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Rahul Kumar Mishra

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# Université Pierre et Marie Curie

Ecole doctorale 515 - Complexité du vivant

Institut de Biologie de l'ENS – CNRS UMR8197, INSERM U1024

*Equipe “Expression of eukaryotic mRNAs”*

## **STUDY OF EXON JUNCTION COMPLEX IN MOUSE NEURAL STEM CELLS.**

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Thèse de doctorat de :

**BIOCHIMIE ET BIOLOGIE MOLECULAIRE DE LA CELLULE**

Dirigée par Dr. Hervé Le Hir

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## Resume

The Exon Junction Complex (EJC) plays a central role in coupling post-transcriptional processes in metazoans. This multi-protein complex is assembled onto messenger RNAs (mRNAs) by the splicing machinery. Organized around a core complex serving as a platform for numerous factors, EJCs accompany mRNAs to the cytoplasm and is involved in mRNA transport, translation and stability. The physiological importance of the EJC is supported by observations associating defects in EJC component expression to developmental defects and human genetic disorders. Transcriptomic studies revealing the non-ubiquitous deposition of EJCs strengthened the hypothesis that EJCs could participate to gene expression regulation. However, despite a precise picture of the structure of the EJC, functional links between EJC assembly and regulation of specific transcripts under physiological conditions is yet to be established.

During this thesis, I studied the expression of eIF4A3, Y14 and MLN51 three core proteins of the EJC in primary cultures of mouse neural stem cells (NSCs). NSCs can be differentiated into multiciliated ependymal cells that line all brain ventricles and have important physiological functions in brain development. We observed by immunofluorescence that in quiescent NSCs, all three proteins are concentrated in the vicinity of the centrosome at the base of the primary cilia. This localization reflects the presence of fully assembled EJCs as proved by the study of Y14 mutant that prevent EJC core mounting. Remarkably, the intense signals of EJC core proteins in the centrosomal region totally disappeared when quiescent cells are committed toward proliferation or differentiation. The presence of EJCs around centrosome is not restricted to NSCs as identical cellular localizations were observed in quiescent mouse embryonic fibroblasts (MEF) and IMCD3 cell lines. Immunopurification of EJC-bound transcripts residing in the cytoplasm of NSCs coupled to large-scale sequencing revealed that EJCs are enriched onto several transcripts encoding proteins linked to proliferation and differentiation.

Given that cytoplasmic EJCs mark mRNAs that have not yet experienced translation, our data plead for the presence of numerous untranslated transcripts around the centrosome in quiescent cells. The complete disappearance of centrosomal EJCs when cells enter the cell-cycle or into differentiation most likely reflects the translation of EJC-bound transcripts. Together, our results reveal a massive EJC-associated spatio-temporal program of post-transcriptional gene regulation around the division apparatus of quiescent cells concomitant with cellular decisions for proliferation or differentiation.

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# **INTRODUCTION**



## 1.0: mRNPs

Messenger RNAs (mRNA) do not exist "naked" *in vivo*, they are coated and compacted by RNA-binding proteins (RBPs) forming messenger ribonucleoprotein (mRNPs). These mRNPs are the functional units of gene regulation as they control the central affairs of gene expression. From the very first step of transcription, RBPs mediate co-transcriptional 5'-end capping, splicing, and editing; 3'-end cleavage and polyadenylation; and quality control of mRNAs within nascent mRNP complexes (Gerstberger et al. 2014; Singh et al. 2015; Müller-McNicoll & Neugebauer 2013). The recent development of large-scale approaches including mass spectrometry and next-generation sequencing allow to analyze mRNP organization and architecture at transcriptome-wide level. CLIP technology (Crosslinking and Immunoprecipitation) coupled with high throughput sequencing has yielded instant interactions between proteins and RNA up to single-nucleotide resolution (König et al. 2011; Ascano et al. 2012). To date, more than 1500 proteins have been characterized as RBPs (Castello et al. 2012; Baltz et al. 2012; Gerstberger et al. 2014). Among these RBPs, some play a role in mRNA packaging, such as complexes binding to the methylated 5' cap or proteins that recognize the polyA tail located at the 3'-end of mature RNA. Also, during the splicing, the mRNA is bound by exon and intron definition complexes, and splicing snRNPs (Wahl et al. 2009; Bentley 2005). Following intron excision, the spliceosome also leaves several marks on mRNA including exon junction complexes (EJC) and SR proteins to assemble mature mRNPs ready for nuclear export. mRNPs undergo remodeling as they cross the nuclear pore to achieve unidirectional export into the cytoplasm (Singh et al. 2015)). By the virtue of RBPs, some mRNPs are transported to specific regions of subcellular localization (Cody et al. 2013; Martin & Ephrussi 2009; Lécuyer et al. 2007; Medioni et al. 2012). Cytoplasmic mRNPs undergo structural rearrangements in order for translation to occur. Ultimately, most RBPs that were associated with the mRNA during processing and export are replaced by ribosomes on actively translated mRNAs or by mRNA degradation machinery.

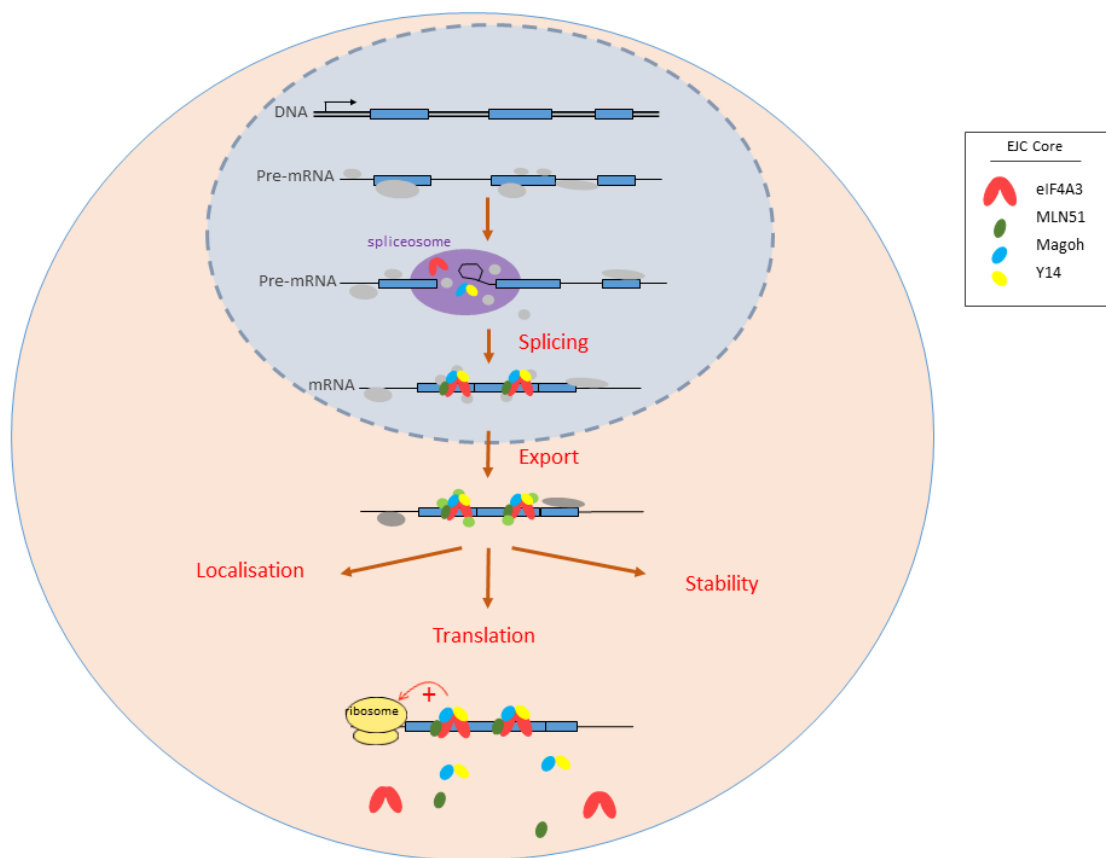
These processes are tightly regulated in time and space to ensure accurate and coordinated functioning.

