REVIEW ARTICLE

Insights into Mechanisms of Central Nervous System Myelination Using Zebrafish

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Myelin is the multi-layered membrane that surrounds most axons and is produced by oligodendrocytes in the central nervous system (CNS). In addition to its important role in enabling rapid nerve conduction, it has become clear in recent years that myelin plays additional vital roles in CNS function. Myelinating oligodendrocytes provide metabolic support to axons and active myelination is even involved in regulating forms of learning and memory formation. However, there are still large gaps in our understanding of how myelination by oligodendrocytes is regulated. The small tropical zebrafish has become an increasingly popular model organism to investigate many aspects of nervous system formation, function, and regeneration. This is mainly due to two approaches for which the zebrafish is an ideally suited vertebrate model—(1) *in vivo* live cell imaging using vital dyes and genetically encoded reporters, and (2) gene and target discovery using unbiased screens. This review summarizes how the use of zebrafish has helped understand mechanisms of oligodendrocyte behavior and myelination *in vivo* and discusses the potential use of zebrafish to shed light on important future questions relating to myelination in the context of CNS development, function and repair.

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Introduction

The formation of myelinated axons is a key feature during the evolution of complex nervous systems. In the central nervous system (CNS) myelin is formed by oligodendrocytes, which do so by iteratively "wrapping" tightly compacted plasma membrane around the axon. This results in electrical insulation of the myelinated axon and leads to fast "salutatory" propagation of the action potential along the short unmyelinated gaps between each myelin sheath, the nodes of Ranvier. While the insulating properties of myelin have long been known, it is now becoming increasingly clear that oligodendrocytes play additional vital roles in regulating CNS function. Myelinating oligodendrocytes metabolically support axons and ensure their long-term survival (Fünfschilling et al., 2012; Lee et al., 2012b; Saab et al., 2013). Moreover, there is recent experimental evidence that myelination is involved in regulating forms of learning and memory. Mice reveal white matter changes and impaired memory performance following social isolation (Liu et al., 2012; Makinodan et al., 2012), and genetic ablation of newly differentiating oligodendrocytes impairs learning of complex motor tasks (McKenzie et al., 2014).

Disruption to myelination results in severe neurological impairments. Impaired nerve conduction and ultimately neurodegeneration due to failure of myelination or damage to myelin are hallmarks of myelin diseases like multiple sclerosis (MS), hereditary leukodystrophies (Pouwels et al., 2014), as well as pathologies such as periventricular leukomalacia (diffuse white matter injury in early born infants) (Volpe et al., 2011). More recently, defects in oligodendrocytes and myelination have also been linked to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Huntingtons disease (HD), which have both long been thought to be due to autonomous defects in neurons (Kang et al., 2013; Philips and Rothstein, 2014; Huang et al., 2015).

Despite the importance of myelin for nervous system function, there are still large gaps in our understanding of the mechanisms that regulate myelination in the CNS. We lack a detailed understanding of the basic cellular principles underlying the formation of myelinated axons. New myelin is formed

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over long time periods well into adulthood (Miller et al., 2012; Young et al., 2013), but how this dynamic is reflected at the cellular level, and where new myelin comes from, is subject to ongoing debate. Some studies suggest only very little turnover of oligodendrocytes and myelin at the global level (Toyama et al., 2013; Yeung et al., 2014). However, cellular analyses provide evidence for myelin turnover in adults (Young et al., 2013). Furthermore, ultrastructural reconstructions recently revealed that cortical neurons often only show intermitted myelination along the length of their axon (Tomassy et al., 2014), leaving space for dynamic myelination. Indeed, a substantial amount of the adult brain (about 5%) consists of undifferentiated oligodendrocyte precursor cells (OPCs, also referred to as NG2 cells) (Dawson et al., 2003). This cell population has been linked to several functions including the production of new myelin. However, how OPCs differentiate to myelinate specific axons is still largely unknown.

The molecular mechanisms of CNS myelination are not well defined. In contrast to the peripheral nervous system (PNS), where axonal Neuregulin 1- Type III signals through cognate ErbB receptors on Schwann cells to initiate axon ensheathment (Michailov et al., 2004; Taveggia et al., 2005), axo-glial signalling molecules essential for myelination by oligodendrocytes are yet to be identified. Numerous extrinsic and intrinsic factors as well as intracellular signalling cascades have been identified to regulate various aspects of oligodendrocyte lineage progression from their specification through to their differentiation [for reviews, see (Emery, 2010; Liu and Casaccia, 2010; Zuchero and Barres, 2013)]. However, in many cases it is unclear how these molecules directly relate to the myelination of target axons *in vivo*.

To understand how myelination is regulated, it is important to (1) gain insight into the cellular principles underlying oligodendrocyte behavior and myelination, and to (2) identify the molecules crucial for mediating this behavior. Two dimensional cell cultures have provided important information on mechanisms of oligodendrocyte proliferation and differentiation, but they have natural limitations when it comes to the study of intricate cellular interactions such as myelination so that *in vivo* studies are required. However, one of the problems with studying myelination *in vivo* is that it occurs relatively late during CNS development and continues over long time periods, which makes investigating oligodendrocyte behavior over time technically challenging in rodent models.

Zebrafish allow circumventing some of the inherent difficulties when studying myelination *in vivo*. They produce large numbers of offspring, which develop rapidly and outside the mother. This made the zebrafish a popular vertebrate model to carry out genetic and chemical screens. In addition, their small size and optical clarity make them a preeminent model for *in vivo* live imaging using genetically encoded markers. This review aims to provide an overview of zebrafish as model system, to summarize insights on mechanisms of myelination that have been obtained from zebrafish, and to discuss how open questions relating to myelinated axon formation can be addressed in the future.

Zebrafish as Vertebrate Model to Study Myelination

Over the past decade the zebrafish has evolved as a popular model organism with which to study myelinating glia. One key characteristic is their rapid early development. Within only 3 days a zebrafish develops from a fertilized egg to a freely swimming animal of no more than five millimeters length (Fig. 1), which undertakes complex behaviors such as prey capture by 5 days of age. Myelination of axons is the last major event during CNS development, both in mammals and in zebrafish. However, while myelination primarily occurs during postnatal stages in mammals, the first OPCs in the zebrafish are already present as early as 48-h post fertilization (hpf) and oligodendrocytes, which express myelin genes such as myelin basic protein (Mbp), appear shortly thereafter by 60 hpf (Brösamle and Halpern, 2002; Buckley et al., 2010a; Park et al., 2002). Despite the early oligodendrocyte specification and onset of myelination this process continues over long time, as is the case in higher vertebrates including humans (Miller et al., 2012). Active oligodendrogenesis in the zebrafish spinal cord has been reported to continue over a period of a month (Park et al., 2007), and myelination progresses continuously into adulthood (Jung et al., 2009). In our own work, we have demonstrated that new myelin sheaths are produced over periods of weeks, concomitant with the emergence of newly differentiating oligodendrocytes [(Czopka et al., 2013), and unpublished observations]. This work also showed that new myelin sheaths were often deposited along the same axon gradually over a period of days, meaning that these axons display transient patchy myelination. The phenomenon of patchy myelination has recently been described along axons of pyramidal neurons in the mouse cortex (Tomassy et al., 2014). Whether this non-uniform pattern of myelination is a sign of mammalian neocortical diversification or rather represents a snapshot of ongoing myelination as observed in the zebrafish is subject to further elucidation. However, work so far suggests that CNS myelination in zebrafish and mammals follow common patterns, but with the difference that myelination in the zebrafish occurs at a much faster rate when the entire animal measures only a few millimeters.

