in *FOXP3* predispose to multi-organ autoimmunity (Sugimoto et al., 2017). In the future, it will be important to investigate whether aberrant selection of immune cells during T-cell maturation or prolonged exposure to self-antigens in combination with genetic and environmental risk factors can be applied to model certain aspects of autoimmune diseases such as type 1 diabetes in zebrafish. The repertoire of zebrafish immune cells is not fully understood and one needs to carefully consider differences in immune cell and cytokine profiles between zebrafish and mammals. However, models are emerging that can be used to monitor T-cell development and migration within their niche (Tian et al., 2017; Aghaallaei and Bajoghli, 2018), and will help to further characterize the zebrafish immune repertoire.

Being able to assess beta-cell activity under metabolic and inflammatory stress is critical to identify small molecules that prevent the loss of its function in diabetes. We propose that some of the above-mentioned tools allowing to quantify the rate of ROS production or the metabolic state of cells can be applied to beta-cells as well. These tools, in conjunction with small molecule screening, can facilitate the discovery of novel therapeutic interventions that intervene at different levels in the cascade responsible for beta-cell stress and dysfunction.

CONCLUSION AND FUTURE APPROACHES

Progress has been made to exploit the advantages of zebrafish for studying the control of energy metabolism at tissue, cellular and subcellular resolutions. Achieving this level of resolution is critical considering the specialization of metabolically active tissues that often show different, and in some cases even opposite, homeostatic responses to metabolic challenges. Performing whole-larval transcriptomics metabolomics or proteomics analyses therefore provides limited information. The community should apply nano- or micro-sampling approaches wherever possible, facilitated by an active exchange of protocols and access to state-of-the art technologies. The same is the case for the use of reporter tools that often require experience and an optimized set-up, but once implemented provide powerful technologies to perform metabolic studies within the context of a live organism in physiological or pathological states.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PERSPECTIVE
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Zebrafish as a Model for the Study of Live *in vivo* Processive Transport in Neurons

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Motor proteins are responsible for transport of vesicles and organelles within the cell cytoplasm. They interact with the actin cytoskeleton and with microtubules to ensure communication and supply throughout the cell. Much work has been done *in vitro* and *in silico* to unravel the key players, including the dynein motor complex, the kinesin and myosin superfamilies, and their interacting regulatory complexes, but there is a clear need for *in vivo* data as recent evidence suggests previous models might not recapitulate physiological conditions. The zebrafish embryo provides an excellent system to study these processes in intact animals due to the ease of genetic manipulation and the optical transparency allowing live imaging. We present here the advantages of the zebrafish embryo as a system to study live *in vivo* processive transport in neurons and provide technical recommendations for successful analysis.

Keywords: neuronal transport, zebrafish, myosin, kinesin, dynein, *in vivo*

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INTRODUCTION

Processive intracellular transport is essential for the distribution of organelles and cellular cargoes within the cell. In the case of neurons, such transport provides communication between different cell compartments and ensures supply to the growing synapse, clearance of detritus and serves as the support for intracellular signaling (Hirokawa et al., 2010). This process relies on the function of motor proteins and their interaction with the cell cytoskeleton, the three components of which play important roles in regulating transport. Indeed, neurofilaments provide structure and regulate axonal caliber, which influences transport metrics. Microtubules are responsible for axonal polarity, a consequence of the stereotyped orientation of their dynamic fast-growing ends, and act as the rails guiding motor proteins within the axon and dendrites. Finally, actin filaments form a structural network supporting the growth cone, pre- and postsynaptic regions, and play an important role in dendrites where they form the spines, essential for synaptic transmission.

Dynein and the kinesin superfamily are the unidirectional molecular motors responsible for transport on microtubules, both in dendrites and axon. In polarized axons, it is split according to the direction relative to the microtubule fast-growing end (+), with kinesins being responsible for 'anterograde' movement (toward the synapse) and dynein for 'retrograde' movement (toward the cell body). Their movement can in turn be categorized as 'slow' or 'fast' depending on their transport rate. Slow axonal transport is mainly used for delivery of cytoskeletal components and associated proteins, with kinetics in the range of 0.2–8 mm/day (Lasek et al., 1984). Fast axonal transport is used for organelles and vesicles, but also for mRNA granules (Maday et al., 2014), with kinetics in

the range of 50–400 mm/day (Lasek et al., 1984). This type of transport, in terms of motor complex involved for different types of cargoes, motor adaptor complexes and transport metrics, has been widely studied and is well reviewed elsewhere (Maday et al., 2014).

Unconventional myosins, molecular motors of the actin cytoskeleton, are commonly associated with dynamic shaping of membranes, as well as organelle formation and transport, but their functions in neuronal transport is not well understood. Among this super-family, Myosin5a, 5b, 6 and 10 have been identified as processive transporters in neurons, and participate in local transport of intracellular cargoes over short-range distances. Processive myosins are likely part of a cooperative mechanism which is based on the coordination of actin and microtubule transporters (Wu et al., 2000). This is nicely illustrated with Myo5a binding directly to kinesins (Huang et al., 1999), suggesting that organelles transported in axons along microtubules may be transported by Myo5a in presynaptic terminals, which lack microtubules (Wu et al., 1998; Bridgman, 1999; Lalli et al., 2003; Nalavadi et al., 2012). Processive myosins are implicated in vesicle endocytosis, recycling and exocytosis, and hence participate in receptor transport and localization, regulating neuronal signaling and axonal pathfinding (Wu et al., 2002; Osterweil et al., 2005; Zhu et al., 2007; Correia et al., 2008; Nash et al., 2010; Lazo et al., 2013; Sui et al., 2015). Moreover, processive myosins take part in transport of mRNAs and RNPs in neurons, as demonstrated for Myo5a (Ohashi et al., 2002; Yoshimura et al., 2006; Balasanyan and Arnold, 2014; Calliari et al., 2014; Lindsay and Mccaffrey, 2014).

From recent evidence, it is apparent that *in vivo* axonal transport data do not recapitulate what has been observed *in vitro* (Gibbs et al., 2016; Klinman and Holzbaur, 2016; Knabbe et al., 2018), emphasizing the need for a more physiological context. With this in mind, excellent work has been published reporting *in vivo* axonal transport (reviewed in Sleigh et al., 2017) in models such as the *Drosophila* wing (Vagnoni and Bullock, 2017) and larvae (Vukoja et al., 2018), as well as the mouse brain (Knabbe et al., 2018) and sciatic nerve (Gibbs et al., 2016). All of these models have advantages and drawbacks: the mouse model is widely used and as a mammal, has a high genetic conservation of genes of interest but is not translucent and only allows access to axonal transport in a restricted area of the targeted cell population by way of surgery. The *Drosophila* is a model with a fantastic genetic manipulation toolbox, however, it is an invertebrate with reduced conservation to human compared to vertebrate models.