Numerous RBPs, including EJC proteins, SR, Cap Binding Proteins and PolyA Binding Proteins are involved in virtually every step of mRNA biogenesis and metabolism. Understanding their precise contribution or function during each step will serve to understand the output of gene expression at all steps of the mRNA life cycle. To date, a major challenge in this field is to determine how myriads of RBPs coordinate in order to modulate every aspect of mRNP life cycle; more importantly, it is also to understand what differentiates individual mRNP despite the fact that they are made of common RBPs.

## 2.0: EJC

Since the beginning of 1990s, several evidences revealed that introns and their excision mRNA precursors enhance gene expression. This outcome originates from an effect of splicing on mRNA transport, translation and degradation based on mRNA length and position (Matsumoto et al. 1998; Luo & Reed 1999; Hentze & Kulozik 1999). It is the study of Nonsense Mediated mRNA Decay (NMD) that strongly suggested that nuclear splicing can have an impact onto post-translational decay of mRNAs. NMD is a quality control process that degrades mRNAs carrying premature translation termination codons (PTC) in order to prevent the synthesis of truncated proteins potentially harmful for the cell (Kervestin & Jacobson 2012). The mechanism of discrimination of PTCs from normal stop codons has been a central question for a long time (Maquat 1995; Brogna et al. 2016). Studies on PTC containing transcripts revealed that in mammals, a PTC triggers efficient mRNA degradation when positioned more than 50 nt upstream of an intron (Cheng et al. 1994; Carter et al. 1996; Thermann et al. 1998; Zhang et al. 1998). This result explained the functional link between splicing and NMD and generated the idea that the nuclear splicing machinery leaves a 'molecular signature' at exon junctions that accompanies the mRNAs until they are translated (Maquat 1995; Hentze & Kulozik 1999; Shyu & Wilkinson 2000). The hypothesis that presence of this mark downstream of a

stop codon would trigger NMD fits perfectly with the notion that the majority of normal stop codons are located in the last exon (Nagy & Maquat 1998). Biochemical characterization of this splicing-related mark led to the discovery of a multiprotein complex, named the exon junction complex (EJC). EJC is a multiprotein complex deposited on mRNA exon junctions as a consequence of splicing (**Figure 1**). EJC binds the mRNA at a conserved position 24 nucleotides upstream of the spliced junction, in a sequence independent manner (Le Hir et al. 2000), and serves as a binding platform for several mRNA processing factors. This complex is assembled in the nucleus, travels with mRNAs to the cytoplasm, and influences in the meantime mRNA cellular localization, translation and stability through NMD. Finally, all EJCs are removed from transcripts after one round of translation (Dostie & Dreyfuss 2002). Thus EJC nicely exemplifies the impact of nuclear history on mRNA lifecycle. Here we discuss the components and functions of EJC in relation with various post-transcriptional processes, including splicing, transport, translation and NMD.



**Figure 1: Scheme showing the splicing mediated assembly of EJC and its cytoplasmic role in mRNA fate.**

## 2.1: The EJC core components

The EJC core is composed of four proteins: eIF4A3 (eukaryotic initiation factor 4A3), MAGOH, Y14 (also known as RNA-binding motif 8A) and MLN51 (metastatic lymph node 51; also known as Barentsz or CASC3) (Ballut et al. 2005; Tange et al. 2005). Throughout the mRNA life cycle between the nucleus and the cytoplasm, this hetero-tetramer remains stably bound to mRNA and serves as a binding platform for many factors that will become temporary partners, and allow EJCs to interact with various cellular machineries. Following, I will present in more details the individual EJC core proteins and their properties.

### 2.1.1: eIF4A3

eIF4A3, also known as DDX48, is a DEAD-box RNA helicase composed of 411 amino-acids. DEAD box proteins are involved in an assortment of metabolic processes as ATP-dependent RNA remodeling enzymes. Helicases of these families contain two globular domains: RecA1 and RecA2 (**Figure 2: A**). RecA domains contain seven highly conserved motifs (I, Ia and II, III, IV, V and VI) that are involved in RNA binding and ATP hydrolysis (Linder & Jankowsky 2011). Human eIF4A3 shares 70% sequence similarity with its mammalian orthologs eIF4A1 and eIF4A2. However, it is functionally distinct from these eIF4A counterparts of the same family. While eIF4A1 and eIF4A2 are bonafide translation initiation factors, eIF4A3 does not play a direct role in translation (Li et al. 1999). In contrast to eIF4A3, eIF4A1 and eIF4A2 are cytoplasmic proteins that are not incorporated into EJC. The most documented function of eIF4A3 concerns its EJC-related roles. However, one study proposed that eIF4A3 is also involved in ribosomal RNA processing (Alexandrov et al. 2011).

### **2.1.2: MAGOH-Y14**

MAGOH and Y14 are two small proteins consisting of 146 and 173 amino acids respectively. These two proteins form a tight heterodimer (Fribourg et al. 2003). The evolutionary patterns of MAGOH and Y14 protein families show that both proteins slowly co-evolved in eukaryotes, demonstrating the requirement of their heterodimerization to maintain their function (Gong et al. 2014). The abolition of their interaction has a functional effect on the metabolism of mRNA, mainly for their location and stability (Hachet & Ephrussi 2001; Fribourg et al. 2003; Tange et al. 2004). The crystallographic analysis of the heterodimer from *Drosophila* revealed that Y14 and MAGOH interact via highly conserved hydrophobic surfaces, supporting that the two proteins act as a single structural and functional unit (Fribourg et al. 2003) (**Figure 2: B**). The central core of MAGOH contains a broad  $\beta$  sheet surrounded by three  $\alpha$  helices. The two largest  $\alpha$  helices form a hydrophobic interaction with Y14 by occupying the RNA binding domain (RBD). This interaction completely inhibits the ability of RBD to interact with RNA and stabilizes the heterodimer (Lau et al. 2003). The wide  $\beta$  sheet of MAGOH then provides a surface for interaction with other proteins, including the other EJC core partners.

In mammals, two MAGOH paralogues exist (MAGOH and MAGOHB) with almost identical amino acid sequence. Both MAGOH and MAGOHB are efficiently incorporated into EJCs (Singh et al. 2013). Their individual contribution in the EJC and downstream events of EJC mediated regulations of mRNA is not distinguishable.

### **2.1.3: MLN51**

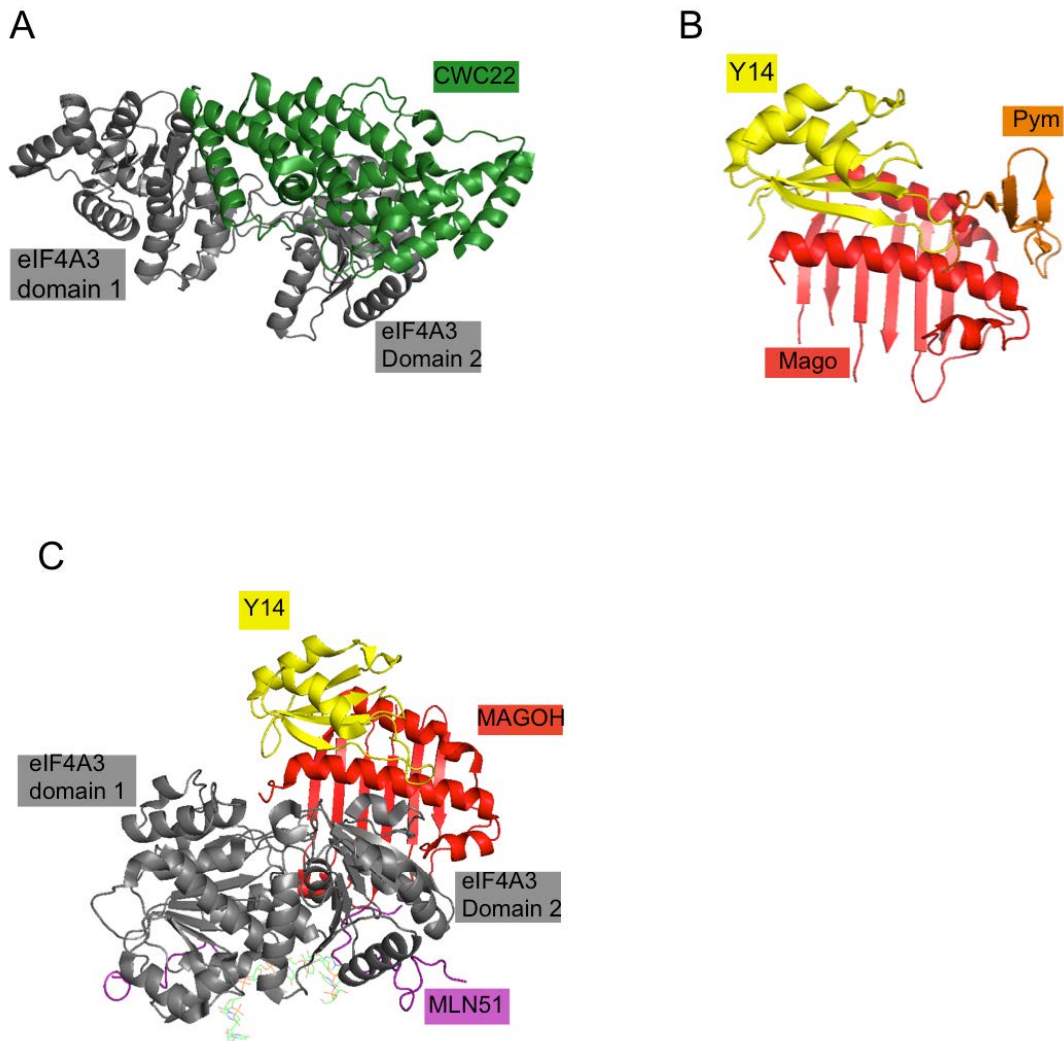
Metastatic Lymph Node 51 (MLN51) also known as Barentsz (Btz) and CASC3 (Cancer Susceptibility Candidate gene 3) is a protein composed of 703 amino acid residues. This protein is conserved in vertebrates and invertebrates showing 41% and 48% similarity respectively with *C. elegans* and *D. melanogaster* homologs. The characterization of the protein by bioinformatics analysis enabled the identification of several modules and motifs in the protein