Myelin is a vertebrate elaboration, which presumably co-evolved with hinged jaws in placoderms (Zalc et al.,

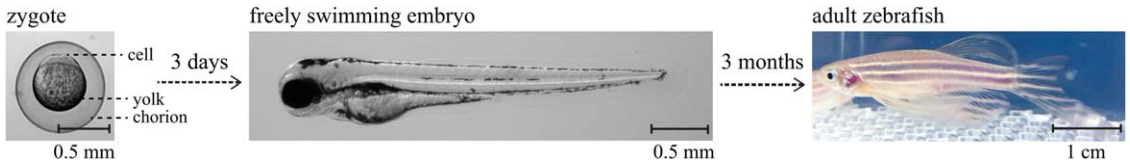

FIGURE 1: Major stages of zebrafish development. Images of a zebrafish at the one cell stage (left), at 3 days post fertilization (middle), and adulthood (right). Note the differences in scale.

2008). Although zebrafish and mammals had hundreds of millions of years of separate evolution, many genes are conserved. All major myelin proteins such as Mbp, proteolipid protein (Plp), and myelin protein zero (Mpz, or P0) are present in fish myelin (Bai et al., 2014; Brösamle and Halpern, 2002; Schweitzer et al., 2003, 2006). In fact, 70% of all human genes have at least one zebrafish paralog and vice versa (Howe et al., 2013). The study by Howe et al. also revealed a comparable number of species specific genes for zebrafish, human and mouse when duplicated genes are regarded as one gene (Howe et al., 2013). Teleost fish have undergone an additional round of whole genome duplication and although many of these duplicated ohnologs got subsequently lost, about 30% of all zebrafish genes still have one (Howe et al., 2013). The degree of conservation changes from gene to gene, and it remains to be determined in many cases whether both or only one of these duplicates are found in a given cell type. For example, two ohnologs of zebrafish *mbp* (*mbpa* and *mbpb*) are expressed in oligodendrocytes, and although their amino acid sequences differ to some extent, their cellular functions, such as membrane association and compaction, remain conserved (Nawaz et al., 2013). Some differences between zebrafish and mammalian myelin have also been reported. Myelin protein zero (Mpz, or P0), which is exclusive to PNS myelin in mammals, has been detected in the PNS and CNS of zebrafish (Bai et al., 2014; Schweitzer et al., 2003). Whether differences in such proteins, whose prime function is often the compaction of myelin membranes, also has functional consequences remains to be investigated. Also, no obvious paralog of the zebrafish myelin protein 36k has yet been identified in mammals (Morris et al., 2004). It will be helpful in the future for the community to have a comparative transcriptome and proteome database for zebrafish glia like the transcriptome databases that have been assembled for mouse CNS cells using microarray analysis (Cahoy et al., 2008), and more recently also RNA sequencing (Zhang et al., 2014).

The vast majority of oligodendrocyte specific markers and transcription factors used in mammals are the same in zebrafish and their roles are highly conserved. As in mammals, OPCs in the zebrafish spinal cord originate from the pMN domain, which also gives rise to motor neurons in a sonic hedgehog and Olig2 dependent manner (Park et al., 2002). Key transcription factors known from mammalian studies, such as Nkx2.2, Sox10, and Olig1, have all been reported to function in oligodendrocyte differentiation in zebrafish as well (Kirby et al., 2006; Kucenas et al., 2008; Li et al., 2007; Schebesta and Serluca, 2009), meaning that patterns and general principles of myelination are conserved between fish and mammals. The function of numerous other molecules on oligodendrocyte development have been studied in zebrafish and some of them have recently been reviewed by Preston and Macklin in great detail (Preston and Macklin, 2015). However, for the purpose of this review I want to focus on the approaches where the properties of zebrafish as a model have been of particular advantage to obtain insights on myelination, and discuss how it can be used to address future questions.

Insights into Oligodendrocyte Development and Myelination In Vivo *Using Live Cell Imaging*

Seeing how cells interact and how they respond to experimental manipulation in a living animal in real time is of great value to understand their behavior. The small size and optical transparency of young zebrafish make them particularly suited to study cellular behaviors using genetically encoded fluorescent proteins and non-invasive optical live imaging methods (Fig. 2). Zebrafish live cell imaging studies have helped our understanding of myelination, including the long standing question of how oligodendrocytes "wrap" their plasma membranes around axons to form a multilayered myelin sheath. Although the ultrastructure of myelin is well described, the cellular basis of myelin morphogenesis remained totally elusive, essentially because of the technical and physical limitations of visualizing myelin dynamics *in vivo*. In an integrative approach using several models and manipulations, Snaidero et al. could show and experimentally test for the first time how myelin layers are assembled in the CNS. In this study, high resolution live imaging in zebrafish was used in combination with *in vivo* and *in vitro* mouse studies using viral labelling of newly integrated myelin membrane, three-dimensional electron microscopic reconstructions and genetic manipulations. Together, this work revealed that new layers of myelin are always added at the innermost tongue of the growing myelin sheath immediately adjacent to the axon, from where they extend laterally to form the multilayered internode

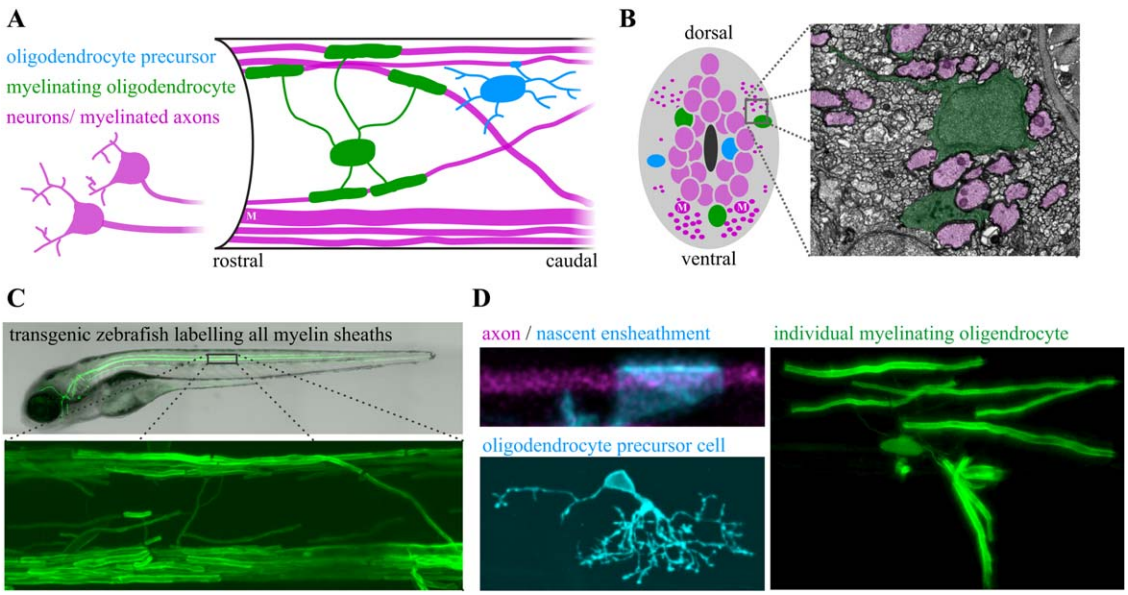

FIGURE 2: Visualising CNS myelination in zebrafish. (A, B) Schematics of lateral (A) and transversal (B) views of the embryonic zebrafish spinal cord showing oligodendrocyte precursor cells (OPCs), myelinating oligodendrocytes, neurons and the position of major axonal tracts. The large caliber Mauthner axons are depicted with M. The electron microscopic image in (B) shows a false coloring of an oligodendrocyte (green) which myelinates axons (magenta) in the zebrafish spinal cord. **(C)** Confocal image of a mbp:EGFP-CAAX transgenic zebrafish at 4 days post fertilization with all myelin sheaths labelled. **(D)** Confocal images of a transgenically labelled nascent myelin sheath, an individual OPC, and an individual myelinating oligodendrocyte in the living zebrafish spinal cord.

with its characteristic paranodal loops (Snaidero et al., 2014) (Fig. 3A). This work very nicely exemplifies how *in vivo* live imaging can help understand fundamental biological principles.

