DEPARTMENT OF MEDICAL BIOCHEMISTRY AND BIOPHYSICS
Karolinska Institutet, Stockholm, Sweden

LONG NON-CODING RNAS IN THE EPIGENETIC REGULATION OF OLIGODENDROCYTE DIFFERENTIATION

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Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7831-526-0

Long non-coding RNAs in the epigenetic regulation of oligodendrocyte differentiation
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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One must imagine Sisyphus happy

- *Albert Camus*

ABSTRACT

Long non-coding RNAs (lncRNAs) constitute a heterogeneous class of RNAs with limited coding potential, united by an arbitrarily placed cut off of >200 ntd. The past decade has seen the emergence of lncRNAs as versatile regulators of gene expression, amidst skepticism regarding the biological usefulness of pervasive genomic transcription and its non-coding RNA products prevalent in most eukaryotes. A significant portion of lncRNAs operate in the development and functioning of the mammalian CNS. Oligodendrocytes (OLs) are the myelinating cells of the CNS that are essential for efficient saltatory conduction and axonal survival. They are derived from OL precursors (OPCs) and progress into transcriptomically heterogeneous OL sub-populations along the differentiation pathway to produce mature OLs, capable of myelination. These epigenetic transitions between different OL subpopulations are carefully regulated, spatially and temporally, by a network of transcription factors, chromatin modulators and lncRNAs. In demyelinating diseases like multiple sclerosis (MS), patients suffer immune mediated attacks against myelin. Eventually, remyelination strategies fail due to deficits in OPC migration and OL differentiation at the site of lesions. Thus, understanding molecular mechanisms governing OL differentiation and myelination is crucial not only for understanding OL function in health but also in disease, in order to develop suitable therapeutic interventions.

The investigations presented in this thesis explore the role of lncRNAs and RNA-binding proteins in neurodevelopment, particularly in embryonic stem cells (ESCs) and cells of the OL lineage.

Article 1 provides a resource for the protein interactome of a key pioneering transcription factor, Sox2, in different nuclear fractions of mouse ESCs. We found Sox2 to be a multi-faceted regulator forming interactions with HP1 family of proteins, whose members perform as both activators and repressors in a context dependent manner. In addition to interacting with RBPs involved in post-transcriptional processes, Sox2 also interacted with *Rn7sk*, a well-known ncRNA involved in the regulation of transcriptional elongation at promoters and enhancers. Although they did not influence each other's recruitment to the chromatin, this interaction opens up the possibility for ncRNA mediated modulation of ES transcriptional programs dependent on Sox2.

Article 2 draws important insights regarding lncRNAs from a broad transcriptomic resource established from single cell- as well as bulk RNA- sequencing of OL lineage cells from different developmental stages. From a subset of lncRNAs which were found to be specific for certain OL subpopulations, we investigated the role of *2610035D17Rik* in modulating the expression of its neighboring gene, *Sox9*, a transcription factor essential for OPC specification. We decoupled the role of lncRNA transcript from its genomic locus using various loss-of-function strategies and found that the regulation of Sox9 was dependent on the regulatory elements and/or ongoing transcription at the *2610035D17Rik* locus, rather than the transcript itself.

In **Article 4**, we investigated a hitherto unexplored RNA-binding function of myelin gene expression factor 2 (Myef2), a known transcriptional repressor of myelin basic protein (MBP). To this end, we uncovered the RNA interactome of Myef2 in a mouse oligodendroglial cell line with individual nucleotide resolution CLIP (iCLIP) followed by sequencing. We show that Myef2 interacts with CUG motifs located within introns and 3'UTRs of protein-coding genes, a finding which implicates Myef2 in post-transcriptional processes like splicing and RNA stability.

Finally, in **Article 3** we have identified disease specific transcriptomic profiles of OL lineage cells through single-cell RNA sequencing of OPCs and OLs derived from experimental autoimmune encephalomyelitis (EAE) mice, a model that recapitulates several aspects of MS. EAE specific OPC and OL clusters were enriched for genes involved in antigen processing and presentation (MHC class I/II). We could demonstrate that OPCs can phagocytose myelin debris and MHC-II-expressing OPCs can activate memory and effector CD4-positive T cells. These findings show OL lineage cells as active participants in MS pathology than passive targets. Further, the findings of Article 2 implicate *2610035D17Rik* as a regulator of immunomodulatory properties of oligodendroglia, as *2610035D17Rik* KO cells showed reduced expression of IFNγ responsive genes and elevated expression of those involved in antigen presentation, compared to the controls, following IFNγ stimulation.

LIST OF SCIENTIFIC PAPERS

I. Interaction of Sox2 with RNA binding proteins in mouse embryonic stem cells. Experimental Cell Research 2019 Aug 1;381(1):129-138
Samudyata, Paulo P. Amaral, Pär G. Engström, Samuel C. Robson, Michael L. Nielsen, Tony Kouzarides, Gonçalo Castelo-Branco

II. Role of long non-coding RNAs *Pcdh17IT* and *2610035D17Rik* in oligodendrocyte lineage progression, manuscript.
Samudyata*, Marek Bartosovic*, David Van Bruggen, Sueli Marques, Darya Vanichkina, Bastienne Zaremba, Simone Codeluppi, Sten Linnarsson, Gonçalo Castelo-Branco

III. Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis. Nature Medicine 2018 Dec;24(12):1837-1844
Ana Mendanha Falcão, David van Bruggen, Sueli Marques, Mandy Meijer, Sarah Jäkel, Eneritz Agirre, **Samudyata**, Elisa M. Floriddia, Darya P. Vanichkina, Charles ffrench-Constant, Anna Williams, André Ortlieb Guerreiro-Cacais and Gonçalo Castelo-Branco

IV. Myef2 interacts with RNAs with CUG motifs in oligodendrocytes, manuscript.
Samudyata, Eneritz Agirre, Ana Falcao, Roman Zubarev, Gonçalo Castelo-Branco

* Equal contribution

CONTENTS

1 INTRODUCTION TO LONG NON-CODING RNAS

1.1 HISTORICAL OVERVIEW

Concepts making up the framework of RNA biology have undergone tremendous changes since its conception; certain fundamental assumptions have been overturned in the process of attaining current insights regarding the pivotal role of RNA in shaping gene expression. The involvement of house-keeping non-coding RNAs (ncRNAs) such as ribosomal RNA (rRNA) and transfer RNAs (tRNAs) in protein synthesis were well established as early as 1950s. The discovery of catalytically active ribozymes and small nuclear RNAs (snRNAs) involved in intron excision (Kruger et al., 1982; Lerner et al., 1980) came nearly three decades after and strengthened the possibility of RNA acting as a catalytic entity of ribosomal unit (Steitz and Jakes, 1975). These discoveries added to the growing speculation that RNA could be something more than a passive purveyor of genetic information. Further, the discovery of a short RNA in *C.elegans* that could base-pair imperfectly with its target mRNA and down-regulate it during development (Lee et al., 1993) and its similarity with the 20-22 ntd RNA-mediated silencing observed in worms and plants (Fire et al., 1998; Hamilton and Baulcombe, 1999), now called miRNAs and siRNAs respectively, gave an unprecedented impetus to the field and paved the way towards understanding a RNA dependent post-transcriptional regulatory mechanism widespread in eukaryotes. Other classes of small ncRNA regulators that have been identified since are small nucleolar RNAs (snoRNAs) that aid in chemical modification of other RNAs (Bachellerie et al., 1995) and piwi-interacting RNAs (piRNAs) that maintain genomic integrity by repressing retrotransposition of repeat elements (Siomi et al., 2011). Most recent addition to this list are a heterogeneous class of ncRNAs called the long non-coding RNAs (lncRNAs) which are distinguished from above said small ncRNAs with an arbitrarily placed cut off of >200 ntd.

1.2 FUNCTIONAL PRODUCT OR TRANSCRIPTIONAL NOISE?

Recent advances in transcriptomic profiling, especially microarray/tiling and deep sequencing technologies, made it possible to look at mammalian RNA species in much greater detail. Global genomics consortia such as FANTOM and ENCODE started in early 2000 revealed protein coding genes accounted for only a small fraction of the genome, making ncRNAs a major component of mammalian transcriptomes (Carninci et al., 2005; ENCODE Project Consortium et al., 2007; Okazaki et al., 2002). Later projects uncovered a comprehensive catalogue of transcription start and termination sites, with promoters also mapped within exons and 3'UTRs of coding genes (Carninci et al., 2006; Katayama et al., 2005). Usage of different combinations of exons and polyA sites seemed commonplace, with even non-coding transcripts producing alternative forms (Ravasi et al., 2006). This unexpected transcriptomic complexity generated an interest regarding the possibility of non-coding RNA based regulatory systems principally driving organismal complexity (Mattick, 2004). But despite pervasive transcription and such diversity, only a small number of non-coding transcripts have known functions, lncRNAs being the least understood products of transcription.

A pertinent question that arises in the light of such a discovery is whether these transcriptional products are biologically meaningful or constitute mere 'transcriptional noise'. It is known that the transcriptional machinery is slippery and open chromatin could be easily transcribed into products that serve no purpose (Struhl, 2007), some of which could also be capped and spliced out of chance and tolerated because of minimal fitness cost to the cell. Some transcripts may be produced coincidentally with the act of transcription that occurs at certain regulatory DNA elements in the genome and may not be functional by themselves (Kornienko et al., 2013). The possibility remains that a substantial fraction of the non-coding transcriptome might indeed be non-functional but with growing lncRNA numbers and their associated functional diversity (Ramilowski et al.,bioArxiv 2019), it seems increasingly likely that they are biologically important.

1.3 CLASSIFICATION

Annotation for a disparate class of molecules united by an arbitrary cut off size of > 200 ntd has just begun and hence there is a need for a universal standardized system of lncRNA classification. The HUGO Gene Nomenclature Committee (HGNC) guidelines (Wright, 2014) recommend naming a lncRNA as an abbreviation of a descriptive name, preferably based on its function (E.g. *NEAT1* - nuclear paraspeckle assembly transcript 1). LncRNAs with no known function are named based on their genomic context, in relation to adjacent protein-coding genes. For example, Overlapping (OT) lncRNAs encompass a protein coding gene or parts of it within them, on the sense or antisense (AS) strand, respectively. Intronic (IT) lncRNAs come from within an intron of a protein coding gene and do not overlap with any exons. Antisense Upstream (AU) or bidirectional lncRNA and a protein coding gene are transcribed divergently from the same or closely situated TSS. Intergenic or Long Intervening ncRNAs (LINC) lie between protein coding loci.

In addition to above classification, other lncRNA classes associated with promoters are found in literature, and are distinguished based on their length. For example, the shortest amongst them (<100 ntd) are called transcription start site associated RNAs (TSSa-RNA) and are likely to be degradation products of nascent RNA (Valen et al., 2011). Longer transcripts produced divergently from a productive Pol II elongation are called promoter upstream transcripts (PROMPTs) and they undergo degradation by nuclear exosome (Kapranov et al., 2007). Transcripts arising from regulatory enhancer regions are enhancer RNAs (eRNAs) and activating ncRNAs (ncRNA-a) (Santa et al., 2010).

1.4 ORIGIN

LncRNAs are thought to originate via several mechanisms. Homologous lncRNAs are born when there is duplication of sequence containing a functional lncRNA locus. It is possible for a duplicated protein coding gene to undergo metamorphosis by accumulating mutations that disrupt the ancestral open reading frame to give rise to a pseudogene, which when transcribed produces a lncRNA (Pei et al., 2012; Zheng et al., 2007). Such pseudogenisation can also occur on unitary protein coding genes resulting in a lncRNA with no apparent protein-coding

homologue (Marques et al., 2012). Yet another mechanism of lncRNA birth is the integration of RNA derived from transposable elements into non-coding genes. Mostly defunct transposable elements are found within lncRNAs, with retrotransposons contributing to almost two thirds of mature mouse and human lncRNA sequences (Kapusta et al., 2013). They are more likely to provide functional cis-elements such as a transcription start site, polyadenylation and splice sites to lncRNA genes compared to protein coding loci (Kapusta et al., 2013). LncRNAs can also arise de-novo when pervasive transcription from non-coding DNA results in cryptic RNAs and if they pass through evolutionary selection, become transcripts with novel functions (Heinen et al., 2009).

1.5 EVOLUTIONARY CONSERVATION

Generally, genomic sequences of lncRNAs have low evolutionary constraints, unlike protein coding genes (Kowalczyk et al., 2012). Criterion based on primary sequence conservation has given rise to considerable skepticism regarding the biological significance of lncRNAs but this consideration alone might be restrictive. Indeed, lncRNAs show more conservation in their exons and splice sites than repeat elements under neutral evolutionary pressure (Ponjavic et al., 2007). In fact, lncRNA promoters were found to be as conserved as those of protein coding genes (Derrien et al., 2012; Guttman et al., 2009). LncRNAs that do share such limited homology amongst vertebrates are the lincRNA subfamily, often found to be actively regulated with similar expression patterns as protein coding genes involved in tissue specific functions (Hezroni et al., 2015; Necsulea et al., 2014). Those with no detectable sequence conservation still show conserved genomic locations or synteny (Ulitsky et al., 2011). Thus low conservation might be a result of rapid turnover of lncRNAs, given their shallow evolutionary origin compared to protein-coding genes or it could be due to evolutionary constraints acting over a small region buried within a long transcript.