(Degot et al. 2004; Degot et al. 2002). Schematically, the MLN51 protein can be divided into two equal halves. The carboxy-terminal region of MLN51 is rich in proline and contains a nuclear export signal (NES). The amino-terminal region of MLN51 protein contains three special domains: A coiled-coil-type structural motif; two sequences of nuclear localization signal (NLS); and a module called SELOR (speckle localizer and RNA-binding module) which is the most conserved portion of the protein among metazoan species. Human SELOR shares 100% similarity in mice, 92% in *Xenopus* and 66% similarity in *Drosophila* (Degot et al. 2004). *In vitro* MLN51 binds to RNA via SELOR domain at a precise location corresponding to EJC binding site (Degot et al. 2004). This domain is shown to be necessary and sufficient for the formation of the EJC core (Degot et al. 2004; Ballut et al. 2005). Independently of its EJC-dependent roles, MLN51 is involved in the assembly of cytoplasmic stress granules (Baguet et al. 2007). Stress granules are bodies located in the cytoplasm corresponding to mRNA storage compartments in which translationally repressed mRNAs are stored following cell stress.

## 2.2: The tetrameric EJC core

The *in vitro* reconstitution of the EJC core with purified recombinant proteins and then, the crystal structure of this recombinant complex by X-ray diffraction revealed the peculiar mode of RNA binding (Ballut et al. 2005; Andersen et al. 2006; Bono et al. 2006) (**Figure 2: C**). The EJC core is built around the RNA helicase eIF4A3. The two RecA domains of eIF4A3 move relatively freely with respect to each other without ATP, but they adopt a closed conformation in the presence of ATP allowing the binding to RNA. The characteristic and conserved RNA helicase motifs of eIF4A3 make contacts exclusively with the sugar-phosphate backbone explaining how it binds 6 nucleotides of RNA in a sequence-independent manner. This RNA binding is lost when the helicase hydrolyses ATP leading to an open conformation (Ballut et al. 2005). Interestingly, the MAGOH-Y14 heterodimer simultaneously binds the two domains of eIF4A3, preventing conformational changes upon ATP hydrolysis hence locking eIF4A3 in a closed conformation on the RNA (Andersen et al. 2006;

Bono et al. 2006; Nielsen et al. 2009). The two most conserved regions of MLN51 surround the two domains of eIF4A3 and stabilize the complex. In the EJC core, MLN51 also binds to MAGOH and contacts one nucleotide of RNA. Thus a stable tetrameric core is assembled on mRNA in which eIF4A3 and MLN51 touch the RNA.

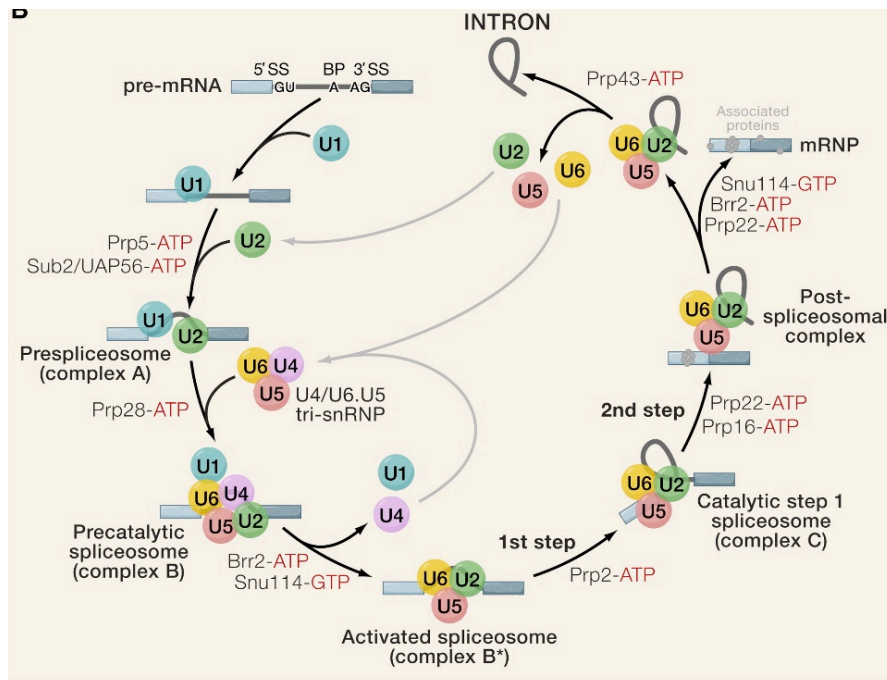


**Figure 2: Structure of EJC core components and associated factors.** **A:** Two RecA domains of eIF4A3 are shown in the heterodimer with human splicing factor CWC22 (complexed with CEF1 22). **B:** The MAGOH–Y14 heterodimer is shown in a trimeric complex with PYM (partners of Y14 and MAGOH) in *Drosophila melanogaster*. **C:** The tetrameric core of the EJC is shown comprising the RNA helicase eIF4A3, MAGOH, Y14 and MLN51. The MAGOH–Y14 heterodimer keeps the two domains of eIF4A3 in a closed conformation. The two most conserved regions of MLN51 contact the two domains of eIF4A3 and further stabilize the complex. (Adapted from Le Hir et al., 2016).

## 2.3: EJC assembly

The EJC core is not a preassembled complex that joins mRNAs after their maturation. In fact, the EJC core components are brought together by the splicing machinery. Spliceosomes are highly dynamic multi-megadalton RNA-protein molecular machines that assemble de novo for each splicing event (**Figure 3**). The main spliceosome assembly process entails the stepwise recruitment of RNA-protein subunits called small nuclear (sn) RNPs U1, U2, U4, U5 and U6 and a multitude of other proteins. In humans, it has been estimated that more than 300 proteins participate to the splicing reaction. The biochemical characterization of splicing reaction sub-divided it into five successive complexes named as E, A, B, B\* and C. Each complex is defined by the presence of different snRNPs and their cofactors. Transition to the following complex is caused by a series of rearrangements and remodelling (Figure 3), (Hoskins & Moore 2012; Will & Lührmann 2011; Wahl et al. 2009). The U1, U2, U4, U5 and U6 snRNPs are the main building blocks of these complexes. During canonical cross-intron assembly, U1 snRNP recognizes the 5' splice site (complex E) and U2 snRNP replaces early-binding SF1 protein at the branch point sequence (BPS), forming complex A. Subsequently, the U4, U5, and U6 snRNPs join as a pre-formed tri-snRNP, giving rise to complex B, which is still inactive. This complex is activated by consecutive release of U1 and U4 to sequentially form the B\* and C complexes, in which the first and second trans-esterifications steps take place leading to intron excision and exon ligation (Will & Lührmann 2011). Following, we discuss the step-wise assembly of EJC core during these successive steps of splicing.