A multitude of zebrafish reporters exist for virtually every cell type and subcellular structure, primarily due to the relative straightforwardness of generating reporter lines. The dominating strategy in recent times is transgenesis via Tol2

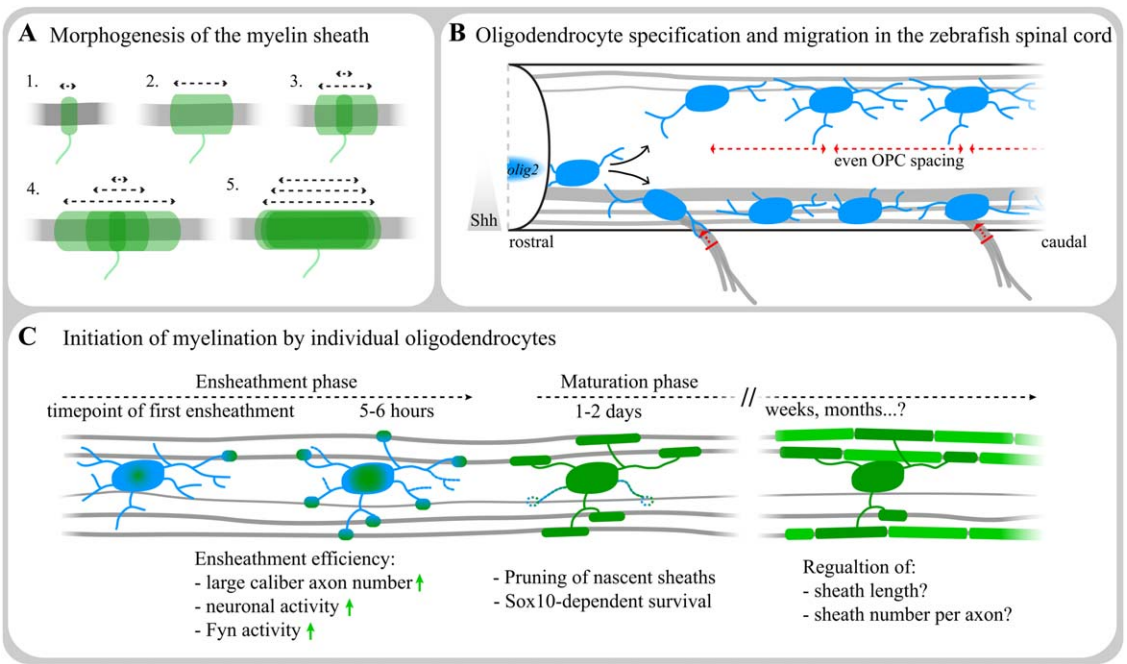

FIGURE 3: Mechanisms of oligodendrocyte development and myelination revealed in zebrafish. (A) Mechanisms of myelin sheath morphogenesis in the CNS. **(B)** Regulation of oligodendrocyte specification and migration in the zebrafish spinal cord. **(D)** Initiation of myelination and regulation of myelin sheath number by individual oligodendrocytes in the zebrafish spinal cord.

TABLE 1: Transgenic Reporter Lines to Label Oligodendrocyte Lineage Cells

Gene	Labelled cell type(s)	Line name, reference	Notes
olig1	Oligodendrocyte lineage cells	Tg(olig1:mEGFP)^{nv150Tg}, Schebesta and Serluca , 2009	
olig2	pMN domain, oligodendrocyte lineage cells, motor neurons	Tg (olig2:EGFP)^{vu12Tg}, Shin et al., 2003 Tg(olig2:dsRed)^{vu19Tg}, Kucenas et al., 2008 Tg(olig2:Kaede)^{vu85Tg}, Zannino and Appel, 2009	
nkx2.2a	Subset of OPCs and early myelinating oligodendrocytes, various non-related cell types	Tg(nkx2.2a:mEGFP)^{vu16Tg}, Ng et al., 2005	
sox10	Oligodendrocyte lineage cells, some interneurons, neural crest derivatives including Schwann cells	Tg(sox10:mRFP)^{vu234Tg}, Kirby et al., 2006 Tg(sox10:EGFP)^{ba4Tg}, Dutton et al., 2008 Tg(sox10:nls-Eos)^{w18Tg}, Prendergast et al., 2012	Length of sox10 promoter fragment varies between lines
mpz (p0)	Myelinating oligodendrocytes, some non-related cell types	Tg(mpz[10kb]:EGFP)^{pt408Tg}, Bai et al., 2014	
plp	Oligodendrocyte lineage cells, some non-related cell types	Tg(Mmu.Plp1:EGFP)^{cc1Tg}, Yoshida and Macklin, 2005	Promoter fragment of mouse *plp* gene
mbp	Myelinating oligodendrocytes and Schwann cells	Tg(mbp:EGFP)^{ck1Tg}, Jung et al., 2009 Tg(mbp:EGFP)^{ue1Tg}, Almeida et al., 2011 Tg(mbp:EGFP-CAAX)^{ue2Tg}, Almeida et al., 2011	
claudinK	Myelinating oligodendrocytes and Schwann cells	Tg(cldk:Gal4)^{ue101Tg}, Münzel et al., 2011	

transposable elements (Kawakami, 2007). Tol2 sites have been used to generate enhancer trap lines in which a reporter (a fluorescent protein or a Gal4 transactivator) with a minimal promoter randomly integrates into the genome. The reporter gene will be expressed when it integrates in the proximity of the enhancer of an endogenous gene (Korzh, 2007; Asakawa and Kawakami, 2009). Collections of such enhancer trap lines like the zTrap database provided by the Kawakami laboratory (http://kawakami.lab.nig.ac.jp/ztrap/) (Kawakami et al., 2010) consist of hundreds of different lines with expression in distinct cell types. Lines with expression in subsets of neurons have been used in several studies to elucidate neuronal functions. Enhancer trap lines that are specific for glial cells have not yet been reported, most likely because systematic screening for these cell types simply has not been carried out so far. The identification of such reporters is thus subject to further investigation and may even lead to the detection of labelled subsets of oligodendrocytes as is the case for neurons, furthering future studies on myelination using zebrafish.