Over the last decades, zebrafish has emerged as a powerful vertebrate model to study the development of the nervous system *in vivo*. Adult zebrafish are small in size and produce a large number of offsprings, with a rapid external development. The embryonic zebrafish are translucent, and recent advances in genetic manipulation have made this model a great option to monitor neurodevelopment by high-resolution live imaging and at single-cell level. In addition, the zebrafish embryo is used extensively for modeling neurodegeneration (Bandmann and Burton, 2010; Kabashi et al., 2010; Santoriello and Zon, 2012; Babin et al., 2014; Patten et al., 2014; Fontana et al., 2018). Some processive motors have been associated with neurological disorders (Chen et al., 2013) and many studies have reported axonal transport defects in the context of neurodegenerative diseases (Chevalier-Larsen and Holzbaur, 2006; Goldstein, 2012; Liu et al., 2012; Millecamps and Julien, 2013), further outlining the interest of this model. In this article, we thus discuss the advantages of the zebrafish model in the study of live *in vivo* intracellular transport, with a particular focus on fast axonal transport.

ADVANTAGES OF THE ZEBRAFISH MODEL

Relevance to Mammalian Models

The genome of *Danio rerio* is fully sequenced and presents at least one ortholog for 70% of human genes (Howe et al., 2013). In particular, kinesin, dynein and myosin molecular motors implicated in neuronal transports are extremely well conserved in eukaryotes and even more in vertebrates (Kim and Endow, 2000; Sittaramane and Chandrasekhar, 2008). These proteins have a higher conservation with the human ortholog in zebrafish compared to *D. melanogaster* for example. Zebrafish and drosophila dynein Dync1h1 show 91% and 72% identity (NCBI Blastp) with the human protein, respectively. Similarly, the processive Myo6 is 85% and 53% identical to the human one in zebrafish and drosophila, respectively. This high degree of conservation provides support for using zebrafish as a model system to investigate the functions of these molecular motors.

Genetic Manipulations

Compared especially to the mouse, the ease of stable or transient genetic manipulations has positioned the zebrafish as an ideal vertebrate model for live *in vivo* imaging.

Transgenesis in zebrafish is routinely and efficiently performed to express fusion proteins, mutated proteins or the *gal4* transcription factor under a tissue-specific promoter thanks to the use of transposon elements. Ease of genetic manipulations in zebrafish has tremendously increased with the development of the CRISPR/Cas9 technology. The generation of knock-out mutants has become extremely powerful (Hwang et al., 2013) and using a Gal4/UAS-based restriction of Cas9 expression makes it possible to induce tissue-specific mutations and restrict the phenotype to a subset of cells (Di Donato et al., 2016). Recent advances based on the fusion of a mutated Cas9 (nickase) with an acetyl deaminase leading to the precise editing of a single nucleic acid (Komor et al., 2016) was also shown to work in zebrafish (Zhang et al., 2017). This technology makes it possible to target a specific protein domain in order to interfere with protein–protein interaction and opens the possibility of reproducing mutations associated with human diseases to elucidate the underlying pathological mechanism.

To recapitulate endogenous expression of a protein of interest, both in terms of pattern and level, bacterial artificial chromosome (BAC) transgenesis, where very large DNA sequence (up to 300 kb) can be inserted into the genome, is used in zebrafish (Lee et al., 2001; Suster et al., 2011). The CRISPR/Cas9 era has now opened the possibility of direct knock-in at a targeted

locus. This strategy has been successful in zebrafish, based on the error-prone non-homologous end-joining DNA damage repair mechanism (Auer et al., 2014) and by short or long homology arm recombination (Hruscha et al., 2013; Hwang et al., 2013; Irion et al., 2014; Zhang et al., 2016). However, the efficiency of the latter technique is still low and locus-dependant. Its optimization will be an important technical advance in the field (Albadri et al., 2017), for example, to allow endogenous expression of a fusion protein of choice for visualization *in vivo,* without overexpression.

Pharmacological Manipulations

Zebrafish embryos are amenable to pharmacological treatment by bath application, allowing for treatment of intact, live embryos with compounds known for the modulation of cytoskeletal dynamics, for instance, targeting microtubules: Colchicine (Roche et al., 1994), vinblastine (Keiter et al., 2016; Yao et al., 2017), vincristine (Mizgirev and Revskoy, 2010; Khan et al., 2012; Holloway et al., 2016), nocodazole (Plucińska et al., 2012; Jayachandran et al., 2016) and paclitaxel (Jayachandran et al., 2016). For the actin cytoskeleton: Cytochalasin D (Nukada et al., 2015; Artelt et al., 2018) and latrunculin A (Artelt et al., 2018), jasplakinolide (Artelt et al., 2018), phalloidin oleate (Dutta and Kumar Sinha, 2015), and the inhibitor of actin–myosin interaction BDM (Norden et al., 2009) have been used with success.

Finally, zebrafish embryos are well suited to high-throughput approaches that have made them an excellent tool in drug discovery by small molecule screening (Zon and Peterson, 2005; Mathias et al., 2012; Miscevic et al., 2012; Tamplin et al., 2012; MacRae and Peterson, 2015).

EXAMPLES AND RECOMMENDATIONS FOR THE ANALYSIS OF *IN VIVO* TRANSPORT IN ZEBRAFISH

To date, a few studies have taken advantage of the zebrafish model to perform *in vivo* axonal transport assays, generating tools to study the movement of mitochondria (Plucińska et al., 2012; Campbell et al., 2014; Paquet et al., 2014; Auer et al., 2015; Drerup et al., 2017), endosomes (Clark et al., 2011; Ponomareva et al., 2014, 2016), autophagosomes (He et al., 2009), lysosomes (Drerup and Nechiporuk, 2013), synaptophysin-containing vesicles (Auer et al., 2015) as well as motor proteins and components of their regulatory complexes (Drerup and Nechiporuk, 2013, 2016). The *in vivo* analysis of myosin-based transport is only starting in zebrafish neuronal development (Liu et al., 2013).