**Figure 3: Stepwise assembly of the spliceosome onto pre-mRNA.** Cross-intron assembly and disassembly cycle of the major spliceosome. The stepwise interaction of the spliceosomal snRNPs (colored circles), but not non-snRNP proteins, in the removal of an intron from a pre-mRNA containing two exons (blue) is depicted. (Adapted from Wahl et al. 2009)

### 2.3.1: The pre-EJC core

The pre-EJC core can be defined as containing three factors MAGOH, Y14 and eIF4A3. Biochemical analyses of the protein contents of splicing complexes assembled *in vitro* revealed that MAGOH, Y14 and eIF4A3 are already present in the B complex and become more stably associated in the C complex before exon ligation (Bessonov et al. 2008; Reichert et al. 2002; Makarov 2002; Merz et al. 2007; Zhang & Krainer 2007). These proteins separately contact the regions of exons in a stable manner where they would be assembled as ‘pre-EJC core’ after the exon ligation (Reichert et al. 2002; Merz et al. 2007; Gehring, Lamprinaki, Hentze, et al. 2009). Since MLN51 was not detected in the splicing complex, it is likely that it joins and stabilizes the pre-EJC core concomitantly with the active release of mRNA from spliceosome (Ballut et al. 2005; Bessonov et al. 2008; Reichert et al. 2002; Zhang & Krainer 2007; Gehring, Lamprinaki, Hentze, et al. 2009).

### 2.3.2: The recruitment of eIF4A3 by CWC22

The loading of EJC by spliceosome-mediated remodeling of mRNPs has recently been highlighted by the discovery of how eIF4A3 is escorted to the spliceosomes. Like other RNA helicases, eIF4A3 does not possess any apparent sequence specificity. Therefore, it was quite intriguing to see how eIF4A3 is recruited to spliceosome, at the right time, to a specific position on mRNA. The biochemical characterization of eIF4A3 helicase demonstrated that it is not required for constitutive pre-mRNA splicing (Zhang & Krainer 2007; Shibuya et al. 2004). In fact, the recruitment of eIF4A3 to the spliceosome is ensured by the splicing factor CWC22 (complexed with CEF1 22; also named NCM or Nucampholin in *Drosophila melanogaster*) with which it forms a stable complex (Alexandrov et al. 2012; Barbosa et al. 2012; Steckelberg et al. 2012). CWC22 is a core splicing factor composed of two conserved domains, MIF4G (middle domain of eIF4G) and MA3. A direct and stable interaction of the MIF4G with RecA-2 domain of eIF4A3 maintains eIF4A3 in an open conformation (**Figure 2: A**), in which RNA- and ATP- binding sites are disjointed, hence preventing eIF4A3 binding to both RNA and MAGOH-Y14 (Barbosa et al. 2012; Buchwald et al. 2013). By blocking the RNA binding activity of eIF4A3, CWC22 most likely shields eIF4A3 from binding to inappropriate RNA molecules before its incorporation into the EJC. Once bound, CWC22 ensures eIF4A3 recruitment to active spliceosomes and its integration into EJC core (Alexandrov, Colognori, Shu & J. a Steitz 2012; Barbosa et al. 2012; Steckelberg et al. 2012). CWC22 and eIF4A3 co-exist in large protein complexes that also contain Prp19 (precursor mRNA (pre-mRNA)-processing factor 19 (PRP19) (Barbosa et al. 2012), a splicing factor that is essential for building active spliceosomes (Makarov 2002; Wahl et al. 2009).

The role of introns in formation of EJC core is highlighted by their association of the RNA helicase IBP160 (intron-binding protein of 160 kDa; also known as Aquarius) (De et al. 2015; Ideue et al. 2007). IBP160 exhibits structural adaptations at the centre of the intron-binding complex (IBC) that binds to pre-mRNAs in association with the U2 snRNP upstream of splicing branch points and

is also necessary for EJC loading. However, the interconnections between eIF4A3–CWC22, PRP19 and IBP160, and the interpretation of their incorporation into spliceosomes, remain unclear. After escorting it to the spliceosome, CWC22 must leave eIF4A3 to allow RNA clamping (Barbosa et al. 2012; Steckelberg et al. 2012; Buchwald et al. 2013). However, it is still unclear when CWC22 does dissociate from the complex. It is also unrecognized how pre-EJC core is assembled onto spliced mRNA. MAGOH-Y14 and eIF4A3-CWC22 are recruited to the spliceosomes independently (Gehring, Lamprinaki, Hentze, et al. 2009; Shibuya et al. 2004; Barbosa et al. 2012) and we do not know if MAGOH-Y14 requires specific partners to enter spliceosome. How EJC loading occurs at 24 nucleotides upstream of exon junctions is also an enigma. It is possible that spatial organization of protein-RNA interactions during splicing may impose some constraints at exon junctions that lead to deposit EJC at a precise distance.

Following the splicing reaction, MLN51 seals the EJC core most likely concomitantly with mRNP release and spliceosome disassembly to form the complete EJC core (Ballut et al. 2005; Zhang & Krainer 2007; Gehring, et al. 2009). So far, there is no evidence that alternative versions of the EJC core exist but we can suppose that EJCs devoid of MLN51 could exist (Le Hir et al. 2016).

## 2.4: EJC peripheral factors

Once locked on mRNA, the EJC core accompanies mRNAs from nucleus to cytoplasm until translation. The EJC serves as binding platform for more than a dozen of proteins called EJC peripheral factors (Tange et al. 2004; Le Hir et al. 2016). The composition of EJCs during this period changes dynamically as many partners join and leave intermittently. *In vitro* reconstitution of the complex and validation of EJC assembly by *in vitro* and *in vivo* studies have helped to initiate a catalogue of EJC peripheral factors (**Figure 3**).

### 2.4.1: Splicing-related peripheral factors

The first peripheral factors to bind to EJC core are most likely the ones known to be present in the spliceosome before exon ligation. This includes

splicing factors Acinus, PININ, RNA-binding protein with Ser-rich domain 1 (RNPS1) and SAP18. RNPS1 and SAP18 are splicing activators (Schwerk et al. 2003; Singh et al. 2010; Sakashita et al. 2004; Blencowe et al. 1998) and ACINUS and PININ are scaffold proteins that use a similar conserved motif to bind to RNPS1 (Murachelli et al. 2012). The alternative interaction with ACINUS or PININ give SAP18 the ability to form two distinct ternary complexes, named ASAP (apoptosis and splicing- associated protein) and PSAP (contains PININ, SAP18 and RNPS1) (Schwerk et al. 2003; Murachelli et al. 2012). ACINUS has three alternatively spliced isoforms, termed Acinus-L, Acinus-S and Acinus-S' (Sahara et al. 1999). All three isoforms are capable of forming a ternary complex (Schwerk et al. 2003). Therefore, ACINUS must be involved in different complexes. To date, we do not know how these trimeric complexes are attached to the EJC core.

## **2.4.2: Export factors**

Among the list of EJC peripheral factors are also conserved mRNA export factors UAP56 (also known as DDX39B), Aly/REF export factor (ALYREF; also known as THOC4), the nuclear export factor 1 (NXF1) and NTF2-related export protein (NXT1). UAP56 is an RNA helicase that facilitates the recruitment of ALYREF to processed mRNAs. Both ALYREF and UAP56 are also part of the TREX (transcription export) complex that bridges pre-mRNA synthesis and export (Tange et al. 2005; Merz et al. 2007; Katahira 2012). NXF1 and NXT1 form a heterodimer and are considered as the general receptor responsible for mRNP export to the cytoplasm (Köhler & Hurt 2007). NXF1-NXT1 interact with mRNAs via several adaptors (including the EJC) on one side and on the other side, directly interact with the nuclear pore complex (NPC)(Köhler & Hurt 2007). Since UAP56 is weakly associated with purified EJCs (Tange et al. 2005; Reichert et al. 2002; Merz et al. 2007), it is possible that its quick release is necessary for the subsequent recruitment of NXF1 and NXT1 by ALYREF and/or by RNPS1 (Le Hir et al. 2001; Lykke-Andersen et al. 2001) (**Figure 3**).