1.6 BIOGENESIS AND PROCESSING

Most eukaryotic lncRNAs are transcribed by RNA pol II, from open chromatin/DNase hypersensitive regions, and require canonical transcriptional machinery. Accordingly, histone signatures include H3K4me3 at linc promoters and H3K36me3 over the transcript body, which is similar to what is observed for protein coding genes (Guttman et al., 2009). LncRNA genes that overlap with enhancers exhibit H3K27ac epigenetic mark and a higher ratio of H3K4me1/ H3K4me3 in their transcription initiation regions (Kim et al., 2015). PROMPTs however are predominant at CpG rich promoters, do not extend into nucleosome depleted regions and are generally devoid of transcription initiation factors such as TAFI, TAFII p250 and E2F1 (Preker et al., 2011).

Many lncRNAs are capped at the 5’ end, although intronic lncRNAs comprise a higher percentage of uncapped transcripts (Ayupe et al., 2015). While mRNAs undergo robust co-transcriptional splicing and polyadenylation, lncRNAs show decreased splicing and are biased towards having two exons and longer introns (Derrien et al., 2012). Decreased splicing in lincRNAs may be due to the presence of weaker 3’ splice signals and less binding by the

splicing factor U2AF65 (Melé et al., 2017). Furthermore, transcription termination mark in the form of phosphorylated Thr4 on RNA pol II CTD seems to be found across entire lincRNA transcriptional units (Schlackow et al., 2017). A study has also reported an asymmetric enrichment in polyadenylation sites (PASs) in antisense lincRNAs transcribed from bidirectional promoters, which would favour their premature transcription termination (Almada et al., 2013). LncRNAs may or may not possess a 3' poly-adenylated tail and in some cases could even be bimorphic and exist in both polyA+ and polyA- forms (Yang et al., 2011). Additionally, they exhibit a wide range of half-lives, with the median lncRNA half-life (3.5 hours) being slightly less than that of mRNAs (5.1 hours) (Clark MB, Genome Res 2012). Amongst different lncRNA classes, intergenic and cis-antisense transcripts were found to be more stable than intronic lncRNAs and PROMPTs (Clark et al., 2012). Thus, splicing, poly-adenylation and specific 3' structures such as the triple helix accord higher stability to lncRNAs (Clark et al., 2012; Wilusz, 2016). LncRNAs are lowly expressed, the median expression being almost a tenth of mRNAs' (Cabili et al., 2011; Ulitsky et al., 2011) but their expression seems more restricted to a particular tissue/cell type or developmental stage (Batista and Chang, 2013).

Most lncRNAs lack robust ORFs (Mercer et al., 2009). Data from ribosome profiling however shows a considerable fraction of lncRNAs to be associated with ribosomes (Bazzini et al., 2014; Ingolia et al., 2011), in a pattern indicative of their potential to produce short peptides. Bioinformatic analysis of ribosome profiling data from two cell-lines has identified translated ORFs in over 500 lncRNAs (Ji et al., 2015). However, it has been pointed out that ribosome occupancy alone cannot distinguish non-coding from protein coding transcripts, as classical ncRNAs also engage the ribosomes at their 5'UTR. Further, non-coding RNAs do not show ribosome release from any of their ORFs, an event that occurs consistently at the stop codons of protein coding genes (Guttman et al., 2013). Although the proportion of lncRNAs that can encode peptides is debatable, it has become clear that some lncRNAs harbour short ORFs (sORFs) that are translatable into micropeptides of < 100 aa (Mumtaz and Couso, 2015), examples of which have recently come to light (Choi et al.).

2 LNCRNA FUNCTION

Only a fraction of lncRNAs have been well characterized; they are very heterogeneous in their mode of action and are known to operate at multiple levels of gene expression regulation.

2.1 TRANSCRIPTIONAL REGULATION

Many lncRNAs localize to the nucleus preferentially (Quinodoz and Guttman, 2014; Vance and Ponting, 2014). Though the global determinants of lncRNA nuclear localization is still unclear, it has been mainly linked to inefficient splicing (Zuckerman and Ulitsky, 2019) and presence of integrated Alu elements along with HNRNPK binding (Lubelsky and Ulitsky, 2018). In addition, specific motifs have been identified in individual lncRNAs that play a role in their nuclear retention (Miyagawa et al., 2012; Zhang et al., 2014a).

The mode of lncRNA action in the nucleus could be dependent on the act of transcription rather than the transcript itself, where transcription of lncRNA induces local chromosomal remodelling affecting the binding of regulatory factors which could in turn influence gene expression in the vicinity (Camblong et al., 2007). Alternatively, the lncRNA transcript could directly recruit multiple regulatory complexes by making use of its discreet domains or larger structure, targeting them to a specific DNA locus. In the latter case, lncRNA can act either in –cis (at/near the site of its transcription) or in –trans (away from the site of its transcription), though the three-dimensional (3D) scape of the genome sometimes blurs this distinction.

2.1.1 Imprinting

During early mammalian gametogenesis, some protein coding genes are epigenetically marked such that their expression occurs in a parent of origin specific manner in a process known as genomic imprinting. LncRNAs expressed from differentially methylated "imprinting control regions" usually enact imprinting on gene clusters via cis-regulatory mechanisms. For example, the expression of nuclear localized lncRNA *Kcnq1ot1* is restricted to the paternal chromosome and upon its transcription recruits chromatin and DNA methyltransferases (G9a, DNMT1) to silence the *Kcnq1/Cdkn1c* gene cluster in cis, while their maternal alleles continue to be expressed (Kanduri, 2016).

One of the best-studied examples of imprinting is that of 17kb lncRNA *Xist* that dramatically silences one of the female X chromosomes (Xi) from which it is expressed (Jonkers et al., 2008), thereby providing dosage compensation. Other lncRNA partners involved in mouse X-chromosome inactivation (XCI) are *Xist* activators, *Jpx* and *Ftx*, along with *Tsix*, a Xist repressor. *Tsix* is transcribed anti-sense to *Xist* on the active X chromosome (Xa), thereby protecting it from ectopic XCI (Gayen et al., 2015). While *Jpx* lncRNA acts in either cis or trans to bind and remove the insulator protein, CTCF, from repressing *Xist* (Sun et al., 2013b), transcription in the *Ftx* lncRNA locus leads to expression of *Xist* in cis (Furlan et al., 2018). This establishment of monoallelic *Xist* expression triggers a cascade of events where *Xist* gradually spreads from its transcription locus by binding to a scaffold attachment factor,

drawing in more distant regions on XI while using its modular domains to recruit multiple epigenetic regulators (HDAC3, PRC2/1, SETDB1) that help in initiating and maintaining transcriptional repression through histone deacetylation and methylation, respectively (Cerase et al., 2015).

2.1.2 Enhancer mediated regulation

Enhancers are decompacted DNA elements capable of binding transcription factors and co-activator complexes; they drive gene expression independently of the distance and orientation of their target promoters and are epigenetically marked with a higher H3K4me1/ H3K4me3 ratio (Li et al., 2016). Active enhancers often give rise to bi-directional, unspliced, non-polyadenylated transcripts (Kim et al., 2015) and sometimes relatively more directional, polyadenylated transcripts (Koch et al., 2011), termed as enhancer RNAs (eRNAs) and activating ncRNAs (ncRNA-a), respectively. The production of eRNAs is highly regulated and often precedes the induction of adjacent mRNAs in response to a certain stimulus (Kim et al., 2015; Li et al., 2016; Wang et al., 2011). However, successful eRNA induction seems to be dependent on the RNA endonuclease activity of the Integrator complex, which is involved in 3' cleavage of the eRNA transcript (Lai et al., 2015).

The activating function of eRNAs sometimes appears to be sequence specific as reported by enhanced gene expression in a plasmid reporter system in which eRNAs were tethered upstream of its minimal promoter (Lam et al., 2013). In other cases, they seem to stabilize and strengthen enhancer-promoter looping through cohesin (Li et al., 2013) and the mediator complex (Kagey et al., 2010; Lai et al., 2015) as well as facilitate RNA pol II loading or productive elongation at the target gene promoter (Mousavi et al., 2013; Schaukowitch et al., 2014). Thus eRNAs and/or active enhancer transcription regulates genomic accessibility, transcription machinery availability, chromatin conformation locally and in highly specific ways to drive cell type and activity dependent expression programs.

2.1.3 Modulation of transcriptional machinery

LncRNAs can directly act on the transcriptional machinery. In one case, a lncRNA generated from short interspersed elements (SINEs) during heat shock blocks transcription by binding to RNA pol II, by entering into transcriptional complexes at promoters (Mariner et al., 2008). Alternatively, lncRNAs can form stable triplexes with promoters and regulate binding of transcriptional activators or inhibitors in trans, as in the case of lncRNA *Khps1* that forms a triplex with a homopurine stretch upstream of *SPHK1* (Sphingosine kinase 1) promoter and activates it by recruiting histone acetyl transferase p300/CBP54 (Postepska-Igielska et al., 2015). They can also interact with transcription factors and help in their recruitment to specific gene sets as in the case of lncRNA *RMST* (rhabdomyosarcoma 2-associated transcript) and SOX2 co-regulating neural stem cell fate (Ng et al., 2013).

2.1.4 Nuclear architecture

Recent studies have shown that lncRNAs actively assemble and maintain nuclear domains. Paraspeckles are transcription dependent dynamic foci that are enriched with RNA-binding proteins (RBPs) involved in RNA splicing, editing and DNA unwinding (Bond and Fox, 2009). Though their function is not entirely known, it is possible that they provide an ordered pool of their component proteins and help in directing their activity when required (Schuldt, 2002). Along with RBPs, they also harbour lncRNA *NEAT1* that is crucial for paraspeckle structural integrity, so much so that knock down of *NEAT1* results in paraspeckle dispersion (Clemson et al., 2009) and an artificial tethering of *NEAT1* to a genomic location is sufficient for paraspeckle formation (Shevtsov and Dundr, 2011). Interestingly, paraspeckle formation also seems to be dependent on ongoing *NEAT1* transcription, and not just the presence of the lncRNA (Mao et al., 2011). Further, some adenosine-to-inosine edited mRNAs, along with those that contain Alu repeats, associate with paraspeckles and it is speculated that *NEAT1* might play a role in their nuclear retention (Chen and Carmichael, 2009). Another lncRNA called *MALAT1/NEAT2* is a part of nuclear speckles; a storehouse for pre-mRNA processing proteins (Lamond and Spector, 2003). Although *MALAT1* is not required for nuclear speckle integrity, it seems to have a role in ensuring proper localization of some nuclear speckle proteins involved in splicing (Tripathi et al., 2010). It has been proposed that *MALAT1* scaffolds actively transcribing genes (West et al., 2014) to nuclear speckles and its contents to influence gene expression (Engreitz et al., 2016)

LncRNAs can also be involved in modulating higher order nuclear architecture. For example, lncRNA *Firre* localises across a X chromosome domain that escapes inactivation and forms high-affinity interactions with DNA loci on different mouse chromosomes (Hacisuleyman et al., 2014). This trans-chromosomal co-localisation brings genes involved in energy metabolism and/or adipogenesis together in 3D space to facilitate their co-regulation (Sun et al., 2013a). The capacity of lncRNAs to passively diffuse while acting as bridging molecules between transcribing DNA and different proteins combined with the ability of certain self-interacting RBPs to segregate into phase separated bodies, most likely drives nuclear compartmentalization (Kato et al., 2012; Yamazaki et al., 2018).

2.2 POST-TRANSCRIPTIONAL REGULATION

lncRNAs also function as effective modulators of post-transcriptional processes like pre-mRNA splicing, mRNA decay, and translation, in addition to their roles as transcriptional regulators.

2.2.1 mRNA splicing and stability

There has been a growing appreciation of the ways in which chromatin status and transcription is coupled to splicing events (Montes et al., 2012) and lncRNAs seem to play a role in bridging these regulatory systems. For example, a nuclear anti-sense lncRNA from the *FGFR2* locus was shown to promote alternative splicing of *FGFR2* mRNA into an epithelial-specific isoform by recruiting polycomb proteins and histone demethylase (KDM2a), which

impair mesenchymal-specific splicing by abrogating the binding of a repressive chromatin-splicing adaptor complex (Gonzalez et al., 2015). In addition, lncRNAs can influence splicing outcomes by sequestering RNA-binding proteins involved in splicing regulation. One such example is a new class of intron-derived lncRNAs, called sno-lncRNAs, which are flanked by small nucleolar RNA (snoRNA) sequences and lack 5' cap and polyA tail. Particularly, those derived from the 15q11-q13 human chromosomal region implicated in Prader-Willi syndrome (Duker et al., 2010), harbour multiple binding sites for the alternative splicing regulator FOX2 and act as a molecular sink to prevent its binding to other mRNA targets (Yin et al., 2012).