Based on published evidence, it is plain to see that the metrics reported for the same cargo visualized *in vivo* in zebrafish display variation between cell types and developmental stages. Indeed, we have observed metrics for mitochondrial anterograde transport in primary motor neurons (MN; axon) and in retinal ganglion cells (RGC; arbor) and while we did not find differences in average run speed, average run length and duration were significantly different in these two cell types. Furthermore, the average run speed detected was approximately 0.4 μm/s (**Figure 1C**), which is consistent with reported data from Campbell et al. (2014) (peripheral sensory neuron arbors, approx. 0.4 μm/s) but inconsistent with data from Plucińska et al. (2012) (peripheral Rohon-Beard sensory neuron axons, approx. 1.2 μm/s 'moving speed' and 0.6 μm/s 'average speed') and from Drerup et al. (2017) (peripheral lateral line axon, approx. 1.0 μm/s). We also found discrepancies between cell types in the transport of recycling endosomes (labeled with Rab11a-GFP), where we observed an average speed of approx. 0.5 μm/s (**Figure 2B**), whereas Ponomareva et al. (2014) report an average speed of approx. 0.18 μm/s/0.03 μm/s (central/peripheral Rohon-Beard sensory neuron axon).

Based on the evidence above outlining the variability of these processes, we will highlight a few key points to take into consideration when designing experiments to characterize transport in zebrafish neurons.

Regulation of Construct Expression and Imaging

Most approaches discussed here rely on the overexpression of fusion proteins, allowing *in vivo* detection of the bound fluorescent protein. This can be achieved injecting DNA constructs to obtain single-cell labeling of cargoes, as shown here (**Figure 1A**), or creating stable transgenic lines, where restriction of expression can be achieved by a combination of Gal4- and UAS-expressing lines. While this technique produces a bright signal well suited to time-lapse imaging, overexpression of protein can lead to deleterious effects by interfering with endogenous expression and triggering stress response mechanisms (Cheng and Lee, 2010). It is therefore essential to ensure that the construct does not lead to toxicity by monitoring cell morphology and embryonic development. The acquisition parameters in time-lapse microscopy are optimized to limit bleaching of fluorescent proteins and damage of the target cell, while still observing the target movement (for instance: high frequency sampling but reduced duration). In the case of the examples presented here, time-lapse imaging of labeled cargo in neurons was performed at 2 Hz for endosomes (5 min duration; **Figure 2B**) and at 1 Hz for mitochondria (10 min duration; **Figures 1B,C**) on a spinning disk confocal microscope.

Single Cell Type

Axonal transport dynamics can be influenced by the varying expression of subunits composing the motor protein complexes or particular cargo adapters, as well as by the axon caliber, due to differences in microtubule density affecting engagement of motors (Yu et al., 2017) and due to activity- and myelination-dependent number of neurofilaments (de Waegh et al., 1992). It is therefore recommended to target one cell type (**Figures 1B,C**), and in the case of spinal cord neurons, to limit observation to a specific region as cell size can fluctuate along the trunk and tail owing to the rostro-caudal developmental wave.