### 2.4.3: NMD related factors

Key factors of NMD are also part of the list of EJC peripheral factors (**Figure 3**) including UPF3A (up-frameshift 3A; also known as RENT3A), UPF3B, UPF2, UPF1 and SMG6 (also known as EST1A). The study of EJC composition in both nuclear and cytoplasmic compartments revealed the successive interaction of these factors (Le Hir et al. 2001a). UPF3A and UPF3B are mainly nuclear and bind to nuclear EJCs. Within EJC core, eIF4A3, MAGOH and Y14 together form a composite binding site for the EJC-binding motif (EBM) of UPF3B (Gehring et al. 2003; Buchwald et al. 2010; Chamieh et al. 2008). UPF2 joins the EJCs in cytoplasm by direct binding to UPF3B while UPF1 joins the complex only in the context of NMD (H Le Hir et al. 2001; Chamieh et al. 2008b; Kashima et al. 2006). Thus, three UPF proteins form a trimeric complex, in which UPF2 bridges UPF1 and UPF3B (Chamieh et al. 2008). The endonuclease SMG6 interacts with the exon junction complex via two conserved EBMs (Kashima et al. 2010). EBMs may serve as a place of contest for different proteins to hook mRNAs via EJC. Thus EJC occupies a centre stage in events involved in mRNP remodelling.

Protein (aliases (UniProt AC, length*))	Cellular localization	Functional characteristics	Cellular functions	Present in spliceosome B and C complexes?	Stable binding to spliced mRNPs	Direct binding to other EJC components	Refs
<i>EJC peripheral factors</i>							
RNPS1 (Q15287, 305)	Shuttling protein; mainly nuclear	RRM and RS	Splicing	Yes	Nuclear and cytoplasmic	ACINUS, PININ, SAP18, UPF3B	13,168,169
ACINUS (Q9UKV3, 1341 (L), 583 (S), 614 (S*))	Nuclear	RRM, RS, and SAP motif	Transcription, splicing and apoptosis	Yes	Nuclear	RNPS1, SAP18	28,63,170
PININ (PNN, desmosome-associated protein) (Q9H307, 716)	Nuclear	RS	Splicing	Yes	Nuclear	RNPS1, SAP18	63,171
SAP18 (O00422, 153)	Shuttling protein; mainly nuclear	Ubiquitin-like domain	Transcription and splicing	Yes	Nuclear and cytoplasmic (?)	ACINUS, PININ, RNPS1	28,62
ALYREF (Aly, Yra1, THOC4) (Q86V81, 256)	Shuttling protein; mainly nuclear	RRM	Transcription and mRNA export	Yes	Nuclear	NXF1, UAP56, Y14	13,17,165, 172,173
UAP56 (DDX39B, Sub2, HEL) (Q13838, 427)	Shuttling protein; mainly nuclear	DEXD-box RNA helicase	mRNA export and splicing	Yes	–	ALYREF	16,174
NXF1 (TAP, Mex67) (Q9UBU9, 618)	Shuttling protein; mainly nuclear	RRM, LRR, NTF2-like and UBA-like	mRNA nuclear export	No	Nuclear	ALYREF, NXT1	15,21,22, 165, 172,175
NXT1 (p15, Mtr2) (Q9UKK6, 139)	Shuttling protein mainly nuclear	NTF2-like	mRNA nuclear export	No	Nuclear	NXF1	15,176
UPF3A (RENT3A) (Q9H1J1, 476 (L), 443 (S))	Shuttling protein; mainly nuclear	RRM and EBM	NMD	No	Nuclear and cytoplasmic	UPF2	21,22,68,177
UPF3B (RENT3B, Upf3x) (Q9BZ17, 483)	Nuclear	RRM and EBM	NMD	No	Nuclear and cytoplasmic	eIF4A3, Y14, MAGOH, UPF2, RNPS1	15,21,22,25, 68–70, 169,178
UPF2 (RENT2) (Q9HAU5, 1272)	Cytoplasmic, perinuclear	MIF4G	NMD	No	Cytoplasmic	UPF3B, UPF3A, UPF1	69,178,179
UPF1 (RENT1) (Q92900, 1129)	Shuttling protein; mainly cytoplasmic	Zinc-knuckle and SF1 RNA helicase	NMD	No	Cytoplasmic	UPF2	69,179
SMG6 (EST1A) (Q86US8, 1419)	Shuttling protein; mainly cytoplasmic	EBM, 14-3-3-like and PIN	NMD	No	?	eIF4A3, Y14, MAGOH	72,73,180

AC, accession number; ALYREF, Aly/REF export factor; EBM, EJC-binding motif; eIF, eukaryotic initiation factor; LRR, Leu-rich repeat; MIF4G, middle domain of eIF4G; MLN51, metastatic lymph node 51; mRNP, messenger ribonucleoprotein particle; NMD, nonsense-mediated mRNA decay; NTF2, nuclear transport factor 2; RNPS1, RNA-binding protein with Ser-rich domain 1; RRM, RNA-recognition motif; RS, domain rich in Arg (R) and Ser (S); UBA, ubiquitin-associated domain; UPF, up frameshift. \*Length of human protein (amino acids).

**Figure 3: List of EJC peripheral factors.** (Adapted from Le Hir et al. 2016).

#### 2.4.4: Other EJC related factors

Apart from the bonafide list of peripheral EJC factors, some other proteins are also related to EJC but their functional role as a part of EJC is not validated. SRm160 (SR-related nuclear matrix protein of 160 kDa) is a coactivator of splicing that has been proposed to enhance mRNA 3' end processing (Le Hir et al. 2000; Blencowe et al. 1998). However, its previously considered role as an EJC protein has not been fully validated. This is also true for SKAR (S6 kinase 1 (S6K1) ALYREF-like; also known as POLDIP3)(Ma et al. 2008) which is a part of the mTOR1–S6K1-signalling pathway that positively regulates translation (Fonseca et al. 2014). Many proteins of SR family also associate with EJC (Singh et al. 2012; Saulière et al. 2012) but there is no establishment of their organized existence as genuine EJC factor. Interestingly, the most abundant SR proteins

bound to EJCs are SR splicing factor 1 (SRSF1), SRSF3 and SRSF7, which are known to continuously shuttle between the nucleus and the cytoplasm (Long & Caceres 2009). By physically interacting, EJCs and SR proteins could constitute the major driving force for mRNP compaction, which might be necessary for proper mRNP transport and translation (Singh et al. 2015; Singh et al. 2012).

## 3.0: EJC life cycle

### 3.1: The localization of EJC core components

Different studies, using diverse strategies, showed that EJCs colocalize with protein components of the spliceosome at nuclear speckles in the nucleoplasm (Custódio et al. 2004; Schmidt et al. 2006). Nuclear speckles, also named “SC35 domains” or “splicing factor compartments,” are nuclear punctuate structures functioning as storage/assembly/modification compartments that supply splicing factors to active transcription sites (Spector & Lamond 2011). *In vivo*, the four components of the EJC core shuttle between the nucleus and the cytoplasm. Three of the core components MAGOH, Y14, and eIF4A3 are mainly nuclear (Ferraiuolo et al. 2004; Kataoka et al. 2000; Hervé Le Hir et al. 2001; Palacios et al. 2004; Daguene et al. 2012). In contrast, MLN51 is predominantly cytoplasmic (Degot et al. 2002; Macchi et al. 2003; Daguene et al. 2012), despite having two nuclear localization domains. The nuclear export signal (NES) in its C-terminal is responsible for its export from nucleus to cytoplasm and predominant cytoplasmic localization. The export of MLN51 was functionally validated by blocking the crm-1 nuclear export pathway using inhibitory drugs, which leads to accumulation of MLN51 in nucleoplasm (Daguene et al. 2012). These four proteins come together to form an EJC core at the periphery of nuclear speckles: a region named “perispeckles”. By using FRET (Fluorescence Resonance Energy Transfer) assays and EJC assembly mutants, Daguene et al. showed that perispeckles do not store free subunits, but instead are enriched for fully assembled EJC core. These results supported the model that perispeckles are apparently the major assembly sites for EJCs on transcripts. The occurrence of perispeckles close to the sites of active RNA Pol-II also gives strong reasoning

that these are the nucleoplasmic locations for co-transcriptional splicing and hence EJC assembly on mRNAs.