LncRNAs can act as both positive and negative regulators of mRNA stability. For example, Alu-repeat containing lncRNAs form imperfect base pairing with the Alu elements in the 3' UTRs of their target mRNAs which is recognized by Staufen 1 (STAU1) to induce staufen-mediated decay (Gong and Maquat, 2011). By contrast, a lncRNA transcribed antisense to beta-secretase producing *BACE1* mRNA, forms a duplex with the mRNA and abrogates miR-485-5p induced repression, thereby stabilizing its target mRNA (Faghihi et al., 2010).

2.2.2 Translation

The mouse ubiquitin carboxyterminal hydrolase L1 (Uchl1) mRNA encodes an enzyme with roles in brain development and shares complementarity at its 5' end with a neuron specific, nuclear enriched, antisense (AS) Uchl1 lncRNA. Upon mTORC1 inhibition, it was found that Uchl1-AS translocates to the cytoplasm, and through overlapping complementarity, enhanced the formation of active polysomes on its target mRNA and therefore its translation (Carrieri et al., 2012).

2.2.3 Encoding small peptides

Some lncRNAs harbor sORFs that are translatable, and a few of the resulting peptides have been reported to be biologically active. SPAR is a lncRNA encoded small peptide <100 aa that inhibits mTORC1 activation necessary for muscle regeneration (Matsumoto et al., 2017). Minion, another sORF peptide was found to be critical for the induction of mononuclear progenitor fusion into multinuclear myotubes during skeletal muscle development (Zhang et al., 2017b). In addition to peptides functioning in muscles, a cancer related peptide, HOXB-AS3, inhibits hnRNPA1 mediated splicing of pyruvate kinase M (PKM) and suppresses glucose metabolism needed for supplying energy to tumors (Huang et al., 2017).

2.2.4 Regulatory sponges

Circular RNAs (circRNAs) are created when a splice donor at the 3' exon end is joined to a 5' splice acceptor of an upstream exon in a non-canonical splicing event called 'backsplicing' (Memczak et al., 2013; Salzman et al., 2012). CircRNAs are stable and accumulate in the cytoplasm without associating with ribosomes (Guo et al., 2014), however exceptions have been reported recently. CircRNAs were found to undergo translation especially in response to stimuli that interferes with cap dependent translation (Legnini et al., 2017; Pamudurti et al.,

2017). Known functions of circRNAs till date include their ability to act as miRNA sponges. For example, cerebellar degeneration-regulated protein 1 anti-sense RNA (*CDR1-AS*) contains more than seventy miR-7 binding sites and is resistant to miRNA mediated destabilization, resulting in an increased expression of miR-7 targets (Hansen et al., 2013).

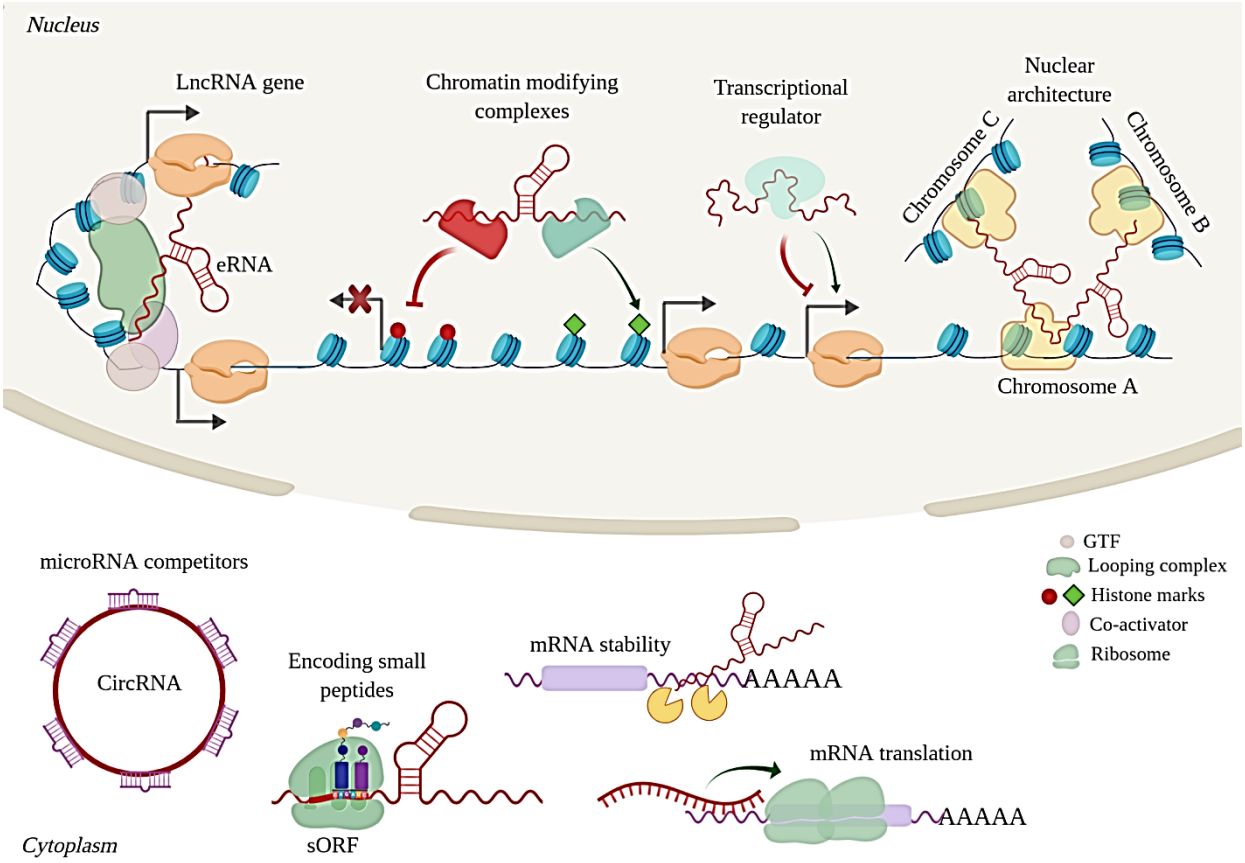

Figure 1**: **Modes of lncRNA action

From left to right– LncRNAs can regulate transcription in the nucleus by mediating enhancer looping, by acting as scaffolds for chromatin modifying complexes, modulating activity of transcription factors and by influencing spatial conformation of chromosomes. In the cytoplasm, circRNAs can sequester miRNAs, some cytoplasmic lncRNAs carry small ORFs that can be translated into biologically active small peptides and they can regulate mRNA stability or translation. (Adapted from Moralando et al.,2015)

3 TECHNOLOGICAL ADVANCES

3.1 IDENTIFICATION OF LNCRNAS

Deep sequencing methodologies in the last decade have revolutionized the way we understand the transcriptomic output of cells and consequently their functional states. Predictably, the most commonly employed technique to discover novel lncRNAs and quantify their expression is bulk RNA-sequencing (RNA-seq) Usually rRNA-depleted transcriptomes from large cell populations are reverse transcribed with random primers into a pool of cDNAs, which are then sequenced. The total RNA-seq libraries thus obtained are enriched for both polyadenylated and non-polyadenylated transcripts and the resulting data is representative of average gene expression pattern across millions of cells. Given that heterogeneity of gene expression is observed even within same cell populations and could harbour biologically relevant information, single-cell RNA sequencing (scRNA-seq) was developed to address this concern (2014). Broadly, this technique involves isolating single cells via fluorescence activated cell sorting (FACS) or microfluidics followed by capturing and barcoding their transcripts. Reverse transcription and amplification of cDNAs then generate libraries in which the transcripts can be mapped back to individual cells. Cap analysis of gene expression (CAGE) is another high-throughput method that relies on the presence of 5' cap on RNAs to identify active promoter regions and TSS driven by Pol II, as such excludes non-capped transcripts and circRNAs (Shiraki et al., 2003). While RNA-seq and CAGE measure steady state RNA levels, methods like global run-on sequencing (GRO-seq) target nascent RNA transcription from actively engaged polymerases (Lopes et al., 2017). This genome wide snapshot of transcription is particularly suited for lncRNAs that have a high decay rate (Chu et al., 2015).

3.2 LNCRNA FUNCTION

Subcellular localization of lncRNAs can potentially hold clues about their function. *In-situ* hybridization (ISH) techniques can detect and localize target RNAs within cells by using complementary probes. This is followed by visualization with fluorescence microscopy, either with direct labeling of probes with fluorophores (single molecule RNA FISH) (Kwon, 2013) or with signal amplification schemes (RNAscope) (Wang et al., 2012). Recently developed technologies like fluorescent in situ RNA-seq (FISSEQ) (Lee et al., 2014) have combined transcriptomic data from sequencing with spatial information from ISH to achieve cell or tissue specific visualization of lncRNA expression and localization.

As discussed above, many lncRNAs function by interacting with protein partners. One of the methods to investigate RNA-protein complexes in-vivo with high positional resolution is to use UV mediated crosslinking and immunoprecipitation followed by high throughput sequencing (CLIP-seq) (Ule et al., 2003). Any interacting RNA covalently fixed to the protein of interest is partially digested and this complex is pulled down using an antibody against the protein. Following immunoprecipitation, RNA is reverse transcribed and the protein content is digested before the preparation of cDNA libraries for sequencing. One of

the limitations of CLIP is the loss of a large fraction of truncated cDNAs lacking the 5' adapter due to reverse transcriptase stalling at the peptide residue left at the site of cross-linking while proceeding from the 3' adapter end. A particular variant of CLIP called individual nucleotide resolution CLIP (iCLIP) (König et al., 2010) resolves this problem by circularizing cDNAs containing cleavable adapters which when linearized generates templates that can be captured for PCR amplification. These techniques have been widely used to generate transcriptome wide binding maps for many RBPs, particularly those involved in post-transcriptional gene regulation, in order to characterize their molecular function.

LncRNAs also function by interacting with DNA directly or through protein intermediates. Thus many genomic-context based methodologies have been developed to unravel lncRNA chromatin occupancy that differ in their cross-linking strategies and probe design. Cells are cross-linked with glutaraldehyde or formaldehyde or both, sonicated to generate smaller DNA fragments, hybridized with biotin conjugated DNA probes complementary to RNA of interest, and then the tertiary protein-DNA-RNA complex is purified with streptavidin beads. Upon cross-link reversals, the resulting DNA can be sequenced or the associated proteins could be analyzed by mass spectrometry. Specifically, chromatin isolation and purification (ChIRP) (Chu et al., 2011) uses a pool of 20nt long probes that tile the entire length of the lncRNA while capture hybridization analysis of RNA targets (CHART) (Simon et al., 2011) relies on using probes designed to target accessible binding sites with the help of RNase H mapping. Similarly, RNA-RNA interactions can be studied by using a variety of cross-linking chemicals that target both direct and indirect interactions, namely 4'aminomethyltrioxalen (AMT) that captures direct RNA-RNA interactions through uridine cross-links and a combined treatment of formaldehyde and disuccinimidyl glutarate (FA-DSG) that captures RNA interactions occurring via multiple protein intermediates (Engreitz et al., 2014)

3.3 LNCRNA STRUCTURE

LncRNAs could harbour relatively more conserved secondary structures that are important for their function. Despite challenges in structural analyses due to their size, RNA structural profiling methods that utilize chemical and enzymatic probing techniques have been developed. For example, selective 2′ -hydroxyl acylation by primer extension (SHAPE) (Wilkinson et al., 2006) relies on certain chemicals to modify more accessible regions of RNA like single strands and loops by adding 2'-O-adducts while the highly structured regions are shielded. Modified regions are extended by target specific primers until the adduct and the extension length is used to build a secondary structure map. Using specific nucleases that cleave single stranded regions and then sequencing the resulting RNA fragments allows for a genome wide reconstruction of RNA secondary structure (Kertesz et al., 2010; Underwood et al., 2010). Therefore, these techniques hold a lot of potential in terms of linking lncRNA structure with its functionality.