Toolsets to express any gene in a defined tissue and cell type have also been developed. The most commonly used system is the Tol2Kit developed by the Chien laboratory (Kwan et al., 2007). This combines Tol2 transgenesis with recombination based Gateway cloning to generate transgenic expression constructs of any gene of interest under the control of a desired upstream regulatory sequence in a simple overnight reaction. Injection into fertilized zebrafish eggs results in mosaic expression of the transgene, which allows analysis of morphology and dynamics of single cells. Growing up injected animals followed by screening for germline transmission of the transgene to the next generation can generate stable full transgenic lines. Suites of transgenic reagents have been generated to visualize oligodendrocyte lineage cells at different stages of their differentiation and neuronal subsets using distinct fluorescent proteins (Table 1, Fig. 2). The vast

majority of markers used to drive transgene expression in zebrafish are the same as in mammals. *Nkx2.2a* and *olig2* upstream regulatory sequences label OPCs (Kirby et al., 2006; Ng et al., 2005; Shin et al., 2003), *sox10* and *olig1* all oligodendrocytes along their lineage (Kirby et al., 2006; Schebesta and Serluca, 2009), whereas driver elements of the myelin genes *mbp* and *plp* are only expressed by myelinating cells (Almeida et al., 2011; Jung et al., 2009; Yoshida and Macklin, 2005). Some markers of mammalian oligodendrocytes are not (yet) used in zebrafish glia like the major OPC markers PDGFRα and NG2, and key myelin transcription factors like MYRF (or MRF). Whether these markers cannot be used as glial marker in zebrafish or if they simply have not been established yet is presently not clear.

Control of Oligodendrocyte Development and Myelination by Cellular Interactions. Myelination requires the production of appropriate numbers of oligodendrocytes, their migration into axonal territories, and the timed differentiation and ensheathment of the correct axons. Live cell imaging studies using zebrafish have made important contributions to our understanding of how different cellular interactions regulate oligodendrocyte behavior. OPCs are born in distinct areas (in the spinal cord the majority arises from the pMN domain) from where they migrate into target axonal areas to evenly populate the CNS tissue. Live imaging studies in zebrafish showed that one mechanism to regulate proper OPC migration and spacing is by cell-cell interactions between glial cells [for example (Kirby et al., 2006; Kucenas et al., 2009; Smith et al., 2014), Fig. 3B]. OPCs are highly migratory cells but do not leave the CNS along potential exit points such as motor nerves, due to repulsive interactions with other glial cells at the motor nerve exit points (Kucenas et al., 2009). However, when the glial cells that ensheath motor axons at these exit zones were ablated, OPCs did exit the spinal cord (Kucenas et al., 2009). The exact cellular identity of this glial barrier is not clear, but it has recently been proposed that it is formed by a specialized cell population of CNS derived motor exit point glia (Smith et al., 2014). Another elegant study showed that even spacing of OPCs within the CNS is regulated by repulsive interactions between neighboring OPCs (Kirby et al., 2006) (Fig. 3B). Highly motile OPC processes constantly scan their environment and retract as soon as they encounter processes from a neighboring OPC. Laser-ablation of individual cells resulted in the immediate proliferation of neighboring OPCs, indicating that cell numbers are controlled by OPC-OPC interactions. A later study carried out by the Bergles group confirmed and extended these findings using live cell imaging in the adult mouse cortex showing that OPCs in the adult mammalian CNS behave almost identically to those in the embryonic zebrafish spinal cord (Hughes et al., 2013). Work using rodents further showed that intercellular interactions with neighboring oligodendrocytes are also implicated in controlling oligodendrocyte differentiation as well as the number of myelin sheaths made by individual oligodendrocytes. OPC differentiation can be induced by cellular density *in vitro*, which could even be mimicked with artificial beads (Rosenberg et al., 2008). In addition, oligodendrocytes form fewer myelin sheaths when grown at higher densities *in vitro*, which seemed to be mediated by Nogo-A. In the absence of Nogo-A individual oligodendrocytes produced more myelin sheaths during myelination and also remyelination in mice (Chong et al., 2012). Together, the zebrafish live imaging data along with the mouse studies demonstrate that OPCs recognize one another and sense their density/distribution to regulate migration and differentiation.

Work in zebrafish has also provided insight into control of myelination by axon-oligodendrocyte interactions. Zebrafish are extremely well suited to study this kind of interaction because of their relatively simple nervous system with readily identifiable spinal neurons (and axons) with stereotypic and well described morphology and physiological properties (Hale et al., 2001; Higashijima et al., 2004; McLean and Fetcho, 2008, 2011). The number of axons that surround any single oligodendrocyte greatly exceeds the number it myelinates, meaning that oligodendrocytes have to carefully select which axon to ensheath. There is a clear correlation between the number of myelin sheaths made by any one oligodendrocyte and the diameter of the target axon. Oligodendrocytes either myelinate a small number of large caliber axons or a large number of relatively thin axons. Work that we have published using zebrafish showed that this correlation is also causative and that axonal properties directly regulate the myelinating behavior of individual oligodendrocytes *in vivo* (Almeida et al., 2011). The biggest axon of the zebrafish spinal cord is the unique Mauthner axon, which is also always the first one myelinated. The generation of animals with supernumerary numbers of Mauthner axons revealed that individual oligodendrocytes readily produce more myelin sheaths to also myelinate these additional large diameter axons (Almeida et al., 2011). This showed that oligodendrocytes can produce different amounts of myelin depending on the axonal environment. Whether such larger caliber axons express a positive cue or lack an inhibitory signal remains to be investigated. *In vitro* work suggests that caliber itself is a parameter that regulates myelination. It has been shown that there is no requirement for active axo-glial signaling *in vitro* as oligodendrocytes readily myelinate paraformaldehyde fixed axons (Rosenberg et al., 2008). Furthermore, innovative experiments carried out by Lee et al. showed that oligodendrocytes even wrap artificial polystyrene fibers, as long as they had a caliber

greater than 0.4μm (Lee et al., 2012a). This indicates that size is a simple biophysical property that contributes to regulation of myelination by oligodendrocytes.

Zebrafish live imaging also provided novel information into the cell biology of myelin sheath formation during the lifetime of individual oligodendrocytes. New myelin is formed over long periods of time and the number of myelin sheaths produced by each cell is plastic. However, it remained unclear when oligodendrocytes can exert such plasticity. We could show that, regardless of when and where in the zebrafish spinal cord an OPC differentiated, each individual cell had only a short but very dynamic time window of no more than 5 h to form its full complement of myelin sheaths after having made its first one (Czopka et al., 2013). After this time, a cell made no new myelin sheaths (Fig. 3C). It remains to be seen how long such time window of myelination lasts for any one oligodendrocyte in higher vertebrates, given that mammalian development generally occurs at slower rates. However, there is evidence from *in vitro* studies that such a short time window also exists in rodents. Cultured retinal ganglion cell axons become myelinated when co-cultured with isolated OPCs but not when mature oligodendrocytes were used (Watkins et al., 2008). Time-lapse imaging showed that also these co-cultured OPCs stopped generating new sheaths 12 h after the start of imaging. Furthermore, juvenile mice that were socially isolated during a short critical period showed behavioral deficits and impaired myelination due to reduced myelin sheath numbers per oligodendrocyte (Makinodan et al., 2012). Very interestingly, these defects were long lasting and irreversible, indicating that myelinating oligodendrocytes cannot form new myelin sheaths in mice *in vivo*. Irrespective of the exact duration of myelination competence by an individual oligodendrocyte, it will be interesting to determine what triggers the first ensheathment and which mechanisms set the clock that limit myelination competence. The existence of a short time window for an oligodendrocyte to generate new myelin sheaths means that the decision whether to ensheath an axon or not has to occur during this time. Work in zebrafish has shown that following their myelination phase, oligodendrocyte dynamics are limited to occasional sheath retractions, which has also been termed myelin pruning (Czopka et al., 2013; Liu et al., 2013). The meaning of these myelin sheath retractions is currently unclear, but one possibility is that they reflect regulatory mechanisms to correct for having ensheathed a "wrong" axon. This would be plausible given that each oligodendrocyte can only form new myelin sheaths for a short time, whereas it takes much longer to fully myelinate an axon along its length. Therefore, more myelin sheaths than required could initially be deposited along the length of an axon, which then, as sheaths grow in length, requires pruning of some sheaths to ultimately obtain appropriate nodal spacing and conduction speed.