### 3.2: EJC remodeling and variability

Once mRNAs are properly packed with RBPs in form of mRNPs, they are exported to the cytoplasm. At a moment that is not perfectly defined, mRNP are largely remodeled and see important modification of their protein composition. Many of the nuclear mRNP components are replaced with cytoplasmic ones. One of the important remodeling steps before translation is the replacement of nuclear cap-binding complex (CBC) with the translation initiation factor eIF4E and exchange of nuclear poly(A)-binding protein (PABPN) with its cytoplasmic counterpart poly(A)-binding protein (PABPC), respectively (Singh et al. 2015). While most mRNPs get engaged into translation, some of them are transported to the specific sites of translation (see section 6.3 – mRNA localization). Following this model, the composition of EJC does not remain fixed during the export and major EJC rearrangements occur. Since ACINUS and PININ are restricted to the nucleus, they must detach from EJCs before export. In the absence of these scaffold proteins (Murachelli et al. 2012), RNPS1 and SAP18 could be stabilized by other peripheral factors, such as UPF3, which can interact with RNPS1 (Gehring et al. 2005; Singh et al. 2007; Lykke-Andersen et al. 2001), or potentially other SR proteins. Several mutually exclusive interactions in the EJC support the notion of EJC variability even if there is no clear evidence for it. For example, UPF3b and SMG6 compete for the same binding site of the EJC, with UPF3b having a higher affinity for the EJC. This suggests that UPF3b must dissociate from the EJC to make way for SMG6 to bind (Buchwald et al. 2010; Kashima et al. 2010). Similarly ACINUS and PININ cannot be a part of the same complex as they also compete through a similar binding motif to join RNPS1 (Murachelli et al. 2012). However, the coordination of these changes and their functional implications remain unexplored.



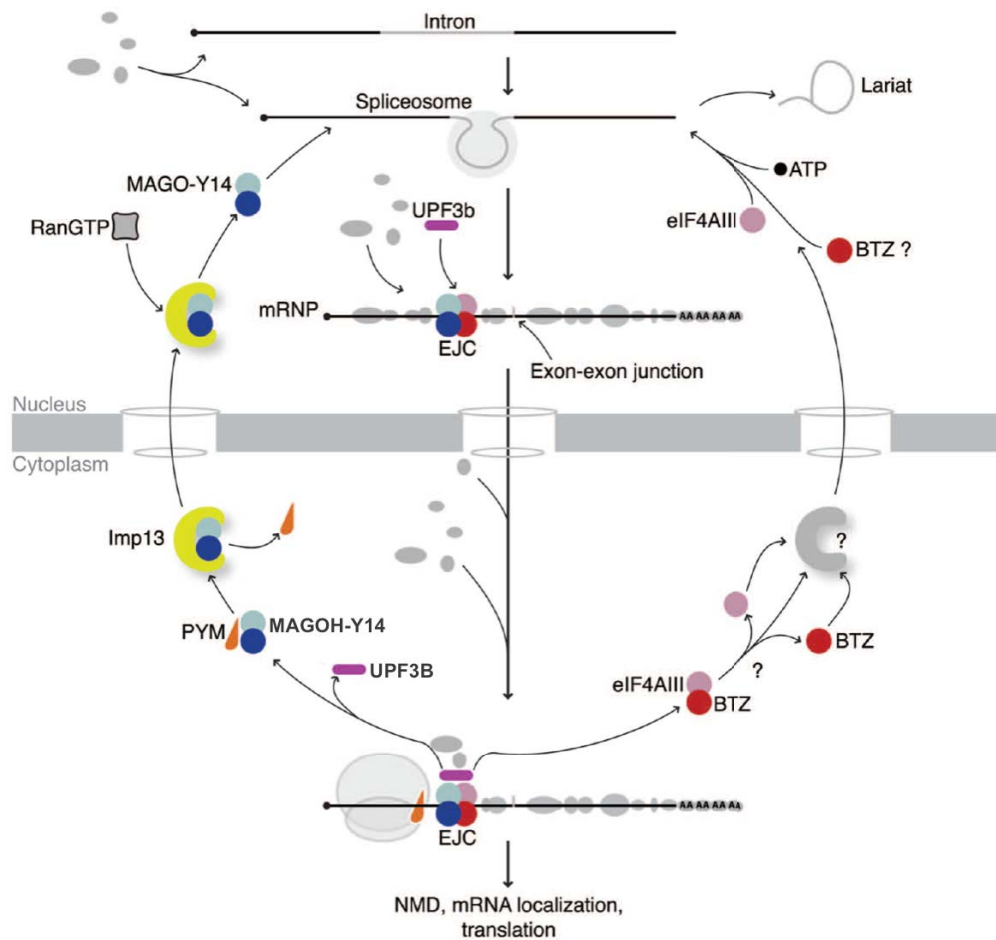
### 3.2.1: EJC disassembly

Initial studies showed that EJC are associated with mRNAs still bound to the nuclear CBC, but not with eIF4E protein suggesting that EJCs are removed during the first round of translation (Lejeune et al. 2002). The study was subsequently complemented with the finding that EJC does not associate with the translating mRNAs and the first ribosome eliminates EJCs contained in the 5'-UTR region and also in the mRNA ORF (Dostie & Dreyfuss 2002). The observations that translation was necessary and sufficient to dissociate EJCs from mRNA, both *in vivo* and *in vitro*, established that EJCs are a mark of untranslated mRNPs. Later, a cytoplasmic RNA-binding protein was identified and proposed as dissociation factor of the EJC, PYM (Partner of Y14 and MAGOH) (Bono et al. 2004; Diem et al. 2007). Structural and biochemical characterization of the PYM and Y14-MAGOH ternary complex (Bono et al. 2004) accompanied by other biochemical investigations (Gehring et al. 2009; Bono et al. 2006; Chamieh et al. 2008a) unveiled that PYM disrupts the EJC assembly and/or stability by stably binding to MAGOH-Y14 at a position that clashes with its binding to eIF4A3. This results in a change in conformation to the open state of eIF4A3 leading to release of mature EJCs from mRNAs. *In vitro*, PYM dissociates specifically the fully assembled EJCs from spliced mRNA but does not inhibit the EJC assembly (Gehring et al. 2009). By doing so, it also inhibits the NMD. In cells, overexpression of PYM leads to a decreased association of EJCs with spliced mRNAs, while PYM inhibition by RNAi leads to an accumulation of EJC components in cytoplasmic cell fractions. The study points out the important phenomenon that PYM inhibition leads to accumulation of EJCs in the cytoplasmic fraction of cells, indicating the importance of PYM in availing free EJCs in the nucleus. Interestingly, PYM function is regulated by its association with ribosomes. Biochemical analysis revealed that the C-terminal of PYM interacts with the 40S subunit of ribosome in 48S pre-initiation complex (Diem et al. 2007). The ribosome mediated disassembly mechanism makes sure that EJCs are not disrupted from the mRNAs until they have fulfilled their functions (e.g., as marker for NMD). However, in *D. melanogaster*, PYM maintains the

homeostasis of not only endogenous mRNA, but also has effects on non-translatable reporter, suggesting a ribosome-independent mode of action (Ghosh et al. 2014). Also in human cells free PYM can disassemble EJCs located outside of the ORFs (Gehring et al. 2009). This confirms a general function of PYM as a self-sufficient EJC disassembly factor. To date, a direct and active role of PYM in EJC dissociation during translation remains to be proved. We can suppose that PYM prevents the reassembly of the EJC core by binding MAGOH-Y14 early after EJC dissociation. In contrast to MAGOH and Y14, we cannot exclude that eIF4A3 and MLN51 that can bind together outside the EJC core, re-bind mRNAs after the first round of translation to further enhance next rounds of translation (Chazal et al. 2013).