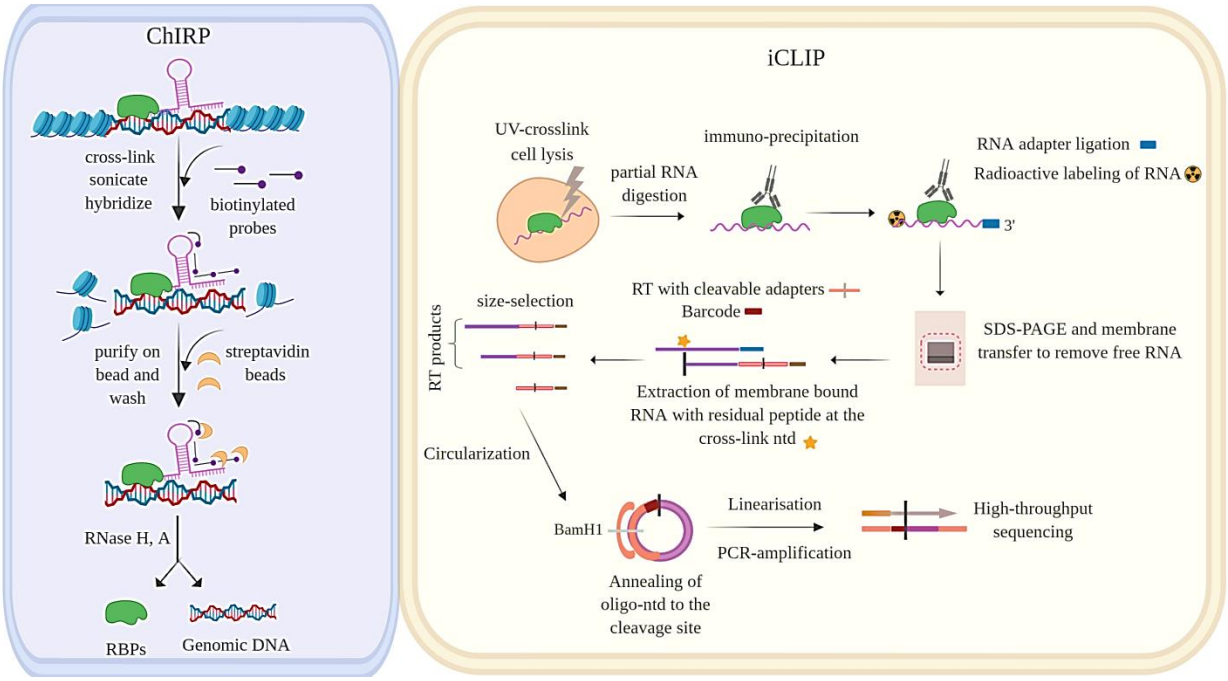

Figure 2**: **Schematics of chromosome isolation by RNA purification (ChIRP) and individual nucleotide resolution, cross-linking and immunoprecipitation (iCLIP).

Workflow of ChIRP involves cross-linking chromatin to RNA:protein in-vivo, tiling the lncRNA of interest with biotinylated probes, using streptavidin beads to bull down the chromatin complex and elute the lncRNA- DNA or protein complex with a mixture of RNase H and A. (Adapted from Chu et al.,2011)

iCLIP workflow involves cross-linking RNA:protein through UV which results in a covalent bond at the site of contact. RNA is partially digested and the protein of interest is immunoprecipitated using an antibody. RNA is ligated with an adapter at 3' end and for visualisation, labelled with radioactivity at the 5' end. After running the complex on a SDS-PAGE gel followed by transfer onto a nitrocellulose membrane, the expected size is cut out and protein is digested with proteinase K. Reverse transcription is performed with two cleavable adapter regions and barcodes. After removing free RT primers through size-selection, cDNA is circularised and an oligonucleotide with a restriction enzyme site is annealed to it. Subsequent cleavage linearizes the cDNA and generates suitable template for PCR amplification and sequencing. (Adapted from Huppertz et al.,2014).

4 NEURODEVELOPMENT

4.1 EMBRYONIC STEM CELLS

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of the pre-implantation blastocyst. These cells would normally develop into the epiblast and eventually produce all adult tissues in-vivo while the outer trophoblast layer generates extra-embryonic tissue like the placenta (Evans and Kaufman, 1981). ESCs are defined by pluripotency and extended self-renewal properties. The molecular network that establishes and maintains these unique properties is driven by the combined action of three pioneering transcription factors essential for early development, namely Sox2, Nanog and Oct4 (Boyer et al., 2005). In addition to being critical for ES cell identity; Sox2 and Oct4 also orchestrate germ layer fate selection into neuroectoderm and mesendoderm, respectively (Thomson et al., 2011). The above-mentioned transcription factors are known to co-occupy hundreds of target loci resulting in feed-forward and auto-regulatory circuits (Boyer et al., 2005; Chen et al., 2008). Some of these multifactor-binding genes are expressed in ESCs but tend to be down regulated upon differentiation (Kim et al., 2008), which points to their significant role in maintaining ESC phenotype. In addition, the transcription factor triad interacts extensively with chromatin modulators such as histone deacetylases (HDACs), polycomb repressive complexes (PRCs) and SWI/SNF ATPase dependent remodeling components (Boland et al., 2014) to use repressive pathways for either silencing differentiation-promoting genes (Petell et al., 2016) or keep them transcriptionally poised with bivalent chromatin marks (Bernstein et al., 2006).

4.2 NEUROGLIOGENESIS

Further during mammalian embryo development, the ICM undergoes differentiation into germ layers consisting of ectoderm, endoderm and mesoderm through the process of gastrulation (Kiecker et al., 2016). While mesoderm gives rise to skeletal muscles, bone, cartilage and connective tissue; endoderm forms visceral organs like stomach and intestines. Ectoderm develops into surface ectoderm (epidermis, hair, nails), peripheral and central nervous systems (Kiecker et al., 2016). Early neurodevelopment comprises of the following sequential events (Zirra et al., 2016): the neuroepithelial cells or neural stem cells (NSCs) of the ectoderm proliferate to specify the neural plate through a process called neural induction. The neural plate eventually invaginates generating the neural tube. Here, the apical-basal polarization of neuroepithelial cells is followed by patterning of the neural tube into spatially and functionally distinct regions along the rostrocaudal (R-C) and dorsoventral (D-C) axes (Stern et al., 2006). Precursors for forebrain, midbrain, hindbrain and spinal cord are formed according to the R-C axis through interplay of different factors, some of which mark the barriers between different regions by their mutually exclusive expression pattern (Wilson and Maden, 2005). On the other hand, primary dorsal morphogenic cues is provided by bone morphogenetic protein 4 (BMP4) and Wnt mediated signaling while sonic hedgehog (Shh) and retinoic acid (RA) signaling is required ventrally and in the intermediate zone of the D-V axis, respectively (Wilson and Maden, 2005). Thus, distinct neuronal subtypes and glial cells are generated from discrete domains formed through opposing D-V morphogenic gradients.

4.3 OLIGODENDROGENESIS

The glial population of the vertebrate nervous system consists of oligodendrocytes, astrocytes and microglia. Oligodendrocytes (OL) spirally wrap neuronal axons through their myelin containing membranes. Insulating properties of myelin allows for fast and efficient impulse transmission between neurons through saltatory conduction, which is crucial for meeting the energy demands of a higher order nervous system (Simons and Nave, 2015). In addition, OLs aid long term axonal integrity and survival by supplying energy metabolites to axons (Fünfschilling et al., 2012).

4.3.1 Oligodendrocyte specification

OLs are derived from oligodendrocyte precursor cells (OPCs), which in turn, arise from neuroepithelial cells lining the spinal cord canal and the ventricles of the embryonic brain (Richardson et al., 2006).

Specifically, the first wave of precursors from a discrete ventral pMN domain in the spinal cord gives rise to motor neurons (E9-10.5) and subsequently (E12.5) to platelet-derived growth factor alpha-receptor (PDGFRα) positive OPCs (Richardson et al., 2000). As discussed above, Shh signaling is necessary for the establishment of ventrally derived OPC fate and acts through homeodomain transcription factors (Nkx6) and basic helix-loop-helix proteins (Olig2/1) (Lu et al., 2000; Vallstedt et al., 2005). Later during development (E15), a second wave of dorsally derived OPCs occurs, presumably through a Shh independent route (Cai et al., 2005). Ventrally derived OPCs are highly migratory and account for approximately 80% of spinal cord OPCs, while the remainder comes from dorsally derived OPCs (Fogarty et al., 2005; Vallstedt et al., 2005).

The forebrain develops from the embryonic telencephalon where the OPCs arise sequentially in three spatio-temporal waves (Kessaris et al., 2006). The first wave appears at E12.5 in the ventricular/sub-ventricular zone from the medial ganglionic eminence (MGE) defined by the expression of homeobox protein, Nkx2.1. The second one marked by homeobox protein, Gsx2, occurs in the lateral-to-caudal ganglionic eminences (LGE/CGE) at E15.5 and the final wave expressing homeobox protein, Emx1, occurs postnatally in the cortex. Although, these precursors account for nearly all OL lineage cells in telencephalon at birth, Nkx2.1 expressing precursors plummet and eventually disappear in adulthood (Kessaris et al., 2006).

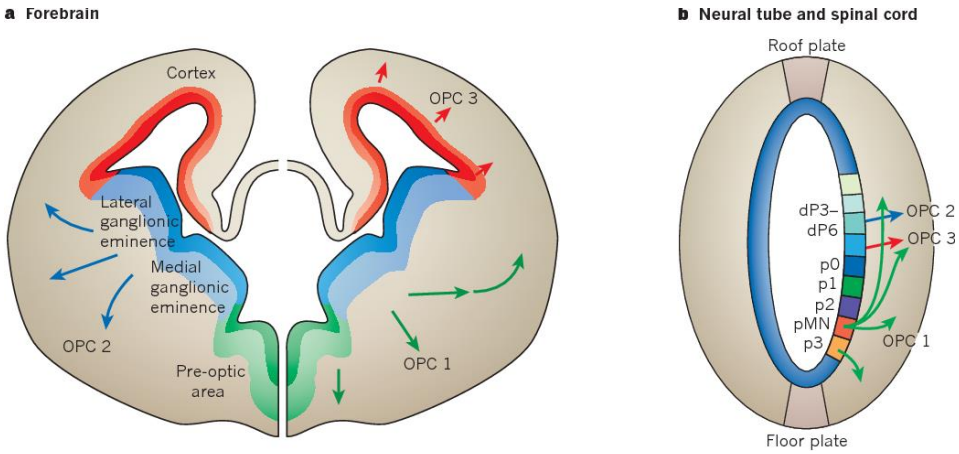

***Figure 3**: Multiple waves of OPCs are specified during the development of mammalian CNS*

a) Three OPC waves arise in the forebrain ventricular zone during development; OPC 1 at E12.5 in the medial ganglionic eminence, OPC2 at E15.5 from lateral-to-caudal ganglionic eminences and OPC3 at birth from the cortex. b) Similarly, in the ventrally located pMN domain of the developing spinal cord, the first OPC1 wave arises at E12.5. The second OPC2 wave occurs dorsally at E15.5. A third OPC3 wave occurs at birth but its origin remains unclear. (Adapted from Rowitch and Kriegstein, 2010)

4.3.2 Neuron-Glial switch

During CNS development, gliogenesis requires a developmental switch from neurogenic programs to those that produce OLs and astrocytes. In the pMN domain, this is mainly facilitated by ongoing activity of Shh and Olig2 (Ravanelli and Appel, 2015) with Olig2 presumably switching binding partners to initiate transition from motor neuron to OPC production (Li et al., 2011). This is accompanied by down regulation of proneural factors like neurogenin 2 (Ngn2) and promotion of a gliogenic phase by delta-like-NOTCH signaling (Zhou et al., 2001) with concomitant induction of transcriptional programs regulated by SRY-box 9 (Sox9) and nuclear factor I (NF1) proteins (Kang et al., 2012). In the embryonic forebrain, the precise domain of OPC specification is not well understood. Proneural factors, Ngn2 and Mash1 show mutual exclusion in the dorsal and ventral telencephalon, respectively (Ma et al., 1997). Astrocytic progenitor cells seem to derive from Ngn2 positive dorsal domain while early OPCs might develop from a MGE region with overlapping expression of Dlx2, Mash1 and Olig2/1, with an eventual co-operation of the latter two proteins (Parras et al., 2007).

4.3.3 Myelination

Following specification, OPCs proliferate and migrate throughout the CNS during early postnatal period, which is characterized by prolific myelination. Peptide mitogens like homodimeric PDGF-AA synthesized by neurons and astrocytes along with insulin growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF-2) enhance OPC proliferation and migration (Gard and Pfeiffer, 1993; Milner et al., 1997; Pang et al., 2007) by activating pathways that inhibit apoptosis and aid OPC survival (Frederick TJ., 2007). OPCs use spatial gradients of signaling molecules like BMP, Shh and Wnt to chemotactically migrate to their destination (Choe et al., 2014; Simpson and Armstrong, 1999), in addition to other local cues

of axonal guidance (Okada et al., 2007; Zhang et al., 2004), extracellular matrix components (Bribián et al., 2008; Garcion et al., 2001) and vascularization (Tsai et al., 2016). Notch and Wnt signals also inhibit premature OPC differentiation (Givogri et al., 2002; Guo et al., 2015) through downstream transcription factors like inhibitor of differentiation 2 (Id2), Id4 and Hes5.

Proliferating OPCs exit cell cycle and start differentiating into oligodendrocytes due to both intrinsic and extrinsic cues. Intrinsic myelination programs involve successful induction of transcription factors that promote differentiation (Sox10, YY1, Tcf7l2, Myrf) and simultaneous dis-inhibition of differentiation (Id2, Hes5, Sox6) (Emery and Lu, 2015). Certain chromatin remodelers and transcription factors act as both repressors and activators of differentiation, depending on the developmental stage. For example, Tcf7l2 partners up with β-catenin during the late OPC stage to form a transcriptional complex that inhibits OL differentiation (Ye et al., 2009). Following downregulation of Wnt signaling (and β-catenin), Tcf7l2 interacts with histone deacetylases 1/2 (HDAC1/2) to repress the expression of OL differentiation inhibitors (Ye et al., 2009). Similarly, HDAC2 in conjunction with co-repressor, NCOR, represses expression of Sox10 in NSCs (Castelo-Branco et al., 2014), while HDAC1 promotes differentiation by acting alongside transcription factor YY1 to relieve OL differentiation inhibition in OPCs (He et al., 2007).