Which signals tell right from wrong axon at a given time? Axon caliber is a positive regulator of myelination fate. However, in contrast to the PNS where only axons with a cross-sectional diameter of $\geq$1 μm are myelinated, no such threshold exists in the CNS where axons as thin as 200 nm can get myelinated, indicating that other regulatory mechanisms are likely to also play a role. There is accumulating evidence that neuronal activity is one mechanism underlying control of axonal myelination fate. Several rodent *in vitro* and *in vivo* studies could demonstrate changes in OPC proliferation, differentiation and myelination upon interference with neuronal firing (Barres and Raff, 1993; Demerens et al., 1996; Fields, 2010; Gibson et al., 2014; Wake et al., 2011). Two very recent zebrafish publications provided the first evidence that neuronal activity directly affects myelination behavior of oligodendrocytes *in vivo* (Hines et al., 2015; Mensch et al., 2015). Blocking synaptic vesicle release using tetanus toxin reduced myelination in zebrafish embryos, primarily because individual oligodendrocytes generated fewer myelin sheaths per cell during their time window of active ensheathment (Mensch et al., 2015). When neuronal firing was increased by pharmacological disinhibition, oligodendrocytes produced more myelin sheaths. In addition to these global loss- and gain of function approaches of neuronal activity, myelination fate of individual axons was also impaired when synaptic vesicle release was blocked in individual axons (Hines et al., 2015; Mensch et al., 2015). The study by Hines et al. further revealed that nascent sheaths that did form on such single silenced axons were also shorter in length. Very interestingly, this reduced sheath length was rescued by suppressing the activity of neighboring axons, indicating mechanisms of activity dependent competition for myelination between axons (Hines et al., 2015). Time-lapse analysis also showed that nascent sheaths were formed on silenced axons at similar rates, but then failed to grow and retracted more frequently, meaning that it is the stabilization and maintenance of a newly formed ensheathment rather than its initiation that is regulated by synaptic vesicle release (Hines et al., 2015). Previous work in rodents demonstrated the existence of synapse-like structures between OPCs and axons, and it has also been shown that OPCs respond to glutamate and other transmitters with inward currents (Bergles et al., 2000, 2010; Maldonado and Angulo, 2015), indicating active communication between axon and oligodendrocyte prior to ensheathment. The precise role of these axon-OPC synapses in regulating myelination is not known. NMDA-type glutamate receptors in oligodendrocytes are dispensable for myelination in mice (De Biase et al., 2011). In contrast, it was shown that glutamate release can stimulate

oligodendrocyte differentiation and myelination in a co-culture system of OPCs and dorsal root ganglion neurons (Wake et al., 2011). Wake et al. also demonstrated that this glutamate mediated increase in myelin expression involved activation of Fyn, a tyrosine kinase in oligodendrocytes which acts as glial integrator of axonal cues by regulating cytoskeletal dynamics as well as local *mbp* mRNA translation, at least *in vitro* (Klein et al., 2002; Krämer-Albers and White, 2011; White et al., 2008a). Very interestingly, in a separate study in zebrafish, we could show that increasing Fyn activity in oligodendrocytes also increased myelin sheath number per cell, whereas knock-down of Fyn had the opposite effect (Czopka et al., 2013), very similar to what has been seen after manipulation of neuronal activity by Mensch et al. It is unclear if these effects are indeed mediated by glutamate and Fyn as *in vitro* studies suggest, or by other neurotransmitters that are known to act on oligodendrocytes, for example GABA and ATP (Karadottir and Attwell, 2007). However, zebrafish with their identifiable neurons with defined neurotransmitter phenotypes can be used to study individual myelination events of neuronal subtypes. Optogenetic manipulation of defined neurons is relatively straightforward in zebrafish allowing for direct control of their activity. Therefore, zebrafish represent a useful model for further and detailed elucidation of the connection between neuronal firing and myelination in the CNS.

Zebrafish as Screening Model Organism to Discover New Molecular Functions

Forward Genetic Screening. Historically the zebrafish owes its popularity to its suitability to carry out gene discovery screens. It was the first vertebrate model organism in which large scale forward genetic screens has been carried out. Two parallel screens for disrupted tissue morphogenesis, one carried out by the Nüsslein-Volhard group (Haffter et al., 1996) and another one by the Driever group (Driever et al., 1996), led to the discovery of thousands of mutants with unique phenotypes. With the analysis and identification of the causative mutations in subsequent years the zebrafish has become well-established as a prime vertebrate model to carry out genetic screens, a method that is still unchallenged for the unbiased discovery of new gene functions for many biological questions. The principle of a genetic screen is based on the induction of random mutations, followed by phenotypic screening, and the subsequent identification of the causative lesion in mutants of interest [reviewed by (Patton and Zon, 2001)]. In most genetic screens, random point mutations are induced by *N*-ethyl-*N*-nitrosourea (ENU) in F0 animals and then bred to homozygosity in a three-generation breeding regime (Fig. 4). In such a breeding scheme, each F1 animal

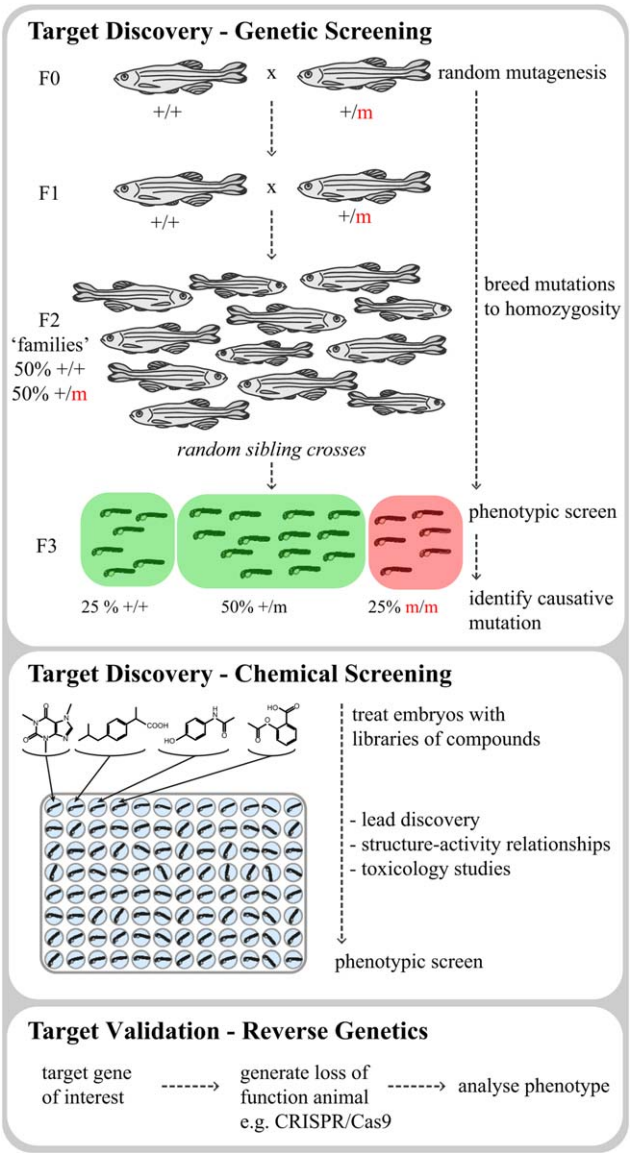

FIGURE 4: Methods of target discovery and validation in zebrafish. The schematics show how new molecular targets can be discovered by genetic and chemical screening in zebrafish, followed by the subsequent validation using reverse genetic approaches.

that emerged from a mutagenized F0 parent represents a unique mutagenized genome and is the foundation of F2 "families." Intercrossing F2 individuals heterozygous for any mutation will give 25% homozygous F3 animals. Screening thousands of F3 animals derived from many different F2 families will allow to discover mutants with a phenotype of interest (such as disrupted myelination). The causative mutation in mutants of interest is identified retrospectively by PCR based linkage mapping analysis and DNA sequencing (Postlethwait and Talbot, 1997), and since more recently whole genome/whole exome and RNA sequencing, which have become efficient and affordable ways to map genetic

lesions (Bowen et al., 2012; Henke et al., 2013; Miller et al., 2013).