### **3.2.2: Re-cycling of EJCs to the nucleus**

EJC proteins are expressed in a quantity that is not sufficient to be present onto all exons in the cell. The quantification of endogenous proteins by Western blotting in cell extracts shows a large difference between expression level of the EJC proteins and the number of exon junctions: 12000 eIF4A3 and 40000 MAGOH-Y14 against over 400,000 exon-exon junctions (Gehring et al. 2009). Therefore, the recycling of EJC from cytoplasm to nucleus must be an effective phenomenon. The mechanism of recycling of Magoh-Y14 is well understood. In this process, the Importin 13 (IMP13) plays an essential role. This nuclear receptor of karyopherin family imports specifically the MAGOH-Y14 dimer into the nucleus in a RanGTP dependent manner (Mingot et al. 2001). Binding of Importin 13 to MAGOH-Y14 dimer is at the interaction site of the PYM protein showing a mutually exclusive binding (Bono et al. 2010). Similarly, MAGOH-Y14 is sterically inaccessible to IMP13 when in the EJC (Bono et al., 2010). Thus, IMP13 binds to MAGOH-Y14 after the disassembly of the EJC core by PYM and transports it to the nucleus. Once in the nucleus, binding of one molecule of GTP to the Ran GTPase-linked leads to the dissociation of Importin 13 of its substrate, allowing the release of MAGOH-Y14 in the nucleus and its incorporation in new EJCs (**Figure 4** (Cook & Conti 2010)). However, the mechanisms of recycling and import of eIF4A3 and MLN51 to the nucleus are still unknown to this day.

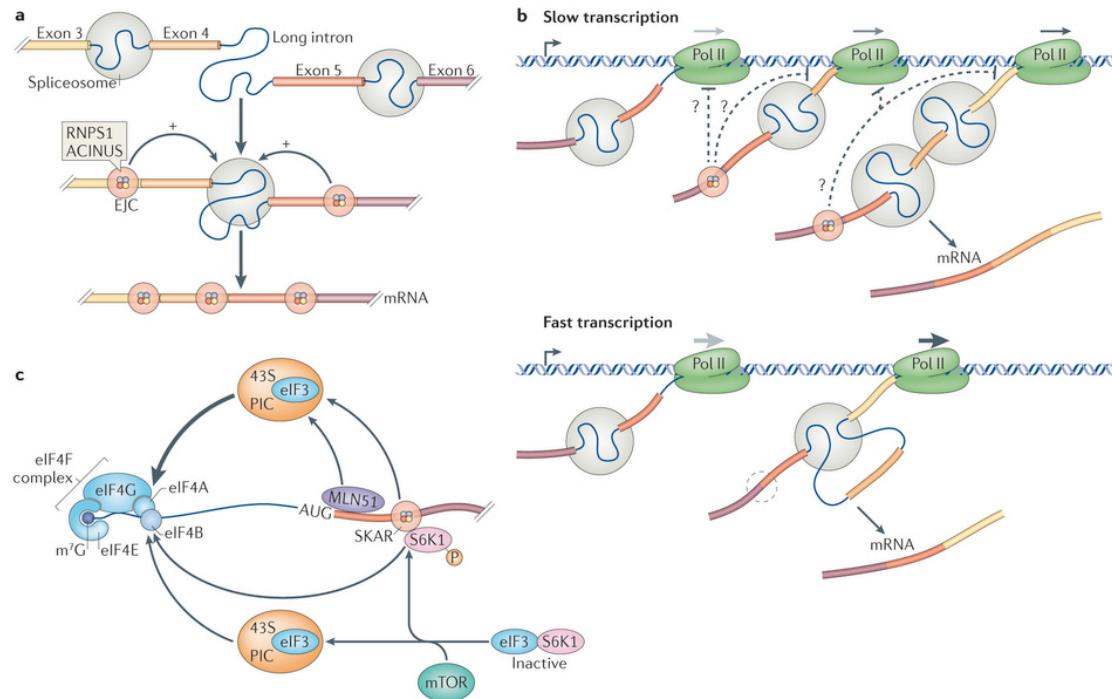


**Figure 4: Model of EJC recycling:** After the export of mRNA into cytoplasm, PYM (orange) is involved in the dissociation of EJCs in the cytoplasm by (i) interacting with the small ribosomal subunit via its C-terminus and (ii) interacting with MAGOH-Y14 via its N- terminal. The EJC core is thus dissociated into a PYM-MAGOH-Y14 trimer and eIF4A3, MLN51 proteins alone or in combination. MAGOH-Y14 is then joined by Importin 13 in the cytoplasm and imported back to the nucleus through the nuclear pores. The cargo is then discharged by the arrival of the Ran GTPase (Adapted from Bono and Gehring 2011).

## 4.0: EJC functions

The EJC is a dynamic complex whose composition changes over mRNA travel in the cell. The capacity of the EJC to recruit diverse peripheral factors grants it the ability to coordinate multiple processes related to maturation and expression of transcripts. By various mechanisms, the EJC can affect pre-mRNA splicing as well as mRNA transport, translation and mRNA surveillance by NMD (**Figure 5**). The importance of EJC-functions in regulation of cell physiology is

highlighted by recent discoveries about EJC-related developmental defects and diseases. We will discuss here particular examples related to different roles of the EJC, which make this 'RBP' a central element of the mRNA fate.



**Figure 5: The function of the exon junction complex (EJC) in splicing and translation.** a| EJCs deposited after the splicing of flanking introns can facilitate (+) the splicing of a neighbouring weak intron. The activation may be promoted by the EJC peripheral factors ACINUS and RNPS1. b| EJCs deposited during co-transcriptional splicing can slow down Pol II elongation rate allowing more time for correct splicing. In the absence of the EJC, the increased Pol II speed will cause more exon skipping. c| Role of EJC in translation activation in mTOR signaling. EJC core protein MLN51 binds to eIF3 directly. The recruitment by MLN51 of eIF3 as part of the 43S PIC enhances translation initiation. (Adapted from Le Hir et al. 2016)

## 4.1: EJC modulates splicing

The EJC core is assembled on transcripts by the splicing machinery. Most molecular processes that depend on the EJC are post-splicing events. However, recent studies revealed that EJC can affect pre-mRNA splicing itself. A genetic screen for mutations affecting photoreceptor in fly showed that mutation and knock-down of EJC core components (eIF4A3, MAGOH and Y14) causes skipping of several exons of *mapk* pre-mRNA containing long introns (Ashton-Beaucage et al. 2010; Roignant & Treisman 2010). The specific skipping of exons in long intron containing pre-mRNAs illustrates the downstream application of EJC

loading on neighboring exon junctions (Ashton-Beaucage & Therrien 2011). Similarly, the nuclear EJC is required for splicing of intron4 of piwi transcript (Hayashi et al. 2014a; Malone et al. 2014). Knockdown of any of Y14, RNPS1 or ACINUS caused intron retention of weak intron 4 of piwi transcripts in 100% cases. In this case, the splicing of flanking exons plays role in enhancing the splicing of this weak intron. EJC core and the accessory factors RNPS1 and ACINUS deposited in close proximity aid in definition and efficient splicing of neighboring weak introns (**Figure 5a**). However, whether the fully assembled EJC or only EJC components were necessary is unknown. The enhancer function of EJC could also be through effectively recruiting SR proteins to enhance exon definition, or through interaction with snRNPs to efficiently recognize neighboring weaker intron. Eventhough intron-retention and exon-skipping are both EJC dependent, EJC-dependent retained introns are generally not very long compared with those found in the previous studies (Ashton-Beaucage et al. 2010; Roignant & Treisman 2010).This indicates that exon skipping of long intron-containing transcripts and intron retention involve different mechanisms that have not yet been characterized (Hayashi et al. 2014a).

The EJC also affects pre-mRNA splicing in other organisms. In *Xenopus laevis*, eIF4A3 is required for proper splicing of ryanodine receptor (ryr1) pre-mRNA (Haremaki & Weinstein 2012). In the absence of expression of eIF4A3, the expression of the RYR1 protein is drastically reduced due to a defect in splicing of its transcript. These data suggest that eIF4A3, potentially through the EJC, binds to ryr-1 pre-messenger and causes the retention of one or more introns.

#### **4.1.1: EJC dependent regulation of splicing in human cells**

In human cells, EJC components (eIF4A3, Y14, Acinus, SAP18 and RNPS1), rather than the assembled complex function as regulators of splicing of apoptosis regulator BCL-X gene (also known as BCL2L1) pre-mRNA (Michelle et al. 2012). Recently, transcriptome-wide analysis from the lab revealed that the impact of EJC on alternative splicing is broader than previously expected (Wang et al. 2014). The reduction of EJC causes large numbers of splicing changes for variety of transcripts and for several of them the fully assembled EJC core is required for

this effect. Interestingly, several constitutive exons were excluded in EJC depleted cells suggesting that EJCs contribute to the recognition of normal splicing events. The mechanism of splicing regulation in human cells can be different than that of *Drosophila* cells as most of the EJC dependent splicing changes are not dependent on ACINUS or SR proteins. Interestingly, RNA polymerase II (Pol II) transcription accelerates when the EJC proteins amount is reduced suggesting that some splicing changes can be attributed to the faster transcription rate. Variation of transcription elongation rates can change the time available for the recognition of competitive splice sites and thus can regulate alternative splicing (Kornblihtt et al. 2013). By slowing down the transcription elongation rate, EJC may allow more time for splicing factors to recognize alternative exons. How EJCs communicate with the transcription machinery is still a matter of investigation. However, we cannot exclude that EJCs in human also serve in some cases as direct splicing regulators as observed in *Drosophila*. The mechanisms underlying the effect of EJC on specific splicing events may also rely on other direct or indirect factors, which are still unknown.