OL mediated myelination is capable of responding to external clues such as both axonal number and size (Almeida et al., 2011; Lee et al., 2012). Indiscriminate myelination is mostly avoided with inhibitory cues present on the axon and they include ligands such as jagged, PSA-NCAM and LINGO-1 (Jakovcevski et al., 2007; Mi et al., 2005). In addition to inhibitory axonal ligands, myelination could also be driven directly (Gautier et al., 2015; Stevens et al., 2002) or indirectly (Ishibashi et al., 2006) by neuronal activity. However, in the absence of neuronal activity, myelination still occurs but at slower rates (Lundgaard et al., 2013).

OPCs continue to cycle in the adult brain, post developmental myelination. Apart from maintaining a steady stem cell pool (Hughes et al., 2013), these OPCs play important roles in adaptive myelination or during injury to correct myelin deficits. Recent studies have shown that OPCs indeed generate new oligodendrocytes in response to neuronal plasticity that requires remodeling existing myelinated axons or production of new myelin (Gibson et al., 2014; Sampaio-Baptista et al., 2013; Young et al., 2013. Further, OPCs proliferate and are capable of migrating to demyelinating lesions to ensure an efficient replacement of myelinating OLs (Gudi et al., 2014).

Thus myelination is a process that harnesses OLs' underlying capacity to produce myelin and fine-tunes it by integrating a variety of region specific environmental cues in the CNS.

4.3.4 Oligodendrocyte lineage heterogeneity

As discussed above, it is clear that OPC populations exhibit a spatiotemporal developmental heterogeneity. However, ablation of each of the OPC population resulted in the remaining

OPCs repopulating the CNS without significant differences in myelination (Kessaris et al., 2006). This finding was supported by a recent transcriptomic study, which reported a transcriptional convergence of post-natal PDGFRα positive OPCs irrespective of their developmental origin (Marques et al., 2018). Although this indicates a gross functional and transcriptomic equivalence between the populations, it is still unclear if they continue to maintain heterogeneity in other more nuanced ways to suit their local environmental needs. For example, postnatal OPCs from spinal cord produce higher myelin sheet length than those from the cortex (Bechler et al., 2015). Further, CNS is made up of two kinds of tissue: white matter (WM) which largely consists of long-range myelinated axons and grey matter (GM) which is made up of neuronal cell bodies, synapses and relatively fewer myelinated axons. Consistent with this observation, adult OPCs in white matter and grey matter exhibit different self-renewal capacities (Hill et al., 2013). The former has shorter cell cycle time and rapid proliferative capacity (Maki, 2017). WM OPCs were also found to have a higher propensity for differentiation into mature OLs compared to GM OPCs (Dimou et al., 2008). Furthermore, OPCs express glutamate receptors and can receive depolarizing synaptic inputs from axons (Káradóttir et al., 2005; Kukley et al., 2010; Spitzer et al., 2016). They also express voltage-gated sodium and potassium channels (FIELDS, 2008). The onset and expression of such electrophysiological properties in OPCs are reported to vary, both within and between brain regions and age (Chittajallu et al., 2004; Spitzer et al., 2019). In addition, electrophysiological properties seem to make OPCs more vulnerable to ischemic injury (Káradóttir et al., 2008).

Although Rio Hortega recorded morphological diversity within OLs nearly a century ago, the underlying transcriptional landscape instructing different OL states along the differentiation pathway only became clear with the advent of large scale transcriptomic and proteomic studies in the last decade (Cahoy et al., 2008; Sharma et al., 2015; Zhang et al., 2014b). More clarity was achieved when the first single-cell transcriptomic study unveiled six distinct subpopulations of OLs in the mouse brain (Zeisel et al., 2015). This was further complemented by a second study in which twelve distinct clusters could be transcriptomically ordered to recapitulate the OL differentiation process in mouse juvenile and adult CNS (Marques et al., 2016). Though post-mitotic committed OPCs (COPs) and newly formed OLs (NFOLs) were present in all regions, mature OLs (MOLs) showed age and location dependant distribution (Marques et al., 2016). Whether the abovementioned MOL subpopulations correspond to functional sub-types in the CNS, is yet to be determined.

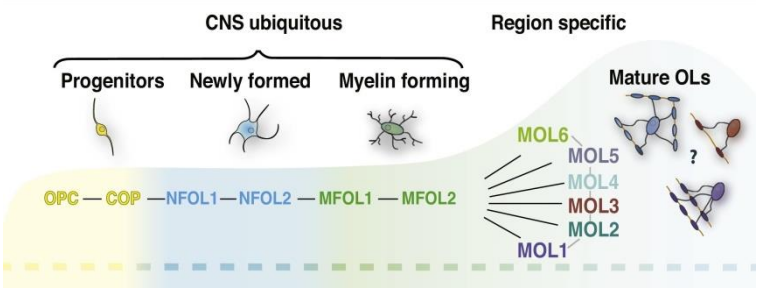

Figure 4: Model of OL lineage progression based on single cell RNA-seq datasets

OL differentiation process is sequential and uniform in the CNS, until the myelin forming stage. Mature populations are heterogeneous and exhibit region specificity, with MOL1-4 common in juvenile mouse and MOL5/6 mostly present in the adult mouse. (Adapted from Marques et al., 2016)

5 MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disorder of CNS affecting approximately 2.3 million people worldwide (Browne et al., 2014), predominantly young adults (Kobelt et al., 2017). Though the underlying cause of the disease is not well understood, studies point towards multiple factors involving complex gene-environmental interactions (Ascherio, 2013; International Multiple Sclerosis Genetics Consortium (IMSGC) et al., 2013; Ramagopalan et al., 2010).

MS is usually viewed as a two-stage disease: an early inflammation phase resulting in demyelinated lesions, characterized by reversible neurological deficits (relapse-remitting MS) followed by a delayed neurodegeneration phase causing progressive, permanent clinical disability (secondary progressive MS) (Leray et al., 2010). Accordingly, demyelination is followed by proliferation and activation of resident OPCs into a regenerative phenotype (Fancy et al., 2004). The activated OPCs migrate to the site of lesion, make axonal contacts and differentiate into remyelinating OLs (Franklin and Ffrench-Constant, 2008). Eventual remyelination failure is linked to depletion of OPCs in plaques as well as reduced OPC migration and differentiation (Boyd et al., 2013; Hartley et al., 2014; Kuhlmann et al., 2008), owing to a dysregulated pathological environment (Franklin, 2002).

The debates surrounding MS aetiology are centered on whether the disease starts with primary autoimmune attack as is traditionally believed (outside-in hypothesis) or if the initial trigger arises from within the CNS in the form of cellular and/or myelination dysregulation (inside-out hypothesis).

Outside-in hypothesis: The strongest genetic risk for MS is within the MHC locus (International Multiple Sclerosis Genetics Consortium (IMSGC) et al., 2013) plus MS lesions are characterized by large infiltrates of immune cells (Frischer et al., 2009). Therefore, the historical view is that MS is principally mediated by auto reactive, pro-inflammatory effector T cells which make their way into CNS through a breakdown of the blood-brain barrier. In health, auto reactive T cells that escape thymic education are kept in check at the periphery by a unique population of FoxP3 positive regulatory T cells (Sakaguchi et al., 2007), while in MS this mechanism is rendered insufficient (Viglietta et al., 2004). Auto reactive T cells are then re-activated by antigen presentation from B cells and myeloid cells in the periphery and CNS (Jelcic et al., 2018), with suspected myelin related antigens. Widely implicated are the IL-17 producing effector CD4+ (T_H17) and CD8+ T cells in establishing inflammatory lesions and OL damage, by recruiting additional T cells and macrophages (Huber et al., 2013; Kebir et al., 2007)).

A commonly used outside-in experimental model of MS is autoimmune encephalomyelitis (EAE). EAE models are of different types. The passive T-cell transfer involves transfer of T-cells from animals sensitized with brain tissue to induce neuroinflammatory response in naïve recipients (Paterson, 1960), mainly via CD-4^+ T-cells. Since the T-cells have already undergone expansion in the host animal, this model does not need immune activation in the

peripheral lymphatic tissue to induce an inflammatory response. Co-transfer models involve inducing brain inflammation by passive transfer of auto-reactive T cells, followed by injection of auto-antibodies (Linington et al., 1988). Here, the inflammatory reaction is also associated with widespread primary demyelination. On the other hand, active sensitization models require immunization with a CNS antigen, usually with the MOG_{35-55} peptide, together with a strong adjuvant (Mendel et al., 1995). In this model, acute or chronic inflammatory encephalopathy results with primary axonal injury, but this $CD\text{-}4^+$ auto-reactive T-cell mediated pathology is restricted to the spinal cord (Kim et al., 2006).

Consistent with this view, therapies have been focused on correcting or limiting contributions of effector T cells to the disease but anti-CD20 mediated depletion of B cells have resulted in lower rates of disease activity in relapse MS patients (Hauser et al., 2017; Kappos et al., 2011; Sorensen et al., 2014). Since antibody secreting plasma cells are unharmed in these therapies, B cells are thought to play an important role in triggering new MS relapses through their antibody-independent functions. For example, B cells are able to present protein antigens more efficiently at low antigen levels (Pierce et al., 1988) and EAE mice with B cell specific MHC-II KO have been found to be resistant to disease induction via recombinant MOG (Molnarfi et al., 2013). Apart from antigen presentation, B cells also express a number of co-stimulatory molecules essential for defining primary and secondary T cell responses (O'Neill et al., 2007) along with co-inhibitory molecules involved in dampening effector T cell response (Bodhankar et al., 2013). B cells present abnormal pro-inflammatory cytokine profiles in untreated MS patients (Bar-Or et al., 2010; Duddy et al., 2007; Li et al., 2015a). Resulting elevated levels of IL-6 and GM-CSF are involved in generating T_H17 and enhanced myeloid inflammatory responses (Barr et al., 2012; Li et al., 2015b), respectively. Thus an updated view of MS pathogenesis accounts for the above mentioned B cell contributions.

Inside-out hypothesis: According to this hypothesis, MS could be triggered from oligodendrocyte death which results in myelin damage. Myelin debris could enter the immune system as antigens following which the immune system erroneously perceives this as foreign and begins a full scale attack on myelin, thus initiating MS (Stys et al., 2012). Recently, a study demonstrated this possibility by ablating oligodendrocytes specifically with diphtheria toxin in a young *Plp1-CreER^T;ROSA26-eGFP-DTA* (DTA) mouse model (Traka et al., 2016). Following oligodendrocyte loss and demyelination that resulted in $CD\text{-}4^+$ T-cell infiltration into the CNS, the DTA mice developed a late onset secondary disease characterized by severe myelin and axonal loss. Subsequent studies have strengthened the possibility of brain intrinsic degenerative mechanisms inducing immune activation (Baxi et al., 2015; Scheld et al., 2016), including altered myelin structure (Caprariello et al., 2018). In a cuprizone autoimmune encephalitis (CAE) model, brief cuprizone treatment was used to disrupt myelin ultrastructure without overt demyelination, following which an immune stimulus was administered without the MOG peptide (Caprariello et al., 2018). CAE mice developed demyelinating immune response comparable to MS, which was significantly driven by biochemical alterations of myelin in the form of citrullination by peptidyl arginine deiminases (PADs). However, in other contradicting studies oligodendrocyte death was not

sufficient to induce immune activation, despite axonal damage, microglial-macrophage activation and draining of myelin material into lymph nodes (Locatelli et al., 2012; Pohl et al., 2011).

Although, the pathological responses vary with different models, it seems like a combination of primary oligodendrocyte damage and a permissive immune system is required for MS to manifest.

6 LNCRNAS IN NEURODEVELOPMENT

The mammalian CNS houses trillions of cells of neuronal and glial origin which are intricately integrated to form the functional circuitry of the brain. Generating such diverse cellular architecture during neurodevelopment and maintaining it to facilitate successful adaptation during growth requires a co-ordinated expression of functional components of the genome. This is achieved by a complex interplay of transcriptional and epigenetic control mechanisms mediated by transcription factors and chromatin remodellers. A third and a relatively novel component of this regulatory network are lncRNAs. The greatest fraction of lncRNA expression is intriguingly observed in the brain and testis (Necsulea et al., 2014; Soumillon et al., 2013), which perhaps underlies the organisational complexity of these tissues.

Indeed, lncRNA loci are preferentially found in the vicinity of protein coding genes that are highly expressed in the brain and are involved in CNS development (Ponjavic et al., 2009), suggesting a possible functional co-operation between the pairs. Many lncRNAs are dynamically regulated within the developing brain (Mercer et al., 2008) and show a striking cell-specific localization, along with restricted expression patterns within distinct neuroanatomical loci (Belgard et al., 2011; Goff et al., 2015; Sone et al., 2007). Thus it is not surprising many emerging studies have reported lncRNAs to play important roles in pluripotency, lineage specification, fate transition and neuronal and glial cell type elaboration.