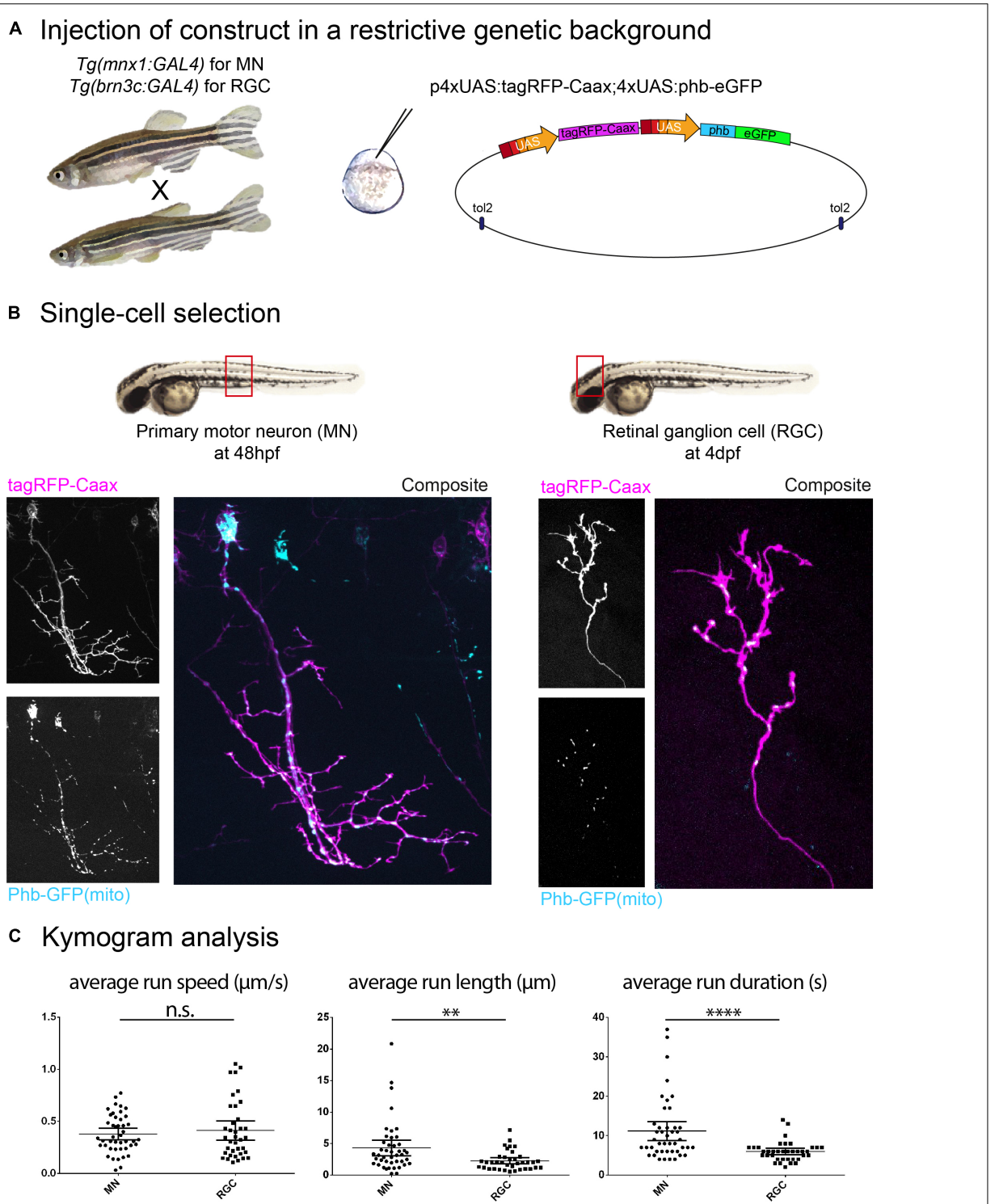

FIGURE 1 | Construct expression and cell type selection. **(A)** Injection of DNA constructs coding for fusion proteins can be restricted to a single cell type by use of the Gal4/UAS system. **(B)** In this example, we injected a UAS construct labeling mitochondria (phb, prohibitin-GFP see schematics in **A**) combined with a membrane reporter (tagRFP-Caax). We obtained labeling of a single primary motor neuron (MN; in the *Tg(mnx1:gal4)* background) and a single retinal ganglion cell (RGC; in the *Tg(brn3c:gal4)* background), respectively, in the embryonic spinal cord (48 hpf) and in the larval optic tectum (4 dpf). **(C)** Time-lapse imaging of mitochondria (1 Hz for 10 min) was performed on these cell types, and transport dynamics were calculated from kymograms. Here, we show example of the disparity in transport metrics that can arise when comparing different cell types for a single cargo (MN $n = 7$ cells/44 anterograde runs; RGC $n = 7$ cells/37 anterograde runs). $^{**}p < 0.01$, $^{****}p < 0.0001$.

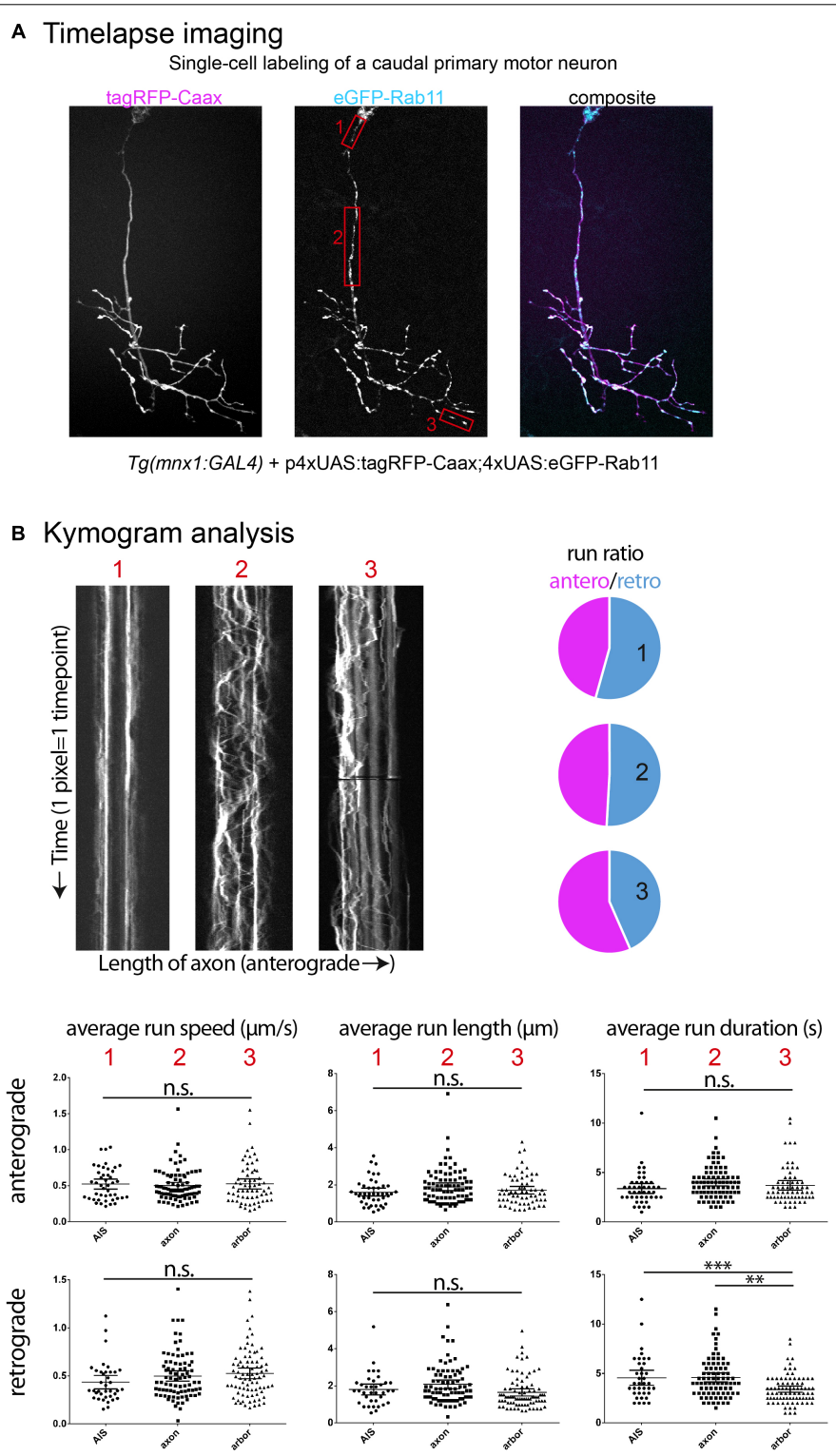

FIGURE 2 | Examples of time-lapse analysis. **(A)** As described in **Figure 1A**, single cell labeling of primary motor neurons was obtained for recycling endosomes (Rab11a-eGFP), combined with a membrane reporter (tagRFP-Caax) to identify cell type. Red boxes: Three cell compartments were imaged (2 Hz for 5 min), 1-axon initial segment, 2-mid-axonal segment, 3-axonal arbor segment. **(B)** Kymograms were generated from the time-lapses acquired (Kymograph tool, ImageJ) and a variety of transport metrics can be calculated manually (compiled in Excel, statistics in Graphpad Prism6). In this example, significant differences between neuronal segments are detected for the transport direction ratio (anterograde/retrograde), and retrograde run duration (n = 3 cells; AIS n = 47/54 anterograde/retrograde runs; mid-axon n = 85/82 anterograde/retrograde runs; arbor n = 63/86 anterograde/retrograde runs). $^{**}p < 0.01$, $^{***}p < 0.001$.