Screening zebrafish for mutations that disrupt oligodendrocyte development also represents a powerful approach to obtain insights into the molecular control of myelination *in vivo*. Screens for disrupted myelination have been carried out in the past by either re-screening already existing mutants with known mutations from other screens (Kazakova et al., 2006), as well as by generating new mutants. An ENU screen carried out by the Appel group screened olig2:EGFP transgenic animals, which visualizes cell bodies of OPCs and oligodendrocytes, while a separate screen by the Talbot group used *in situ* hybridization for *mbp* mRNA to identify mutants with defects in myelin gene expression (Pogoda et al., 2006). Consequently both screens led to the discovery of different mutants with distinct phenotpyes. Mutants derived from the former screen include some that regulate oligodendrocyte numbers and positioning. For example, mutations in the ubiquitin ligase Fbxw7 increases oligodendrocyte numbers in the spinal cord, which was mediated by elevated notch signaling leading to increased OPC production from neuroepithelial precursors (Snyder et al., 2012). Another mutant showed reduced oligodendrocyte numbers resulting from mutations in pescadillo, a protein involved in ribosome biogenesis and cell cycle progression (Simmons and Appel, 2012). Zebrafish with mutations in 3-hydroxy-3-methylglutaryl CoA synthase I (Hmgcs1), an enzyme critical for the synthesis of isoprenoids and cholesterol, showed defects in OPCs migration and in myelin gene expression (Mathews et al., 2014). Isoprenoids are a source for protein prenylation to mediate membrane targeting, while cholesterol is an important regulator of plasma membrane fluidity and particularly enriched in myelin (van Meer et al., 2008). Closer analysis of Hmgcs1 mutants revealed that only cholesterol was essential for axon ensheathment/myelination, which is also known from mouse studies (Saher et al., 2005). In contrast, only isoprenoids but not cholesterol were responsible for the failure of OPC migration arrest observed in Hmgcs1 mutants, revealing crucial roles for lipid modifications in regulating OPC migration *in vivo*.

The Talbot group carried out an ENU screen for disrupted *mbp* expression (Pogoda et al., 2006; Monk and Talbot, 2009). Although this screen was primarily focused on PNS myelination, it also revealed important new genes critical for the formation of myelinated axons in the CNS, such as the identification of the kinesin motor Kif1b as crucial regulator for mRNA localization in oligodendrocytes (Lyons et al., 2009). It is known that *mbp* mRNA is localized to the distal processes of oligodendrocytes (Colman et al., 1982), but the underlying mechanism for this localization remained unclear. Lyons *et al*. showed that Kif1b is required for the transport of *mbp* mRNA molecules in oligodendrocytes *in vivo* and that failure of this transport mechanism results in ectopic compaction of oligodendrocyte plasma membrane. Further evidence for roles of transport along microtubules in regulating myelination comes from two other zebrafish mutants, one with a mutation in a microtubule associated molecule, tubulin alpha 8 like 3a (*tuba8l3*) (Larson et al., 2010), and one in the heavy chain subunit of cytoplasmic dynein (*dync1h1*), which is required for minus end directed transport along microtubules (Yang et al., 2015). Because microtubule transport is essential for many aspects of biology including axonal transport, all of the described mutants also show axonal pathologies. To dissect the precise role of cytoskeletal regulators in oligodendrocytes during myelination, cell type specific mutants are required. For example, by generating genetic chimeras of wildtype and *kif1b* mutant animals, Lyons et al. showed that Kif1b function is cell autonomously required in oligodendrocytes (Lyons et al., 2009). An important follow up technology will be the generation of cell type specific knock-outs in zebrafish in order to investigate the precise functions of candidate molecules of such newly identified pathways. However, zebrafish screens have revealed new regulators of oligodendrocyte biology and opened new avenues to study their precise roles in the future.

Perhaps the most important mutant that emerged from a zebrafish myelination screen led to the identification of the G protein coupled receptor 126 (Gpr126) as absolutely essential for myelination in the PNS (Monk et al., 2009). PNS Schwann cells sort axons but fail to proceed to myelination in the absence of Gpr126 in zebrafish and mice (Monk et al., 2009, 2011). Gpr126 was an orphan receptor at the time and not known in the context of PNS myelination. Recent work showed that Laminin-211 and Collagen type IV, which are both components of the Schwann cell basal lamina, are crucial ligands for Gpr126 function (Paavola et al., 2014; Petersen et al., 2015). The discovery of Gpr126 as essential regulator of myelination, the subsequent identification of its ligands and insights into mechanism of action, are a formidable example to demonstrate how genetic screens led to the identification of entirely new and unknown molecular functions.

While requirements of key surface receptors for PNS myelination like Gpr126, as well as axo-glial signalling via Nrg1-III and cognate ErbB receptors (Michailov et al., 2004; Taveggia et al., 2005), are well known, the molecules that regulate axo-glial signalling in the CNS still need to be identified. Both Gpr126 and ErbB receptors are dispensable for CNS myelination, although Nrg signalling has been reported to be involved in myelination in mice (Lundgaard et al., 2013; Makinodan et al., 2012; Taveggia et al., 2008). Other identified molecules at the axon-oligodendrocyte interface have modulatory functions but are not absolutely required for

mediating ensheathment, like Lingo-1 (Lee et al., 2007; Mi et al., 2005) and PSA-NCAM (Charles et al., 2000; Fewou et al., 2007). Future genetic screens in zebrafish that are designed to identify mutations that specifically disrupt CNS myelination are a promising approach to identify new and essential molecules to regulate axon ensheathment by oligodendrocytes. However, approximately 30% of all zebrafish genes exist as duplicated ohnologs and it is known that some of them are co-expressed in the same cell where they fulfill the same function. Thus, it cannot be excluded that mutagenesis screens in zebrafish will not reveal some genes with important roles for CNS myelination. It also remains possible that there might not exist a single molecule/receptor/ligand that is absolutely required for oligodendrocytes to myelinate, but that it is rather a combination of molecules, a protein complex, or even multiple pathways that regulate whether to myelinate an axon or not. For these reasons other screening approaches will be required to identify new targets important to myelination.