6.1 REGULATION OF PLURIPOTENCY

The chromatin landscape of ES cells is globally more open and transcriptionally permissive (Efroni et al., 2008), yet only a subset of genes are robustly expressed. Further, promoters of many genes, particularly those involved in lineage specification, are kept poised for transcription, and carry bivalent activatory and inhibitory histone marks (Mikkelsen et al., 2007). The most prevalent cellular method of transcriptional control is RNA pol II pausing (Adelman and Lis, 2012) and the small nuclear non-coding RNA 7SK plays a central role in this process. The highly conserved 330 nt ncRNA (Marz et al., 2009) associates with proteins such as La-related protein 7 (LARP7), hexamethylene bis-acetamide inducible 1 mRNA (HEXIM) 1/2 and methylphosphate capping enzyme (Mecpe) to form a canonical small ribonucleoprotein (snRNP) complex (Peterlin and Price, 2006). The resulting, conformationally altered, 7SK snRNP is known to sequester positive transcription elongation factor b (P-TEFb), thereby preventing phosphorylation of RNA pol II needed for successful elongation (Prasanth et al., 2010). Rather than being a passive reservoir of P-TEFb in the nucleoplasm, several studies raised the possibility that there could be chromatin proximal release of P-TEFb in response to appropriate stimuli (D'Orso, 2016; McNamara et al., 2013). Particularly, in ES cells, 7SK was found to supress a specific cohort of lowly expressed genes, enriched for bivalent and active chromatin marks (Castelo-Branco et al., 2013). Surprisingly, it was also found to be important for transcriptional termination and regulation of promoter bidirectionality. Further insights came from investigating genome wide binding of 7SK and its *in-vivo* protein partners. 7SK was found to associate with the nucleosome

remodelling BAF complex at enhancers to limit enhancer RNA initiation and synthesis, while engaging in RNA pol II pausing at promoters through its canonical partners (Flynn et al., 2016). Thus 7SK facilitates distinct molecular mechanisms to operate at different regulatory DNA elements in order to maintain the unique transcriptional landscape of ES cells necessary for pluripotency.

Apart from 7SK, other lncRNAs employ diverse mechanisms to co-operate with transcription factors, RNA-binding proteins, epigenetic modulators and small RNAs to regulate and fine tune the maintenance of ES cell identity. A large scale functional study identified more than 130 lincRNAs whose perturbation led to loss of ES cell pluripotency and many others which were essential for repressing lineage specific genes (Guttman et al., 2011). Not surprisingly, some of the lincRNAs were regulated by the core pluripotency transcription factors and about 30% interacted with chromatin remodellers. Another functional study identified Tcl1 upstream neuron associated lincRNA (*TUNA*), amongst others necessary for pluripotency, and found that the lincRNA associates with RNA-binding proteins to interact with promoters of *Nanog*, *Sox2* and *Fgf4* (Lin et al., 2014). LincRNA, regulator of reprogramming (*Linc-ROR*), is a cytoplasmic RNA that is able to sequester mir-145 and de-repress the translation of critical pluripotency factors in human ES cells (Wang et al., 2013). Similarly, growth arrest specific transcript 5 (*Gas5*) regulated by Oct4 and Sox2, maintains TGFb signaling by protecting its receptor from miRNA mediated degradation to promote mouse ES cell self-renewal (Tu et al., 2018). The histone modifier Wdr5 also interacts with many lncRNAs including those involved in maintaining self-renewal of ES cells, moreover its RNA-binding pocket has been reported to be crucial for enforcing active chromatin state (Yang et al., 2014).

6.2 REGULATION OF NEUROGENESIS

Numerous studies have identified a dynamic change in chromatin status and expression for thousands of lncRNAs all the way from specification and differentiation of NSCs to glial fate specification and oligodendrocyte maturation

6.2.1 Neural stem cell maintenance and differentiation

Previously discussed lncRNA mediated modes of regulation like working with key transcription factors, chromatin remodelers and RNA-binding proteins to enact specific transcriptional programs can be found in this context as well. Brain specific rhabdomyosarcoma 2 associated transcript (*RMST*) was reported to interact with SOX2 and was necessary to recruit the transcription factor to a host of genes implicated in neurogenesis (Ng et al., 2013). Some embryonically expressed lncRNAs like *TUNA* also control neural lineage commitment by targeting neural gene promoters in differentiating ES cells (Lin et al., 2014). LncRNAs like *Dali* and *Paupar* are transcribed in the vicinity of transcription factors, Pou3f3 and Pax6, respectively. In addition to regulating their transcription locally, the lncRNAs also physically interact with these transcription factors to influence the expression of distal genes involved in neural differentiation (Chalei et al., 2014; Vance et al., 2014).

In contrast, the nuclear enriched lncRNA *Pnky* restrains neurogenesis from both embryonic and post-natal NSCs in several ways. During development, NSCs produce transit amplifying cells whose division gives rise to neuroblasts from which interneurons are generated in the olfactory bulb (Lim and Alvarez-Buylla, 2014). Ablation of *Pnky* results in increased neuroblast formation and a drastic decrease of NSCs (Ramos et al., 2015). Furthermore, *Pnky* interacts with the splicing regulator, PTBP1, and represses the inclusion of neuronal exons in transcripts involved in maintaining a specific cellular phenotype (Ramos et al., 2015).

6.2.2 Regulation of brain circuitry and function

A balance of excitatory and inhibitory neurons is essential for brain function. The Dlx class of homeobox proteins regulates the developmental pathway of producing inhibitory GABAergic neurons from the MGE. LncRNA *Evf2* is transcribed downstream to *Dlx5* in response to Shh signalling, and encompasses *Dlx6* and an ultraconserved intergenic regulatory region important for controlling the *Dlx5/6* bigene cluster. Initially, *Evf2* was thought to act as a transcriptional co-activator with Dlx2 to activate the *Dlx5/6* enhancer (Feng et al., 2006). However, when *Evf2* was prematurely terminated with polyA insertion, there was an increase in Dlx5/6 levels even though the *Evf2* KO mice showed reduced GABAergic interneurons in the embryonic hippocampus and loss of synaptic inhibition in the adult hippocampus (Bond et al., 2009). In addition to the expected loss of Dlx2 recruitment at the enhancer, there was also reduced recruitment of the transcriptional repressor, MECP2. *Evf2* was also found to exist in a large ribonucleoprotein complex with Brg1 and inhibit its ATPase mediated chromatin remodelling activity (Cajigas et al., 2015), further contributing to the repressive outcome. Thus *Evf2* recruits both activators and repressors to modulate the activity of the enhancer and the expression of Dlx proteins.

Functional interactions between neurons require axon and dendritic growth to establish synaptic connections which is modulated by neuronal activity. Neuronal activity can restructure the epigenetic landscape to induce activity dependant genes through the release of neurotrophic factors (Su et al., 2017). Brain-derived neurotrophic factor (BDNF) promotes neuronal differentiation, synaptic growth and is essential for long-term memory (Hu and Russek, 2008). BDNF locus is under the control of a conserved lncRNA *BDNF-AS*, which negatively regulates BDNF expression by recruiting PRC2 to its locus (Modarresi et al., 2012). Ion channels affect neuronal excitability, similarly lncRNA *KCNA2-AS* negatively regulates *KCNA2* that encodes a core potassium channel subunit in DRG neurons (Zhao et al., 2013). Additionally, specific mRNAs need to be transported away from the neuronal cell body to dendritic microdomains where their translation can be controlled in response to neuronal activity (Job and Eberwine, 2001; Steward and Schuman, 2001). The lncRNA *BC1* transported to dendrites upon its synthesis represses translation in the synaptic microdomain by interacting with the initiation factor eIF4A and polyA binding protein (PABP), thereby preventing the association of 48S pre-initiation complex assembly with dendritic mRNAs (Wang et al., 2005).

6.3 REGULATION OF OLIGODENDROGENESIS

Studies assessing the contribution of lncRNAs to OL biology have only begun in the last decade. The first systematic study in this field used microarray to study expression profiles of coding and non-coding RNAs during differentiation of NSCs in the embryonic forebrain (Mercer et al., 2010). Their developmental model examined NSC fate restriction to produce bipotent progenitors, oligodendrocytes lineage specification from Shh responsive progenitors and subsequent oligodendrocyte maturation into myelinating OLs. Similar to previous observations, some of the differentially expressed lncRNAs during these developmental transitions exhibited co-ordinated expression patterns with protein coding genes with well-defined roles in glial lineage specification and elaboration. Further, histone deacetylation has been shown to be necessary for OL lineage progression (Marin-Husstege et al., 2002). Accordingly, following treatment with a HDAC inhibitor, majority of lncRNAs with specific expression profiles during oligodendrocyte differentiation were downregulated, which indicated their integration into a broader epigenetically regulated OL developmental program. A subsequent study integrated transcription factor occupancy data with glial and neuronal transcriptomes generated through RNA sequencing from mouse cortex to catalogue lncRNAs involved in OPC formation (Dong et al., 2015). This led to the identification of an OPC specific lncRNA, lnc-OPC, which had Olig2 binding in its upstream regulatory region. Further, loss of lnc-OPC resulted in reduced OPC numbers *in-vitro*, indicating its essential role in oligodendrogenesis.

The first inclusive high quality catalog of lncRNAs in OLs came from deep sequencing primary mouse OPCs and oligodendrocytes that were differentiated with triiodothyronine (T3) for 1 day (immature OLs) and 3 days (mature OLs), respectively (He et al., 2017). This uncovered over 2000 oligodendrocyte specific lncRNAs, with more than 500 unannotated ones. Subsequent epigenetic profiling for histone marks and genomic occupancy of Sox10, an oligodendrocyte program inducting transcription factor, revealed these lncRNAs are dynamically regulated over the course of oligodendrocyte lineage progression. This led to the functional characterisation of lncOL1 (also termed as *Pcdh17IT* in Article 2) whose expression was highly correlated with the myeliogenic program. Accordingly, lncOL1 deficient mice had lower OLs around birth and exhibited severe myelination deficits at the peak of myelination. Additionally, these deficits were also observed during remyelination following white matter injury. Mechanistically, lncOL1 was found to associate with Suz12 mediated PRC2 complex to silence developmental programs that were antagonistic to OL differentiation, thereby allowing differentiation to proceed.

Since this study, both bulk and single cell transcriptomic studies have expanded the repertoire of lncRNAs expressed in OL lineage cells (Marques et al., 2016, 2018; Zeisel et al., 2015) and have opened up the possibility to explore their functional contribution to glial biology.

7 SUMMARY OF ARTICLES INCLUDED IN THE THESIS

7.1 INTERACTION OF SOX2 WITH RNA-BINDING PROTEINS IN MOUSE EMBRYONIC STEM CELLS

Findings

In this study, we determined the protein interactome of one of the key core pluripotency transcription factor, Sox2, in the chromatin and nucleoplasm fractions of mouse embryonic stem cells. By using Stable Isotope Labelling by Aminoacids in Cell culture (SILAC) technology coupled with immunoprecipitation and mass spectrometry-based quantitative proteomics in a mouse embryonic cell line, we found Sox2 to be predictably interacting with other partner transcription factors and chromatin remodellers involved in stem cell maintenance. Interestingly, Sox2 also interacted with the heterochromatin 1 (HP1) family of highly conserved proteins, HP1 α, β, γ (Cbx5, 1 and 3) in both chromatin and nucleoplasm fractions. Further, this interaction was confirmed with human recombinant Sox2 or ES cell nucleoplasm extracts and with different modular domains of HP1.

Gene ontology of Sox2 interactors in both the nuclear compartments indicated an enrichment of transcriptional regulators, a subset of which was RNA-binding proteins with known roles in post-transcriptional processes like splicing. Although it was shown previously that some Sox2 interactors like HP1a show RNA dependency in binding to chromatin (Muchardt et al., 2002), we saw the interaction between Sox2 and HP1α/β persisted upon RNAse A treatment, indicating that the observed interaction is not dependent on RNA.

In order to investigate the RNA interactome of Sox2 in the light of above findings, we performed immunoprecipation of Sox2 from formaldehyde cross-linked J1 ES cells, followed by poly(A)-neutral RNA-seq. While no lncRNAs were pulled down, there was an enrichment of a restricted subset of RNAs including ncRNAs, snRNA 7sk and snoRNA Snord34. Because 7SK regulates transcription at gene regulatory regions and Sox2 is a known pioneer transcription factor, we hypothesized that interaction between 7sk and Sox2 could play a role in their recruitment to the chromatin. To assess whether genomic recruitment of 7sk is altered in the absence of Sox2, we performed Chromatin Isolation by RNA Purification (ChIRP) with even and odd sets of probes against 7sk (Flynn et al., 2016) in a doxycycline inducible Sox2-knock out mES cell line and compared it with controls treated with DMSO. While specific and efficient pull down of 7sk was confirmed, we could not detect any changes in 7sk binding following doxycycline mediated Sox2 KO.

We then investigated whether 7sk could influence Sox2 binding and to this end performed Chromatin Immunoprecipitation (ChIP) with an endogenous Sox2 antibody in 7sk depleted mES cells with the help of an antisense oligonucleotide (ASO). Some of the genes that were found in the overlapping set between Sox2 and 7sk occupied regions (*Kdm2b*, *Celf2* and *Klf12*) along with others known to be occupied by Sox2 (*Pouf51* and *Nanog*) or shown to be

regulated upon 7SK knock down (*Dll1*), were chosen for qPCR assessment following ChIP. Though we observed specific Sox2 occupancy at these regulatory genes, knockdown of 7sk did not lead to significant changes in Sox2 binding.