Single Cell Compartment

Different cell compartments have different cytoskeletal composition and regulatory mechanisms that can affect the composition and expression of motor proteins and their adaptor proteins, hence influencing the regulation of axonal transport (de Waegh et al., 1992; Yu et al., 2017; Gumy and Hoogenraad, 2018). As shown here (**Figure 2**), time-lapse imaging in different compartments of the same neuron for the same cargo can yield significant differences in some metrics of transport dynamics, but not others. The selection of a compartment most suited to the research hypothesis and consistency in the segment imaged across individual embryos and larva appears thus crucial, as well as the analysis of a variety of metrics such as run length and duration, average speed, pause frequency, average pause duration, switching behavior, area flux and transport rates of cargoes, both in the anterograde and retrograde directions.

PERSPECTIVES FOR FUTURE RESEARCH

Alternative Labeling

Nanoparticles are inorganic semiconductors representing an attractive alternative for fluorescent labeling in live imaging applications because of their high spatial resolution and photostability. In addition, it is possible to tune their emission wavelength by varying their size and chemical composition. Because of this, and their broad absorption profile, it is possible to excite multiple colors at once, which is useful to reduce sample phototoxicity (Gao et al., 2005). In contrast to genetically encoded fluorescent protein tags, however, they need to be efficiently targeted to their biologically relevant endpoint. This has so far relied on surface modifications and solubilization strategies that led to very large particles better suited to high-sensitivity detection of low number of targets, such as single-molecule detection (Pinaud et al., 2006). Of note, this approach has allowed for real-time visualization of single-molecules in living cells (Dahan et al., 2003). Conjugation to biomolecules is, however, an interesting avenue to allow precise targeting and while still requiring the expression of a genetically encoded protein adaptor (Gao et al., 2005; Howarth et al., 2005), would provide the advantageous optical properties of nanoparticles over traditional fluorescent proteins.

Microscopy Improvements

Advances in imaging technology in the last years have yielded many optimized systems applicable to the study of *in vivo* axonal transport in the zebrafish embryo. Indeed, a great example of this is the swept field confocal microscope, which permits higher frame-rate capture when compared with spinning disk confocal and allows the rapid acquisition of z-stack time-lapses or high speed imaging (upward of 1,000 fps) of movement in single-plane (Castellano-Muñoz et al., 2012). Other systems circumvent classic caveats to an *in vivo* approach, such as photodamage, single-plan and temporal restriction and low signal, for instance: 2-photon microscopy (Renninger and Orger, 2013), light-sheet microscopy (Huisken et al., 2004; Panier et al., 2013; Park et al., 2015; Tomer et al., 2015; Fu et al., 2016). In the context of *in vivo* imaging of axonal transport, these strategies could allow the tracking of cargo and motors with exquisite temporal resolution, while also permitting 3D tracking in a whole embryo over long periods of time; considerable advantages over *in vitro* and other *in vivo* models.

Automated Detection and Analysis

The generation of kymograms as a 2D representation of time-lapse imaging is a common tool for the analysis of axonal transport, where the tracked target often moves on a single focal plane, in a linear trajectory. When analyzing movement in more complex environment, however, single particle tracking becomes a necessity, which renders manual analysis an arduous task. In the past years, many options have become available for automated detection and tracking, both commercially (Imaris, Metamorph, Igor Pro, etc.) and *via* open-source programs (MATLAB, ImageJ, etc.). Still, time-lapse videos obtained *in vivo* from intact animals are often noisier by nature than their cell culture counterpart, and since these samples are prone to photodamage, lead to undersampled data. This in turn impedes automatic detection and requires manual check of extracted metrics, while possibly omitting crucial information. Further advances in detection algorithms, based on *in vivo* data estimating how cargoes should behave, will surely be of benefit to researchers facing the tedious task of manual tracking.

CONCLUSION

The zebrafish embryo has emerged as an excellent model to pursue the characterization of processive transport *in vivo* as it can meet the need for more inclusive models, where the contribution of neuronal activity, glia and the cell cytoskeleton are taken into account. We outlined here some advantages and technical hints to use the zebrafish model for this type of analysis. Considering the recent breakthroughs in genetic manipulations and imaging technologies, this vertebrate is gaining attention in the field of neurodegenerative disease modeling, where axonal transport deficits are common hallmarks. In addition, a new emerging model sharing the same subfamily as zebrafish, *Danionella translucida*, which remains transparent throughout its life, will further expand the possibilities of adult neuronal imaging *in vivo* (Schulze et al., 2018). It is thus only a matter of time before axonal transport assays in zebrafish embryos become widespread for the study of physiological and pathological conditions.

ETHICS STATEMENT

As the EU directive 2010_63 explicitly states only "independently feeding larval forms" must be classified as animal experiments, therefore only zebrafish larvae past 120 h post fertilization should be subject to the regulations of European animal protection guidelines. For our experiments, we did not use larvae that

have reached an "independent feeding" stage and therefore we did not have to submit an ethical approval to the competent local/national ethical/legal bodies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Advantages and Challenges of Cardiovascular and Lymphatic Studies in Zebrafish Research

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Since its introduction, the zebrafish has provided an important reference system to model and study cardiovascular development as well as lymphangiogenesis in vertebrates. A scientific workshop, held at the 2018 European Zebrafish Principal Investigators Meeting in Trento (Italy) and chaired by Massimo Santoro, focused on the most recent methods and studies on cardiac, vascular and lymphatic development. Daniela Panáková and Natascia Tiso described new molecular mechanisms and signaling pathways involved in cardiac differentiation and disease. Arndt Siekmann and Wiebke Herzog discussed novel roles for Wnt and VEGF signaling in brain angiogenesis. In addition, Brant Weinstein's lab presented data concerning the discovery of endothelium-derived macrophage-like perivascular cells in the zebrafish brain, while Monica Beltrame's studies refined the role of Sox transcription factors in vascular and lymphatic development. In this article, we will summarize the details of these recent discoveries in support of the overall value of the zebrafish model system not only to study normal development, but also associated disease states.