Chemical Screening. Like genetic screening, chemical screening enables high throughput phenotyping of many different manipulations on a specific biological question. In genetic screens, however, function disrupting mutations are always assessed in a single gene at a time. In contrast, small molecule compounds used in chemical screens can simultaneously target multiple proteins (including duplicated ones) and signalling pathways by e.g., acting on common binding sites required for function. Chemical screening also provides temporal conditional flexibility of drug application, offering the ability to identify distinct effects at early and late stages of development which one would miss by genetic disruption at all stages. Zebrafish are a pre-eminent model to carry out chemical screens for driving discovery of new targets, investigation of structure activity relationships, and toxicology studies *in vivo* (Zon and Peterson, 2005). The external development, aquatic nature, and small size of embryonic zebrafish allows simple drug application in small volumes. There is certainly no other vertebrate model with which to screen hundreds to thousands of different compounds in living animals in 96-well formats (Figure 4). Over 60 chemical screens on different aspects of vertebrate development have been reported over the past 15 years [for a review, see (Rennekamp and Peterson, 2015)]. Some led to the discovery of new targets that are currently being tested to treat human diseases. For example, a zebrafish chemical screen revealed that compounds acting on Prostaglandin E2 synthesis regulate hematopoietic stem cell homeostasis (North et al., 2007). A respective drug called ProHema is currently in phase II clinical trials for treatment of patients with leukemia and lymphoma (Hagedorn et al., 2014). This is the first example of a drug initially discovered in a zebrafish screen that advanced to use in humans.

Enhancing oligodendrocyte differentiation is an often proposed strategy to improve functional recovery and to prevent axon degeneration following demyelination after injury or in diseases like MS (Franklin and ffrench-Constant, 2008). Three independent rodent *in vitro* chemical screens have recently been carried out leading to the identification of compounds that enhance oligodendrocyte differentiation, myelination and remyelination (Deshmukh et al., 2013; Mei et al., 2014; Najm et al., 2015). Identified compounds from two studies act on muscarinic acetylcholine receptors (Deshmukh et al., 2013; Mei et al., 2014), whereas compounds from the third study involved glucocorticoid receptor signalling to increase oligodendrocyte differentiation (Najm et al., 2015). While glucocorticoids are known for regulating oligodendrocyte development (Barres et al., 1994), roles for muscarinic acetylcholine receptors in regulating oligodendrocyte differentiation had not been reported previously, which exemplifies the power of unbiased screening to discover novel targets.

Chemical screens in zebrafish are an attractive strategy to identify compounds that regulate myelination in a more complex environment. *In vivo* screening allows immediate detection of potentially deleterious side effects, which provide important information on the specificity and toxicity of a given compound. Buckley et al. have previously carried a drug re-profiling screen for pro-myelinating compounds in zebrafish (Buckley et al., 2010b). Screening of an olig2:EGFP transgenic line showed that 2% of all screened compounds altered the number of dorsally migrated olig2 expressing cells in the spinal cord. Subsequent secondary and tertiary screens revealed that also myelination was altered by some of these compounds, for example, the Src family kinase inhibitor PP2, showing that screening zebrafish for compounds that alter myelination is possible. There is a continuously growing number of available chemical libraries, some of known bioactivity, some which are already approved by the US Food and Drug Administration (FDA) to be safe for use in humans, and even natural compound libraries. Screening such diverse libraries will increase the spectrum to find novel targets and pathways to regulate myelination. Moreover, automated screening platforms such as the VAST Bioimager (for Vertebrate Automated Screening Technology) which automatically loads larval zebrafish from multiwell plates and positions them under a microscope have been developed (Pardo-Martin et al., 2010; Tamplin and Zon, 2010). The availability of such automated platforms in combination with myelin specific zebrafish reporter lines may allow to carry out high throughput *in vivo* screening and aid discover novel targets that regulate myelination in the future. Following screening,

identified candidate targets of such chemicals should be confirmed genetically by loss of function analyses.

Reverse Genetics. For a long time the zebrafish field has lagged behind with technologies for targeted inactivation of a desired gene. The use of synthetic antisense morpholinos to either block mRNA splicing or translation of a protein of interest was the most common approach for many years (Bill et al., 2009; Nasevicius and Ekker, 2000). Morpholinos can be a simple and efficient way to study gene functions; however, they are prone to give misleading results. It was recently shown that 80% of published morphant phenotypes were not recapitulated in zebrafish with function disrupting mutations in the respective gene (Kok et al., 2015; Stainier et al., 2015). While it remains to be seen to what extent this discrepancy can be due to compensatory mechanisms in mutants, it is clear (and not novel) that antisense morpholino approaches always require careful controls [see for example (Eisen and Smith, 2008)]. Furthermore, various reverse genetic approaches are meanwhile available in zebrafish. Community-wide efforts like the zebrafish mutation project at the Sanger institute (Cambridge, UK) aim to generate mutant lines for every protein coding gene and many mutants are already available (Kettleborough et al., 2014). In addition, the development of genome editing technologies using Zinc finger nucleases, TALENs, and most recently CRISPR/Cas9 now make it possible to generate functional knock-out and knock-in zebrafish [for review, (Gaj et al., 2013)]. Very recently, also the generation of cell type specific knockout zebrafish has been reported using CRISPR/Cas9 (Ablain et al., 2015). However, more work is needed in the future to address potential pitfalls in the generation of such knock-outs.

The quick and straightforward generation of loss of function animals will likely be the future standard to validate roles of candidate molecules that emerged from genetic and chemical screens, and enable to genetically test the involvement of candidate pathways in regulating distinct aspects of myelination. Zebrafish knock-outs in desired genes will also allow for more detailed analysis of cell biological principles underlying reported myelin phenotypes from rodent studies. For example, Sox10 mutant mice do not have myelinating oligodendrocytes (Stolt et al., 2002). Live imaging studies in *colorless* mutant zebrafish, which lack functional Sox10 protein (Dutton et al., 2001), have shown that oligodendrocytes lacking Sox10 develop normally but die shortly after they ensheathed target axons (Takada et al., 2010). This suggests that the role of Sox10 function in oligodendrocytes is different prior to and after initiation of myelination and extends our understanding of Sox10 function. In the future, the availability of (cell type specific) function disrupting mutants for any protein coding gene will make zebrafish an even more attractive vertebrate model to study cellular and genetic control of myelination.

Zebrafish as Model to Study Regeneration

The damaged mammalian CNS does not regenerate properly due to two confounding factors. The first is that most differentiated cells have an intrinsic inability to launch a regenerative programme. The second factor is the formation of an inhibitory environment surrounding the lesion, which prevents regeneration from newly differentiating precursor cells. In contrast to mammals, zebrafish do regenerate rather well and mechanisms of tissue regeneration in zebrafish are widely studied in many organs including heart, fin, and also the CNS (Becker and Becker, 2014; Gemberling et al., 2013; Kizil et al., 2012). Zebrafish do not form an inhibitory scar like the glial scar in the injured CNS, which allows the study of cellular responses to injury in an environment that is permissive for regeneration (scarless healing). However, in contrast to amphibians, which easily regenerate entire appendages, zebrafish also have regenerative limitations. For example, not all axons in the zebrafish spinal cord regenerate equally. The large Mauthner axon does not regenerate after transection although neighboring axons in the same tract do, indicating that the Mauthner axon is situated in an environment permissive for regeneration. Bhatt et al. took advantage of this circumstance to disentangle intrinsic limitations of axon regeneration from environmental ones and revealed that elevated cyclic AMP levels are sufficient to induce an intrinsic axonal growth program (Bhatt, 2004), an approach initially shown in mammalian models to promote axon regeneration (Cai et al., 1999).