Therefore, snRNA 7sk and Sox2 though present in the same complex, do not influence each other's recruitment to the chromatin.

Discussion

Sox2 exists in multi-protein complexes, the composition of which is highly dependant on the cell type and purification technique. Our data, consisting of 124 proteins, provides a resource for the interactome of Sox2 in mESCs in different nuclear fractions.

Some of the Sox2 interactors in the study belong to the heterochromatin 1 (HP1) family. While it is well known that HP1 co-localises with H3K9 methyl-transferase, Su(var)3-9, in heterochromatic regions to confer transcriptional repression (Hiragami and Festenstein, 2005), it can also trigger repressive chromatin structures at specific promoters within euchromatic regions (Vandel et al., 2001). Further, HP1 and H3K9me3 were found on active genes within coding regions, where the localisation of HP1 was dependant on RNA pol II elongation (Vakoc et al., 2005). In the context of pluripotency, a study found that knock down of HP1γ enhances reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) (Sridharan et al., 2013). Accordingly, HP1γ was found to bind upstream of *Nanog* TSS in intermediate pre-iPSCs and repress its expression, in conjunction with H3K9me3 function. In contrast, HP1γ binding was also found within highly transcribed genes in both pre-iPSCs and ES cells, albeit with a distinct binding profile between the two cell types. Thus, there is a possibility wherein Sox2 can aid HP1's specificity in binding to the genome or participate in stabilization of transcription or elongation indicated to be supportive of *in-vivo* HP1 binding within active euchromatic region in ES cells.

Our data also indicates Sox2 to interact with ncRNAs, 7sk and Snord34. Interestingly, snoRNAs have been recently shown to exhibit genomic occupancy (Sridhar et al., 2017) and regulate chromatin/nuclear structure (Schubert et al., 2012). While Sox2 and 7sk might not modulate each other's recruitment to chromatin, they could be associated functionally in other ways. For example, poly ADP-ribose polymerase 1 (PARP-1), a Sox2 interactor in our study, was not only shown to stabilize Sox2 binding to chromatin (Liu and Kraus, 2017), but also inhibited the negative elongation factor (NELF) by post-translational modification, thereby allowing transcriptional elongation to proceed (Gibson et al., 2016). The interaction between Sox2 and 7sk could be mediated by RBPs, namely Srsf1 and hnRNAPA2/B1, which are Sox2 interacting partners. While Srsf proteins recruit 7sk to promoters and mediate transcriptional pause release (Ji et al., 2013), hnRNPA2/B1 is involved in dynamic remodeling of 7SK snRNP in the nucleoplasm (Barrandon et al., 2007; Van Herreweghe et al., 2007).

Alternatively, it remains a possibility that the association between Sox2 and the RNAs reported here is a consequence of their proximity on DNA and nucleoplasm and not

necessarily due to any functional relationship. Future investigations might unveil whether the presence of Sox2 in ribonucleoprotein complex carries any significance either to the functionality of Sox2 or its partner RNAs.

7.2 ROLE OF LNCRNAS *PCDH17IT* AND *2610035D17RIK* IN OLIGODENDROCYTE LINEAGE PROGRESSION

Findings

LncRNAs are known to carry out diverse roles in cellular functions, yet their role in OL function remains unexplored. We have established a broad transcriptomic resource from two datasets. They correspond to single cell RNA sequencing on 5072 cells of the OL lineage from mouse juvenile and adult CNS together with both bulk and single cell RNA sequencing on Pdfgrα positive cells from E13.5 and P7 mouse brain and spinal cord. As discussed earlier, these datasets revealed a spatial and transcriptomic convergence of Pdfgrα positive OPCs during development (Marques et al., 2018) and also identified several well defined populations of OL cells reflecting unique stages along the process of differentiation and myelination (Marques et al., 2016). Therefore, we took advantage of these datasets and identified 938 annotated ncRNAs to be differentially regulated in the bulk RNA-seq dataset, between postnatal OPCs in the brain/spinal cord versus those from embryonic 13.5 brain/spinal cord. Analysis of the single-cell RNA-sequencing dataset led to the identification of 267 ncRNAs differentially expressed between different OL sub populations, with a subset of these overlapping between the two datasets. Co-expression analysis between protein coding genes and non-coding RNAs resulted in four distinct clusters enriched for gene ontological terms associated with OL biology and function, such as myelination, synapse assembly or cell division.

Further, we identified cluster 1 specific lncRNA *9630013A20Rik* (also referred to as *lncOL1* in He et al., 2017) to be specific for the post-mitotic committed OPC (COP) and newly formed oligodendrocyte (NFOL) sub-populations. We named it *Pcdh17IT* (localised to the intron of *Pcdh17* gene) in accordance with the HUGO nomenclature (Wright, 2014). While a knock down of *Pcdh17IT* in the mouse oligodendrocyte lineage cell-line olineu did not change the expression of *Pcdh17* gene, it led to the downregulation of *Tcf7l2*, a transcription factor specifically expressed in NFOLs. In line with previous results, we also observed decreased expression of *Mog*, which is expressed in later stages of oligodendrocyte differentiation and an increase of *Egr1*, a repressor of oligodendrocyte differentiation that is downregulated in COPs.

Similarly, another COP and mostly NFOL specific lincRNA *2610035D17Rik* was found to co-localise with *Itpr2*, a marker for COPs, but not with cd140a, which largely labels OPCs in primary mouse OPC cultures differentiated for 48h. This lincRNA is transcribed from a region between *Sox9* and *Slc39a11* genes on chrm 11. Sox9 is a transcription factor with an important role in OL specification (Stolt et al., 2003) and more importantly, we found the expression of *Sox9* mRNA and *2610035D17Rik* transcript to be anti-correlated in postnatal primary OPCs. Because lincRNAs are known to regulate their neighboring genes, often through *cis* mediated mechanisms, we decided to further investigate any role *2610035D17Rik* might have in Sox9 regulation.

To this end, we performed Chromatin Isolation by RNA Purification (ChIRP) with even and odd sets of probes against *2610035D17Rik* transcript in oli-neu resulting in a successful pulldown of *2610035D17Rik*. qRT-PCR of ChIRP DNA indicated that *2610035D17Rik* interacts with the *Sox9* genomic locus. However, siRNA mediated knockdown of both *2610035D17Rik* and its positionally conserved human homologue, *Linc00673 (SLNCR1)*, did not significantly affect the expression of *SOX9* mRNA. Thus the partial loss of *2610035D17Rik* RNA or its human homolog does not affect the expression of their neighboring gene *Sox9*, despite our finding that this lincRNA interacts with the *Sox9* locus.

In order to differentiate between the effects of transcription/DNA locus from the transcript itself, we created a *2610035D17Rik* KO oli-neu cell line by deleting a portion corresponding to lincRNA exon 1 using CRISPR-Cas technology. Strikingly, we also observed an upregulation of *Sox9* mRNA and Sox9 protein in the KO cell lines, while no changes were observed on the expression of the other neighboring gene, *Slc39a11*. Further, ectopic expression of *2610035D17Rik* from a plasmid in the KO cells did not return *Sox9* to its baseline expression. Chromatin immunoprecipitation (ChIP) with H3K4me3 showed an enrichment of the active transcriptional epigenetic mark on *Sox9* promoter compared to the control. Thus the observed upregulation of Sox9 in the KO cell line is partly achieved through epigenetic modification of *Sox9* promoter via H3K4me3.

In order to understand the global transcriptional effects of the KO of *2610035D17Rik*, we performed total RNA-sequencing on control and three *2610035D17Rik* KO clones. Differential gene expression and gene ontology analysis indicated an enrichment of upregulated genes involved in interferon response while there was trend for those involved in antigen processing and presentation to be downregulated in the KO cells compared to the control. We have recently shown that interferon gamma (IFNγ) can induce oligodendroglia to acquire immunological properties (Falcão et al., 2018). Accordingly, stimulation of KO and control oli-neu cells with IFNγ for 24h followed by qRT-PCR confirmed downregulation of Interferon Regulatory Factor I (*IRF1*) and Interferon Induced with Helicase C Domain 1 (*Ifih1*) genes in KO oli-neu. In contrast, induction of genes associated with MHC-II (histocompatibility 2, class II antigen A (*H2-aa*)) and class II major histocompatibility complex transactivator (*Ciita*) was stronger in KO oli-neu cells in comparison with the control. These results suggest *2610035D17Rik* also has a role in regulating immune responses of oligodenroglia in response to IFNγ stimulation.

In sum, we have drawn on insights regarding lncRNAs from a broad transcriptomic resource established from single cell- as well as bulk RNA- sequencing of OL lineage cells from different developmental stages. We have pursued candidate lncRNAs whose expression was found to be specific for certain OL subpopulations and report two lncRNAs with distinct roles in OL lineage progression.

Discussion

LncRNAs from this study were found to be dynamically expressed throughout the process of OL differentiation, with a cohort showing highly restricted expression to specific OL subpopulations, of which *Pcdh17IT* and *2610035D17Rik* are examples. *Pcdh17IT* was shown to regulate *Tcf7l2*, a transcription factor with similar expression profile as the lncRNA. Further, knockdown of Pcdh17IT led to decrease of *Mog*, a myelin component, and increase of *Egr1*, an OL differentiation repressor, which is consistent with its role in regulating the onset of myelination program as reported in He et al., 2017. LincRNA *2610035D17Rik* is transcribed 200kb downstream of the transcription factor *Sox9* and is anti-correlated with its expression. We show that rather than the *2610035D17Rik* transcript, the DNA locus from which it is transcribed or the act of transcription is involved in regulating Sox9 expression in oli-neu cells. Consistent with this observation is the presence of H3K27ac, a hallmark of active enhancers, within the *2610035D17Rik* locus.

Further, models of gene expression regulation have graduated from centering only transcription factors and regulatory DNA elements to include the full context of chromatin. Therefore, chromatin interactions occurring in three dimensional nuclear space, along with architectural proteins and RNAs that facilitate them have gained further focus. The CCCTC-binding factor (CTCF) is a unique DNA binding protein that can insulate DNA regulatory regions from one another (Bell et al., 1999). It can also be seen demarcating the boundaries between distinct active and repressive chromatin states (Cuddapah et al., 2009) as well as self-interacting, highly conserved, genomic regions called topologically associated domains (TADs). TADs are thought to facilitate interactions between regulatory elements within, than across TADs (Dixon et al., 2012). Another architectural protein that co-localises with CTCF and mediates long range genomic interactions is cohesin (Parelho et al., 2008; Rubio et al., 2008). The heterodimeric subunits of cohesin form a ring-shaped structure that can encompass two chromatin fibres (Nasmyth and Haering, 2009). While strong cohesin sites overlap with CTCF binding, there are many weaker sites that overlap with active promoters, enhancers along with mediator complexes and tissue-specific transcription factors bound to these regions (Faure et al., 2012; Schmidt et al., 2010). Further, transcription has been reported to evict CTCF from chromatin (Lefevre et al., 2008) and displace cohesin by making it slide along the gene (Borrie et al., 2017).

We find CTCF binding sites within *2610035D17Rik* intron and preliminary investigation of Hi-C data in NSCs (Bonev et al., 2017) indicates the presence of a TAD that encompasses the region between *2610035D17Rik* intron and *Sox9*. Given how the act of ncRNA transcription along with accompanying chromatin modifications can profoundly affect chromatin conformation and hence gene expression (Hirota et al., 2008), we are currently exploring a possible interplay between enhancers within the *2610035D17Rik* locus and architectural proteins, CTCF and cohesin, in regulating Sox9 expression. During development, Sox9 is expressed in neural and OPCs before being downregulated at the onset of OL differentiation (data from Marques et al., 2016; Rosenberg et al., 2018) when *2610035D17Rik* starts to be expressed. Accordingly, we hypothesize that chromatin contacts are made within the

abovementioned TAD, between enhancers at the *2610035D17Rik* locus and *Sox9*, to facilitate Sox9 expression in NSCs and during OPC specification. Stable transcription of *2610035D17Rik* in NFOLs during differentiation, either evicts CTCF from the lincRNA locus or results in a local displacement of cohesin thereby disrupting the enhancer-*Sox9* contacts, leading to downregulation of Sox9.