Keywords: zebrafish models, vascular, lymphatic, heart, trangenesis

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INTRODUCTION

Zebrafish has been utilized over the past years to successfully study cellular and genetic processes regulating the development of cardiac, vascular, and lymphatic development (Liu and Stainier, 2012; Hogan and Schulte-Merker, 2017; Mauri et al., 2018). Among the several advantages of the zebrafish model system is the large number of embryos that each mating generates (an extraordinary resource for further experimental needs), external development (e.g., easy accessibility for experimental manipulation and imaging), and optical clarity. Distinctively, the optical clarity of zebrafish embryos allows to easily perform live imaging of cells and to track them in the context of a whole organism. An advantage of this feature is the use of transgenic zebrafish animals marking cardiac, blood, endothelial, and lymphatic endothelial cells with genetically encoded markers. Within the last decade, a considerable number of zebrafish fluorescent transgenic

lines have been generated to better characterize different components of the vascular system, such as myocardial and endocardial cells, arteries, veins, and lymphatic tissues. The availability of tissue-specific transgenic lines and advanced microscopy techniques (i.e., light sheet and confocal microscopy), have permitted high-resolution time-lapse imaging of developing heart structures, lymphatic and blood vessels, as well as perivascular cells at the subcellular level (Isogai et al., 2001; Huisken and Stainier, 2009; Betz et al., 2016). Such dynamic imaging allows for the dissection of the mechanisms underlying heart and blood/lymphatic vessel patterning, branching, and their regulation.

Furthermore, zebrafish are amenable to both forward genetic approaches (e.g., ENU mutagenesis screens), and reverse genetic studies (such as TALEN and CRISPR/Cas9) (El-Brolosy and Stainier, 2017; Housden et al., 2017). Cre/lox recombinase-based genetics to delete genes of interest in a tissue-specific manner has also been successfully applied to study cardiovascular development and homeostasis (Carney and Mosimann, 2018). Here, we will review the most recent methods and studies on cardiac, vascular, and lymphatic development presented at the recent 2018 European Zebrafish Principal Investigators Meeting in Trento, Italy.

RECENT ADVANCEMENTS ON CARDIAC, VASCULAR, AND LYMPHATIC RESEARCH IN ZEBRAFISH

Heart Studies

The heart is the first functional organ to form in the vertebrate embryo. While already pumping and maintaining constant blood flow, the heart undergoes profound morphogenetic changes to acquire its final shape. The periodic changes in the mechanical forces as well as intracellular calcium concentrations contribute considerably to the final organ form and function. From the specification of cardiac progenitors, the genetic programs controlling heart formation and function, to cell biological processes of heart morphogenesis, researchers in the zebrafish field have contributed significantly to our understanding of heart development over the last decade (Bakkers, 2011; Staudt and Stainier, 2012). Although several mutants affecting cardiac function have already been isolated during the first large-scale mutagenesis screens (Stainier et al., 1996), the functional patterning of the developing myocardium is a less explored area of research. This is partially due to the lack of *in vivo* techniques to study action potential propagation or changes in intracellular calcium concentrations during each heartbeat. High-speed optical mapping techniques are currently limited to an *ex vivo* analysis (Panáková et al., 2010), however, the growing number of fast genetically encoded voltage and calcium indicators, together with the recent advances *in vivo* imaging (Weber et al., 2017), will allow researchers to close this gap of knowledge in the very near future.

Pertaining to the functional patterning of the developing myocardium, Dr. D. Panáková expanded on already reported data showing the Wnt11 non-canonical pathway regulating the intercellular electrical coupling in the developing zebrafish heart through attenuation of the L-type calcium channel (LTCC) conductance (Panáková et al., 2010). The LTCC has an important function in cardiac biology. Its conductance is regulated by membrane depolarization or by the β-adrenergic/Protein kinase A (PKA) pathway, and mediates Ca^{2+} influx. The molecular mechanisms by which Wnt11 signaling might modify LTCC, and thus regulate its activity, were unclear. The research group of Dr. Panáková found that Wnt11 signaling regulates the LTCC through its effects on the A-kinase anchoring protein (AKAP)/PKA signalosome. They further showed that the AKAP/PKA signalosome is an essential component of Wnt11 signaling regulating channel function during the establishment of intercellular electrical coupling gradients in the developing zebrafish heart.

The field of arrhythmias is indeed another exciting area where zebrafish models have been successfully applied, as demonstrated by the pioneering work of the MacRae and Saffitz groups (Milan and MacRae, 2008; MacRae, 2010; Asimaki et al., 2014). In the specific context of inherited arrhythmogenic cardiomyopathies (AC), mostly linked to defects in desmosomal components (Pilichou et al., 2016), Dr. Tiso's group presented the first zebrafish model for human AC type 8 (AC8), a form of AC caused by mutations in the desmosomal protein desmoplakin (Rampazzo et al., 2002). By knocking down both zebrafish desmoplakin genes (*dspa* and *dspb*) Tiso's team could specifically phenocopy the ultrastructural features and cardiac frequency defects of AC8. Moreover, the availability of zebrafish lines, which are able to report *in vivo* the activation of a series of signaling pathways (Moro et al., 2013), was exploited in the study to analyze a set of signals known to be involved in cardiac function.

The screen allowed to identify three pathways (Wnt/beta-catenin, Hippo/YAP-TAZ, and TGFbeta), out of nine considered, that are specifically altered in AC8-like conditions (Giuliodori et al., 2018). Interestingly, in chronic organ injury, a possible cross-talk among these signals has been recently hypothesized (Piersma et al., 2015). This discovery, made in a zebrafish setup, is thus offering a new, network-wide view of AC conditions, and suggesting a set of evolutionary conserved signaling mechanisms worth to be considered in the design of a molecularly-targeted therapy for AC.

Vascular Studies

As a model system, the zebrafish offers unique advantages for studying vascular development *in vivo* (Hogan and Schulte-Merker, 2017). Recent findings have also shown that the zebrafish possesses a *bona fide* brain vascular system, making it useful for studying the formation of this distinct vascular network in the head. In Trento, Dr. Wiebke Herzog and Dr. Arndt Siekmann provided new insights on the mechanisms that govern the formation of the brain vasculature in early zebrafish embryos. It is well established that Vascular Endothelial Growth Factor A (VEGFA) is one of the main signaling molecules important for blood vessel growth and homeostasis (Simons et al., 2016). Work over the last years has provided detailed insights on the downstream signaling events that occur upon

receptor activation (Alvarez-Aznar et al., 2017). However, it has remained enigmatic how endothelial cells balance these diverse signaling outcomes in order to temporally and spatially control initiation of cell migration and proliferation. Instead of a single *VEGFA* gene, zebrafish possess two VEGFA genes, *vegfaa* and *vegfab* (Bahary et al., 2007). Dr. Arndt Siekmann presented data on the different phenotypes of *vegfaa* and *vegfab* mutants. As expected, *vegfaa* mutants displayed severe brain blood vessel defects. Surprisingly, *vegfab* mutations mainly affected endothelial cell proliferation. Blood vessels could still sprout and form a relatively normal vascular architecture, despite a reduction in endothelial cell numbers. These findings indicate that proliferation and migration can be uncoupled during angiogenic blood vessel sprouting. They furthermore suggest an unexpected plasticity in terms of endothelial cell sizes when it comes to establishing vascular networks. The important next steps will be to identify *vegfab* targets mediating cell cycle progression, but not cell migration.