The repair of damaged myelin (re-myelination) after injury and in disease is a regenerative process that can occur in all vertebrates including humans and is one of the few truly regenerative capacities of the CNS (Crawford et al., 2013). However, remyelination is often less efficient than developmental myelination. An often proposed strategy to restore function and to prevent neurodegeneration is to enhance remyelination, which requires that OPCs are available in sufficient numbers and readily myelinate demyelinated axons (Franklin et al., 2012). Block of differentiation as well as lack of OPC availability have been reported in demyelinated lesions and are discussed as possible reasons for remyelination failure, and both can be a result of intrinsic and extrinsic limitations.

Zebrafish can be used as a model to study remyelination in a non-scaring environment. It is known from stab wound lesion paradigms in the adult zebrafish telencephalon that precursor cells dynamically respond to injury and that some of these cells also express the OPC marker Olig2 (Baumgart et al., 2012; Kroehne et al., 2011; März et al., 2011).

Chemical demyelination along optic nerves of adult zebrafish using lysophosphaditylcholine (LPC) results in robust demyelination and subsequent remyelination within 4 weeks (Münzel et al., 2014). A main characteristic of mammalian remyelination is the presence of thinner myelin (Blakemore, 1974). Interestingly, myelin thickness recovered fully in young adults, but not in aged ones (Münzel et al., 2014). Given that zebrafish do not form inhibitory scars, it is possible that 'old' OPCs have an intrinsically reduced capacity to (re-)myelinate. Evidence for cell intrinsic senescence comes from analysis of long-term cultured rat OPCs, which show increased cell cycle and differentiation times with increased time of cultivation, very similar to freshly isolated cells that come from animals of different ages (Tang et al., 2000). However, the study by Münzel et al. also reported that reduced remyelination in aged zebrafish was accompanied by a reduced macrophage response to LPC induced remyelination. Work in rodents has shown that macrophages secrete cytokines that can enhance remyelination (Miron and Franklin, 2014; Miron et al., 2013). In addition, heterochronic parabiosis studies revealed that monocytes derived from the young parabiotic partner enhance remyelination in old mice (Ruckh et al., 2012). It thus remains unclear whether OPCs in older animals/zebrafish have an intrinsically reduced myelination capacity.

Analysis of basic cellular principles of oligodendrocyte behavior following damage and repair to myelinated axons would help address whether these are similar or fundamentally different to early developmental myelination. Models of experimental demyelination by targeted cell ablation have been reported using transgenic expression of nitroreductase in oligodendrocytes in zebrafish (Chung et al., 2013) and also in Xenopus tadpoles (Kaya et al., 2012). Nitroreductase induces cell death by generating a DNA-crosslinking agent upon exposure to metronidazole (Curado et al., 2008). The availability of zebrafish pigmentation mutants like *casper* which are essentially translucent even as adults (White et al., 2008b) allow for long-term *in vivo* live imaging to study basic cellular principles following damage to myelin. It is still unclear how exactly OPCs respond to myelin damage, whether there is for example a minimal threshold of myelin damage to elicit an OPC response, and if the cellular basis of remyelination (secondary myelination, or repeated myelination of the same axon) is the same as during primary myelination. Such studies may help address the long-standing question of whether regeneration is a simple recapitulation of development or if fundamental differences exist.

An increasing number of publications use zebrafish to model human diseases. While this can be a perfectly reasonable approach in monogenetic diseases in which known mutations lead to a clear phenotype, zebrafish (like many other animal models) are most likely not suitable to model more complex human diseases. Myelin diseases like for example MS, to which genetic as well as environmental risk factors contribute to, can almost certainly not be modeled in zebrafish embryos or larvae. The innate immune response is well studied in young zebrafish, very similar to that of mammalians, and has helped a great deal to understand aspects of microglial and macrophage biology (Oosterhof et al., 2014; Sieger and Peri, 2012). Macrophages and microglia play important roles in remyelination and work in zebrafish may help obtaining insight into the interplay between oligodendrocytes and innate immune response following experimental damage to myelin. However, mechanisms of B- and T-cell biology, which are of great relevance for autoimmune diseases like MS, still need to be investigated in greater detail in zebrafish regarding their similarity to higher vertebrates (Iwanami, 2014; Langenau and Zon, 2005). Lastly, the presence of an environment permissive to regeneration as discussed above is a major difference and should be taken into account when using zebrafish as model. It is important to not ignore but rather take advantage of these differences and to use the zebrafish to address open questions that cannot be disentangled in other models otherwise.

Concluding Remarks and Future Directions

The use of zebrafish has made significant contributions to our understanding of myelinating cell biology in the CNS. Live imaging studies have provided fundamental insights into the dynamics of myelination and how oligodendrocyte behavior is regulated by cellular interactions between glia and neurons *in vivo*. We are, however, still only beginning to understand how this leads to the formation of a myelinated CNS as it is and many open questions need to be addressed where the zebrafish will serve as a helpful model.

What triggers oligodendrocyte differentiation and the initiation of axonal ensheathment? Over the last few years, various genetically encoded reporters and indicators have been developed to study cellular physiology using optical imaging methods such as the calcium indicator GCaMP6 (Chen et al., 2013), neurotransmitter sensors like the glutamate indicator GluSnFR (Marvin et al., 2013). These reporters have already proven to be of great help for the study of neuronal function in zebrafish *in vivo* and equally offer the opportunity to study direct communication between oligodendrocytes and neurons to form and maintain a myelinated CNS.

How is myelinating cell behavior regulated by interactions with non-neural cells? Immune cells and the vasculature play important roles during myelination and remyelination but how they directly affect oligodendrocyte behavior is not well understood. Innate immunity as well as vascular biology are intensely studied areas in the zebrafish

and the availability of respective mutant and reporter lines may help elucidate how such intersystems interactions regulate myelination.

New imaging technologies available such as light-sheet microscopy, for which no other vertebrate model is better suited than zebrafish, enables long-term fluorescence imaging of whole organs and even entire animals (Keller and Ahrens, 2015). This will allow investigation of myelination at a systems level with high spatial and temporal resolution and may also help address long-standing questions relating to circuit formation, heterogeneity of oligodendrocytes and their functional diversity within the CNS.

Genetic screens in the zebrafish have led to the discovery of new molecules important to PNS and CNS myelination in the past. However, the molecules that mediate myelinating cell behavior *in vivo* are still to be elucidated. For example, axo-glial signaling molecules absolutely essential for CNS myelination are still not known. Future genetic zebrafish screens for CNS myelination may help identify such essential molecular cues. Chemical screens in zebrafish have been powerful approaches in research areas like hematopoiesis and will likely be of equal potential when studying CNS myelination. Furthermore, chemical screening is less sensitive to genetic redundancy (multiple copies and isoforms) and is thus very complementary to genetic screening. In contrast to chemical and genetic approaches, proteomic analysis in zebrafish still seems understudied. Recently, however, it was shown that zebrafish are well suited for analysis of organ specific proteome maps (Nolte et al., 2014), and also a proteomic screen for molecules involved in fin regeneration using differential metabolic labelling using SILAC (stable isotope labeling by amino acids in cell culture) has been reported (Nolte et al., 2015). Such approaches may help reveal new targets in the context of CNS regeneration and remyelination in the future. Lastly, the development of efficient genome editing technologies now enables generation of even cell type specific knock-outs, making the zebrafish also attractive for reverse genetic approaches.

Together, the zebrafish has been a useful model to study mechanisms of myelinated axon formation in the past and will most likely be of even bigger help in the future by combining *in vivo* live cell analyses with unbiased screening and reverse genetic approaches.

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