7.3 DISEASE SPECIFIC OLIGODENDROCYTE LINEAGE CELLS ARISE IN MULTIPLE SCLEROSIS

Findings

Multiple Sclerosis (MS) is characterized my immune mediated attack on myelin, which is produced by OLs. In order to investigate whether this attack is directed towards specific OL populations, we isolated single cells from the spinal cord of control and EAE mice. OL lineage cells were collected at the peak of disease (score=3) via sorting for GFP+ cells from EAE-induced Pdgfra-H2B-GFP transgenic mice and Pdgfra-Cre-LoxP-GFP7, enriching for OPC and OL populations, respectively. Smart-seq2 single cell RNA-seq and clustering analysis revealed thirteen OL clusters (four OPC, one COP, one NFOL and 8 MOL clusters). A subset of the OPC (OPCcyc, OPC2,3) and OL (MOL1/2, MOL5/6a and b) subpopulations were enriched in EAE mice and showed distinct expression profiles compared to healthy controls. More importantly, decomposition of datasets resulted in two gene modules uniquely associated with EAE, and consisted of genes involved in interferon response pathways, MHC-I and II genes (module 1) and *Plin4*, *Hif3a* and *Fam107a* restricted to MOL5/6 EAE population (module 13). Further, specific isoforms of genes involved in myelination like *Mbp*, *Mobp*, *Pdgfa* and *Ifih1* were found in EAE, some of which have been previously reported in MS associated polymorphisms (Capello et al., 1997; Enevold et al., 2009).

MHC-I molecules are found on surface of cells and are used to display non-self peptides from within the cytosol to cytotoxic T-cells to trigger an immune response (Hewitt, 2003). Some of the genes involved in this process (*H2-K1*, *H2-D1*, *B2m*, *Tap1/2*) were elevated upon EAE induction in OLs as well as OPCs, which makes them a potential target for cytotoxic T-cells. On the other hand, MHC-II expression is restricted to immune cells (microglia, macrophages) and are used to present processed exogenous antigens to CD-4^+ T-cells (Holling et al., 2004). Surprisingly, a subset of OPCs and OLs expressed genes required for a MHC-II mediated response (*H2-aa*, *H2-ab1*, *H2-eb1*, *cd74*) and those involved in interferon response (*Ciita*, *Nlrc5*, *Ifih1* etc).

In order to understand the mechanisms triggering MHC-II expression, OPCs were co-cultured with $CD45^+$ immune cells from spinal cord of EAE mice. 72h of co-culturing resulted in about 4% OPCs expressing MHC-II, pointing towards factors secreted by immune cells influencing MHC-II expression. Since MHC-II induction was previously reported in rat OPCs treated with dexamethasone and IFN γ ((Bergsteindottir et al., 1992), we treated mouse OPCs with IFN γ and/or dexamethasone for 3d and observed that just IFN γ treatment was sufficient to induce MHC-II expression.

Further, we treated OPCs with 1μM fluorescent microspheres for 24h and observed OPCs could internalize several microspheres and this uptake was sensitive to cytochalasin, a phagocytosis inhibitor. This observation was also extended to the uptake of pHrodo-labeled myelin (a pH-sensitive fluorogenic dye that fluoresce red in acidic phagosomes). These findings suggest that OPCs are capable of phagocytosis with implications of myelin debris intake in a disease context.

To assess the impact of MHC-II expressing OPCs on T-cells, OPCs (controls, IFNγ and/or MOG_{35-55} treated) were co-cultured with CD4+ T cells (naïve, memory and effector) derived from 2D2 mice, where T cells express the T cell receptor for the MOG_{35-55} peptide. This was followed with flow cytometric analysis of T cells for survival, proliferation and cytokine production. While there was no effect on naïve T-cells in any of the conditions, we observed enhanced survival and proliferation of memory CD4+ T cells in presence of both non- and pre-IFNγ stimulated OPCs and the MOG peptide. Similarly, we observed that effector CD4+ T cells proliferated more in the presence of both non- and pre- IFNγ stimulated OPCs and MOG peptide. Effects mediated by non-stimulated OPC on T cells in the above mentioned cases could be explained by increased cytokine production in the presence of MOG peptide for memory T-cells and just the presence of OPCs for effector T cells.

In sum, we have identified new biomarkers for EAE specific OL lineage cells and strengthened the possibility of OL lineage cells being active participants in inflammatory demyelinating pathology through immunomodulatory properties like phagocytosis and antigen-presentation.

Discussion

According to inside-out hypothesis, MS could be triggered from within the CNS with oligodendrocyte death or myelin dysregulation as discussed previously (Traka et al., 2016). Whether the EAE specific OL lineage cells presented here play any role in triggering an immunologic attack by antigen-presentation remains to be investigated.

Another complementary study has recently investigated the role of cytokine signaling on OPC function (Leslie K et al., bioRxiv 2018). While both IFNγ and IL-17 (cytokines secreted by effector CD4+ T cells) inhibited OPC differentiation, IFNγ additionally induced the expression of MHC-I and II along with immunoproteasome subunits in-vitro and in-vivo. Immunoproteasome usually expressed by immune cells is involved in processing of antigens for presentation through MHC-I, to facilitate surveillance by the adaptive immune system (Ferrington and Gregerson, 2012). Futher, OPCs were capable of engulfing and processing an exogenous OVA peptide or the whole protein and present it with MHC-I molecule through the transporter associated with antigen processing (TAP1) pathway, following IFNγ stimulation. OPCs with OVA loaded MHC-I complex could also activate OVA specific CD8+ T cells. Consequently, CD8+ T cells induced contact dependent apoptosis of their target OPCs through upregulated Fas ligand on their surface. In our study, we see that even healthy OPCs are capable of internalizing particles from their environment which probably is a part of their homeostatic function. One possibility is the co-option of this function in a complex inflammatory milieu to activate CD8+ T cells in the CNS, thus becoming targets of depletion, as is documented in MS.

The transcriptomic haul that occurs during EAE points towards lncRNAs as being possible contributors to the disease, given their role as gene expression regulators. Indeed, aberrant lncRNA expression has been detected in serum (includes NEAT1, 7SK) and blood cells of

relapse remitting MS patients (Eftekharian et al., 2017; Santoro et al., 2016). LncRNAs have also been reported to be involved in MS pathogenesis in microglia and T-cells (Sun et al., 2017; Zhang et al., 2017a). In article 2 of this thesis, we implicate *2610035D17Rik* lincRNA in regulation of immunomodulatory properties of oli-neu. *2610035D17Rik* KO cells show decreased expression of IFNγ responsive genes and an increased expression of MHC-II related genes, in response to IFNγ stimulation. These two responses have been co-occurring in EAE specific OPCs and OLs, so it remains to be seen what the lincRNA mediated differential regulation of these events means in the disease context. Interestingly, in a transcriptomic profiling of spinal cord contusion (Chen et al., 2013), we observe Sox9 expression increases at 2d post-lesion (dpl) and returns to base-line at 7dpl during regeneration, while the expression of *2610035D17Rik* remains down by about 30% during these periods. Therefore, *2610035D17Rik* mediated Sox9 regulation could also be important in the remyelination process.

7.4 MYEF2 INTERACTS WITH RNAS WITH CUG MOTIFS IN OLIGODENDROCYTES

Findings

Myelin gene expression factor 2 (Myef2) is a transcriptional repressor of Myelin Basic Protein (MBP) and hence plays an important role in regulating the timing of myelination at the onset of oligodendrocyte differentiation. It also harbours three RNA Recognition Motifs (RRMs) whose contribution to protein function in oligodendrocytes is largely unknown. In this study, we investigated the mechanism of action of Myef2 in the oligodendrocyte lineage. Using individual nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP) followed by sequencing, we characterized the RNA interactome of Myef2 in the oligodendrocyte cell line, oli-neu, from the two bands on the autoradiograph — an expected lower band at 70KDa, corresponding to the molecular weight of Myef2 and an unexpected upper band. We obtained 641 and 143 peaks which could be mapped to a single genomic position for Myef2 upper and lower bands, respectively. About 65% and 33% of peaks from Myef2 upper band and lower band were found to be unique. Myef2 was observed to cross-link to 64.58% of transcripts from protein coding genes. Significant peaks from the upper and lower Myef2 bands were found mostly within introns and 3'UTRs of protein-coding genes. Gene Ontology (GO) on genes bound by Myef2 implicates it in biological processes such as cell adhesion, transcriptional regulation and mRNA processing. We could also find a predominant CUG motif within the hexamers from the sequences bound by peaks from both the upper and lower Myef2 bands.

Discussion

Myelin gene expression factor 2 (Myef2) acts as a transcriptional repressor of Myelin Basic Protein (MBP) by binding to its proximal DNA regulatory element and hence plays an important role in regulating the timing of myelination during development. It also harbours RNA binding domains in the form of three RNA Recognition Motifs (RRMs), whose contribution to Myef2 function has not been explored. A study from our lab identified Myef2 in the protein interactome of Peptidyl Arginine Deiminase 2 (PADI2) which was found to play a role in modulating oligodendrocyte differentiation (Falcão et al., 2019). Interestingly, an arginine residue near the second RRM of Myef2 was citrullinated by PADI2. In order to understand the role of Myef2 in oligodendrocytes from the perspective of a RNA binding protein, we used individual nucleotide resolution CLIP (iCLIP) to capture the RNA interactome of Myef2. We find that Myef2 is capable of binding transcriptome-wide, especially within introns and 3'UTRs of protein-coding genes, implicating it in post-transcriptional processes like splicing and RNA stability. Known post-transcriptional regulators like poly-pyrimidine tract-binding protein and hnRNP C are reported to bind to 43% and 55% annotated genes , respectively (König et al., 2010; Xue et al., 2009) indicating a similar post-transcriptional role for Myef2. Furthermore, a CUG motif occurs predominantly amongst hexamers from the sequences bound by Myef2. Currently, we are in the process of sequencing Oli-neu cells in which Myef2 has either been knocked down or

over-expressed to explore the levels or splicing patterns of those genes which Myef2 binds, in our iCLIP data.

8 CONCLUSION

My doctoral thesis has covered a wide range of topics from characterization of long non-coding RNAs and RNA-binding proteins in different cell types using genome wide, high-throughput techniques to single cell transcriptomic analysis of OL lineage cells in a mouse model of Multiple sclerosis (MS).

A couple of insights gained from workings of lncRNAs are as follows: Combined action of a ncRNA involved in generic transcriptional control and a pioneering transcription factor with specialized role in maintaining pluripotency, provides a versatile way to adapt universal gene regulatory programs into cell- specific contexts. Also, a subset of lncRNAs restricted to certain sub-populations within a lineage, mediate transitions between different epigenetic states by forming regulatory networks with transcription factors and chromatin remodelers. Further, it is becoming increasingly clear that long and short range chromatin interactions are not only necessary for genomic organization but such an organization underscores many key gene regulatory principles. As such, lincRNA 2610035D17Rik mediated regulation of Sox9 in OLs could shed more light on the complex teamwork operating between chromatin topology, non-coding loci and induction of development-specific gene expression programs.

OL lineage cells have revealed themselves to be much more versatile in function, both in health and disease, than previously imagined. Immunomodulatory properties of OPCs in EAE mice involve antigen presentation and processing, along with activating memory and effector T cells. The expression of MHC-I/II genes can also be induced *in-vitro* by IFN γ treatment of OPCs. Interestingly, treatment with IFNγ results in the downregulation of lincRNA *2610035D17Rik* in WT OPCs. This dynamic could somewhat change in the *2610035D17Rik* KO cells, which might manifest as immunological properties that are different than the WT/controls. The functional implications of such a change need further investigation.

.Normal OPCs are capable of phagocytosing 1 μm beads in a process that is sensitive to inhibition of actin polymerization, while internalization of myelin debris is intriguingly immune to it. These observations suggest OPCs have multiple ways of up taking material from their environment.. Future studies are needed to address the dynamics of OPC mediated phagocytosis and clearance of the internalized material and its subsequent effect on OPC function, during development and disease.

9 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to **Goncalo** for giving me the opportunity to pursue my PhD in his lab. Thank you for always believing in me and the long non-coding RNAs, despite our stubborn nature, and I hope at least one of us has fared better in your expectations.

Thank you, **Ana** and **Sueli**, for your warm welcome when I arrived in the lab and for your continued support from then onwards. **Marek**, your contribution has been immensely valuable for the completion of this thesis. So, thank you for willing to wrestle with long non-coding RNAs, alongside with me. **Elisa**, your passion for making academia a better and more inclusive space is inspiring, and thank you for the timely input and support regarding my career. **Ale**, your willingness to dress up in fancy clothes for my defense has managed to move me greatly. Your dramatic flair and spontaneous food excursions have spared me many a gloomy day. **Eneritz,** in addition to your quiet patience, I must thank you for all the excellent movie recommendations. My fellow PhD students, **Mandy** and **David,** thank you for the fruitful discussions and support you have given me throughout my stay here. **Tony**, thank you for all the assistance you provide for the lab and I hope the excellent wine tasting sessions you organize will continue to help you face the daily onslaught of order requests. Special thanks to **Petra** and **Natalie** for keeping me well fed during fika and drunk the rest of the time. I hope someday, Petra will stop subtracting letters from my name and Nat is going to give up on the idea of saddling me with a cockatoo for the rest of my life. **Lauri,** you will be well remembered for enabling the conception of "Dead Pets". I am greatly indebted towards all the people of **MolNeuro** for making this a stimulating and fun environment.

All my thanks to **Kunal** for being the well-spring of kindness and acceptance; **Rohit,** for the resilient enthusiasm towards just about anything in life; **Sagar,** for the much needed light-hearted banter and **Andrew** for 'saving me' from somewhat freshly expired food items.

This journey began with me leaving home in search of pursuits unknown, and for that I am eternally grateful to my parents for letting me go.

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