Another signaling pathway regulating brain blood vessel formation is Wnt signaling (Reis and Liebner, 2013). Mutations in the Wnt pathway components Wnt7 (Stenman et al., 2008), GPR124, and Reck (Vanhollebeke et al., 2015; Ulrich et al., 2016) were previously shown to specifically interfere with brain blood vessel formation. Of note, blood vessel formation in other regions of the embryos can proceed relatively unperturbed in these mutants. This suggests that the brain vasculature requires a distinct set of molecules for its formation. One reason for this might be the necessity to also form the blood brain barrier during embryonic development that shields the brain. New results from the laboratory of Dr. Herzog now provide evidence that Wnt signaling is less required for the initial sprouting of brain blood vessels, but is rather necessary in newly connecting blood vessels. Here, Wnt signaling regulates the localization of junctional proteins in order to facilitate blood vessel anastomosis. Thus, Wnt signaling might coordinate the different steps necessary for the proper development and maturation of brain blood vessels. Further experiments will aim at a better understanding of the molecules that mediate these different effects of Wnt signaling.

Lymphatic Studies

Mammals, zebrafish, and other vertebrates possess a second vascular system entirely separate from the blood circulatory system called the lymphatic system. The lymphatic system is a complex, blind-ended vascular network that extends into virtually every part of the body. It is essential for maintaining fluid homeostasis, absorbing dietary lipids from the intestine, and for immune cell production and trafficking (Baluk et al., 2007; Alitalo et al., 2005). Since the first identification of zebrafish lymphatic vessels in 2006 (Kuchler et al., 2006; Yaniv et al., 2006), a number of studies have provided evidence that the zebrafish lymphatic system shares many of the morphological, molecular, and functional characteristics of lymphatic vessels in other vertebrates (Yaniv et al., 2006; Hogan et al., 2009; Nicenboim et al., 2015; Jung et al., 2017). The zebrafish has helped identify important molecular players in lymphangiogenesis such as *cxcr4-cxcl12* chemokine signaling, which provides guidance cues directing early trunk lymphatic development (Cha et al., 2012), and *pkd1* for polarity, elongation, and the adherens junctions for lymphatic endothelial cells that are essential for vessel morphogenesis (Coxam et al., 2014). The Weinstein lab recently generated a valuable new *Tg*(*mrc1a:egfp*)^{y251} transgenic zebrafish line for visualizing and investigating lymphatic vascular development (Jung et al., 2017). The Mannose receptor (MR, CD206 or MRC1) is a transmembrane glycoprotein that belongs to the C-type lectin family, known to be strongly expressed in macrophages, dendritic cells, and prominently in lymphatics (Taylor et al., 2005). MRC1 is involved in lymphocyte migration (Marttila-Ichihara et al., 2008). Zebrafish *mrc1a* is expressed in primitive veins and lymphatics (Wong et al., 2009; Jung et al., 2017), and this new transgenic line has proven extremely useful for studying the growth and assembly of novel major superficial and deep lymphatic vessels in the trunk that form during later stages of development (Jung et al., 2017).

In the course of using the *Tg*(*mrc1a:egfp*)^{y251} transgenic line, the Weinstein lab researchers discovered that in addition to lymphatics, this transgenic line also displays strong EGFP expression in a novel cell population on the surface of the zebrafish brain (Venero Galanternik et al., 2017). Despite expressing *mrc1a:egfp* and other lymphatic transgenes, these newly discovered brain cells are individual non-lumenized perivascular cells, associated with blood vessels located exclusively in the brain meninges. In addition to a macrophage-like morphology, these cells also contain large numbers of internal auto fluorescent vesicles or vacuoles, and they exhibit extremely robust scavenger activity as shown by rapid uptake of India Ink and other "tracers" injected into intracranial spaces or brain ventricles. Careful cell lineage and live imaging studies demonstrated that these cells emerge by transdifferentiation from the venous endothelium of the optic choroidal vascular plexus, and RNA-seq on FAC-sorted Mrc1a-positive cells revealed that despite their macrophage-like morphology and perivascular location, they appear molecularly most similar to lymphatic endothelium, strongly expressing lymphatic markers such as *lyve1, prox1a*, and *flt4* (Venero Galanternik et al., 2017). The expression of a number of different lymphatic transgenes also led two other groups to contemporaneously discover and report on these cells in the zebrafish (Bower et al., 2017; van Lessen et al., 2017). Based on similarities in their morphology, location, and scavenger behavior, these cells appear to be the zebrafish equivalent of cells variably characterized as Fluorescent Granular Perithelial cells (FGPs), perivascular macrophages, or "Mato Cells" in mammals (Mato et al., 1984). In mammals, FGPs are found in the leptomeningeal layers surrounding blood vessels, where they are thought to provide an important pinocytotic protective function. Further study of FGPs in the experimentally and genetically accessible zebrafish model system is sure to yield important new insights into blood-brain barrier establishment, removal of toxic waste from the brain, and the relationship between brain homeostasis and waste clearance and neurodegenerative disorders and protection from brain injury and infection.

A debate has been going on in recent years, within the zebrafish community, on the use of morpholinos to study gene function, based on the published observation that mutants in several genes, obtained through reverse genetic approaches, were not sharing the same phenotypes of the corresponding morphants (Kok et al., 2015). Genetic compensation elicited in mutants, but not in morphants, rather than simply toxicity or off-target effects of morpholinos, might be at the basis of these discrepancies (Rossi et al., 2015), and not only in zebrafish, but also in other model organisms where knockout and knockdown approaches are giving different results (El-Brolosy and Stainier, 2017). Cermenati et al. (2013) showed, through the use of morpholinos, that the transcription factor Sox18 plays a role in early zebrafish lymphatic development, suggesting an evolutionary conservation between fish and mammals. Mutations in *SOX18* underlie both recessive and dominant forms of Hypotrichosis-Lymphedema-Telangiectasia syndrome (Irrthum et al., 2003); the phenotypic spectrum is highly variable among patients and most, but not all of them, present with lymphedema (Moalem et al., 2015; Wunnemann et al., 2016). *Sox18*-null mice develop severe lymphedema in a pure B6 background, but show no signs of lymphatic dysfunction in a mixed background, where the upregulation of the closely related transcription factors Sox7 and Sox17 in the cardinal vein compensates for the lack of Sox18 (Francois et al., 2008; Hosking et al., 2009).

van Impel et al. (2014) reported that in a *sox18* loss-of-function mutant thoracic duct (TD) formation was unaffected and they questioned the role of Sox18 in the regulation of lymphatic development in zebrafish. Dr. Monica Beltrame presented at the meeting data gathered in her lab on an independent *sox18*-null mutant, generated through the Zebrafish Mutation Project at Sanger (Kettleborough et al., 2013). Homozygous *sox18* mutants showed subtle but statistically significant defects in TD formation (Moleri et al., unpublished data). A slight perturbation of VegfC signaling exacerbated TD defects in a genotype-dependent manner, unraveling differences in TD formation even in *sox18* heterozygotes versus wild types. Remarkably, an upregulated expression of *sox7* in the posterior cardinal vein was observed in *sox18* mutants, thus possibly explaining the milder lymphatic phenotypes with respect to the knockdown of *sox18*, which was not affecting *sox7* expression (Cermenati et al., 2013). Overall, a high degree of evolutionary conservation in the regulation of lymphatic development by SoxF TFs is thus confirmed.

REPORTER TOOLS AND GENETIC VERSATILITY FOR CARDIOVASCULAR STUDIES IN THE ZEBRAFISH SYSTEM

In recent years, cardiovascular research in zebrafish greatly benefited from the development of a series of innovative genetic tools, mostly represented by mutant, transgenic, and biosensor lines, allowing to analyze *in vivo* a wide range of components in physiological as well as in pathological contexts (development, signaling, contraction, regeneration, inflammation) occurring in the cardiovascular compartment. To date, several zebrafish lines have been generated to identify and perturb *in vivo* different sub-cardiac compartments, such as the endocardium, myocardium, epicardium, valves, or conduction system as well as specific vascular components and cell types including arteries, veins, lymphatic vessels, mural, and endothelial cells (Santoro et al., 2009; Bakkers, 2011; Gore et al., 2012; Bournele and Beis, 2016).

In this perspective, during the EZPM2018 in Trento, a spectacular series of new interesting systems have been presented, all worth to be reported and added to the available toolkit. In the context of new mutant and transgenic line production, the research group of Dr. Beltrame presented a new allele for Sox18, which allowed to further elucidate the role of this key factor in the development of lymphatic vessels in vertebrates. Dr. Siekmann's research group has produced new mutant lines for the two *VEGFA* paralogs (*vegfaa* and *vegfab*), elegantly demonstrating that both factors act through the same receptor (Kdrl) to induce sprouting, vessel formation, and vascular-specific gene expression. Finally, using the transgenic line *Tg*(*mrc1a:eGFP*), Dr. Weinstein's group could efficiently analyze a new population of perivascular cells *in vivo* (Jung et al., 2017).

In the field of cardiovascular signaling, Dr. Tiso's group presented the systematic application of pathway-specific transgenic lines for the identification of drugs with therapeutic effects in cardiac pathology. Specifically, a form of arrhythmogenic cardiomyopathy was modeled in a set of different zebrafish lines reporting signaling pathways activated in the cardiac compartment. This signaling-based screen allowed identifying the involved cascade, rapidly passing to the test of pathway-specific compounds, and the identification of a Wnt agonist as a potential therapeutic drug (Giuliodori et al., 2018). The role of the canonical Wnt pathway in the vascular compartment has also been studied by Dr. Herzog's group (Hubner et al., 2017). In particular, her team produced and presented a set of transgenic lines able to express in a vascular-specific way a dominant negative form of a Wnt effector (dnTcf), as well as destabilized Wnt-responsive fluorophores. With the combined use of these systems, the team could successfully inhibit Wnt signaling in a temporal and tissue-specific fashion, analyzing the response to the pathway within time frames of about 2 h. This strategy allowed to establish a new role for the Wnt pathway in the anastomosis of the capillaries during the formation of the blood brain barrier.

CHALLENGES AND OUTLOOK

Zebrafish is a long-standing model to study heart morphogenesis. With the growing number of genetic tools and technological advances, probing the functional inputs and their related physical stimuli, such as mechanical or calcium cues in greater detail *in vivo*, will soon ensue. The dysregulation of ion channels, such as LTCC, can result in arrhythmias as well as cardiac hypertrophy, both of which are underlying pathologies leading to sudden

cardiac death. Future studies, in which modulation of functional inputs plays a crucial role, will therefore also accelerate novel avenues for future therapies regarding common cardiac diseases, including cardiac hypertrophy, heart failure, and arrhythmias.

Many questions still remain open in the field of vascular and lymphatic studies in zebrafish. Besides VEGF and Wnt, other signaling pathways are increasingly studied in vascular development, such as the Hippo pathway (Nagasawa-Masuda and Terai, 2017; Nakajima et al., 2017; Astone et al., 2018). Similarly, hemodynamic forces seem to play a critical role in cardiovascular development and differentiation (Chen et al., 2012, 2017; Goetz et al., 2014).

We envision a new interactive atlas of zebrafish vascular and lymphatic anatomy using modern technologies, such as light sheet microscopy and fluorescent markers of different colors. It would be a *desideratum* for researchers working in the field to also include perivascular cells, such as mural cells.

It can be expected that in the coming years the growing application of single-cell sequencing technologies and the use of reporter pathway lines, with conditional modulation, combined with mutant lines for new regulators of cardiac as well as vascular components, will allow a highly specific genetic dissection of each process, with thus far unprecedented precision in the cardiovascular research field. We expect that cutting-edge high throughput approaches, such as single-cell RNA sequencing and metabolomics will be feasible in the near future (Santoro, 2014; Pandey et al., 2018). We envision that the zebrafish model system will continue to help in understanding the genetic and molecular basis of human cardiovascular and lymphatic malformations and diseases.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript. MMS coordinated the work.

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