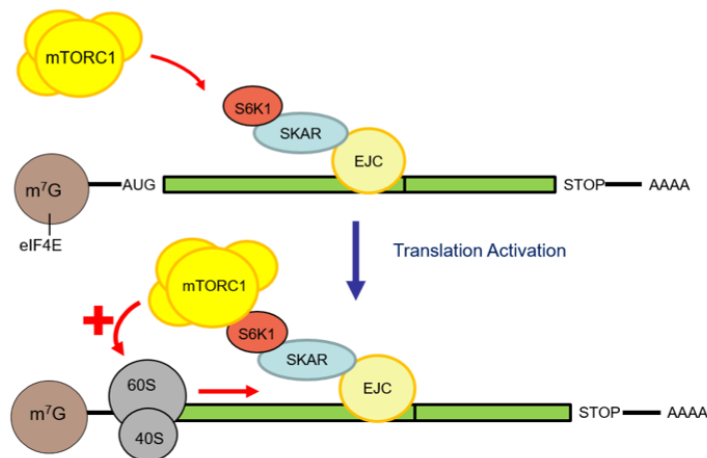
## 4.2: EJC enhances translation

The influence of splicing on protein synthesis is a phenomenon that has been observed in most organisms. Following the discovery of the EJC, which marks the spliced exon junctions, it was a matter of curiosity whether the increased expression of transcripts was related to splicing per se or to the presence of an EJC. Early studies showed that splicing can increase the expression of intron-containing genes compared to their intron-less counterparts, both at the mRNA and protein levels (Nott et al. 2003; Callis et al. 1987). Then, studies of different reporters producing spliced mRNAs associated or not to EJCs allowed to attribute to EJCs a positive effect on translation independently of effects onto expression level (Wiegand et al. 2003; Nott et al. 2004). One study notably showed that EJCs increase the proportion of mRNAs associated to polysomes (Nott et al. 2004). Although EJCs are not part of translation machinery and are removed in the very first round of translation, it is interesting that EJCs offer a selective advantage to mRNAs that have never experienced translation. This effect is most likely important to reduce the time window between gene

activation at the transcriptional level and mRNA translation producing the final product (Nott et al. 2004). The molecular mechanisms linking the EJC and translation machinery are not clearly elucidated. However, few studies have attempted to clarify the role of the complex in the translation.

#### **4.2.1: EJC enhances translation in mTOR pathway**

The first mechanistic insights into EJC role in translation activation is linked to the mTOR pathway (Ma et al. 2008). mTOR is a stress-sensing signaling pathway which enhances translation via kinase S6K1 to promote cell growth. eIF4A3-bound SKAR on mRNAs serves to recruit activated S6K1 to the CBC-mRNP, leading to phosphorylation of ribosomal proteins and translation initiation factors, thus activating translation initiation in mTOR signaling events (**Figure 6**). As EJC is loaded only on newly formed mRNAs but not on older templates, associated SKAR communicates with translation machinery as a signal of newly turned-on gene. Thus mTOR/S6K1 promptly targets the translation of freshly formed transcripts in order to reduce the time lag between transcription and translation in stress conditions. However, most EJC-interactome studies could not identify SKAR as an EJC peripheral factor (Tange et al. 2005; Merz et al. 2007; Singh et al. 2012). This suggests that interaction between SKAR and EJC can be indirect or specific to stress conditions. Alternatively, the EJC-SKAR synergy might be limited to specific transcripts only, making it difficult to detect in general.



**Figure 6: Translation activation via EJC and SKAR protein in mTOR pathway.** SKAR joins the EJC and recruits the kinase S6K1. S6K1, a substrate of mTOR kinase, then recruits the mTORC1 complex to the messenger RNA, allowing activation of the first translation round.

#### 4.2.2: Translation enhancement by MLN51

A more general mechanism to explain the impact of EJC on translation enhancement was revealed through a novel function of the EJC core protein MLN51 (Chazal et al. 2013). Modulation of MLN51 levels *in vitro* or *in vivo* affected both reporter gene and overall translation efficiency. Additionally, downregulation of endogenous MLN51 had a negative impact on general translation efficiency too, which suggests that MLN51 can function as a general translation activator. The SELOR domain of MLN51 contacts eIF3a and eIF3d, two subunits of the multiprotein translation initiation factor eIF3. The initiation factor eIF3 plays multiple roles by recruiting small ribosomal subunits to native mRNA in a position specific manner and assisting in AUG start codon recognition (Hinnebusch 2014). The combination of biochemical and functional studies strongly suggests that MLN51 can enhance translation by recruiting eIF3 either alone by directly interacting with mRNA or as part of the EJC core (Chazal et al. 2013). It has been proposed that once EJCs are disassembled during the first round of translation, MLN51 and eIF3 could re-associate with the translated mRNA to activate further round of translation

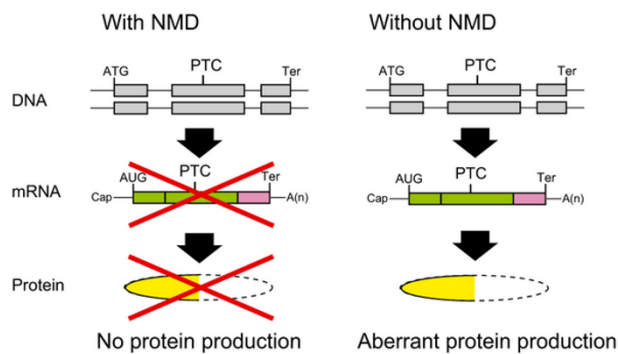


### 4.3: EJC in quality control of mRNAs

The expression of a protein-coding gene is controlled by balancing the synthesis, processing, translation and degradation of its corresponding mRNA. Additionally, mature mRNAs must carry all the information necessary to encode a functional protein. To accomplish this, eukaryotic cells have surveillance systems that scrutinize mRNA integrity (Kervestin & Jacobson 2012; Fatscher et al. 2015). There are several surveillance mechanisms, which ensure the degradation of defective mRNAs before they could be translated. Three major mRNA quality control mechanisms are (i) NSD (Non-Stop Decay) that degrades mRNAs lacking stop codons; (ii) NGD (No-Go Decay) which eliminates the mRNAs in which the ribosomes are blocked; and finally (iii) the NMD (Nonsense-Mediated-Decay), which detects the presence of premature stop codons (PTCs) (Simms et al. 2016). Here, I will simply describe the process of NMD corresponding to the best-documented function of EJCs.

#### 4.3.1: The importance of NMD

In humans, many diseases originate from mutations causing premature termination codons (PTCs) (Khajavi et al. 2006; Kuzmiak & Maquat 2006). A PTC is distinguished by translation machinery from normal termination codon by the presence of EJC or long 3' UTR downstream to it. If translated, these mRNAs would otherwise translate truncated proteins with potentially harmful dominant-negative effects (Nicholson et al. 2010) (**Figure 7**). Approximately one third of all human genetic disorders of known etiology are caused by genes with germline or *de novo* mutations that generate PTCs (Karam et al. 2013). Therefore, NMD plays an essential role in regulation of many human diseases. However, NMD is a double-edged mechanism in the event of such diseases. When synthesized truncated proteins are sufficient for a normal phenotype, the intervention of NMD usually aggravates the clinical manifestations of the disease. In particular, the NMD worsens phenotypes of several diseases related to mutation of the dystrophin gene, Duchenne muscular dystrophy (Kerr et al. 2001). On the contrary, if the produced protein has a deleterious effect on the body, the NMD works as a lifeline.



**Figure 7:** NMD ensures the degradation of transcripts containing premature termination codon (PTC), which would otherwise generate aberrant protein. (Yamashita 2013)

### 4.3.2: NMD components

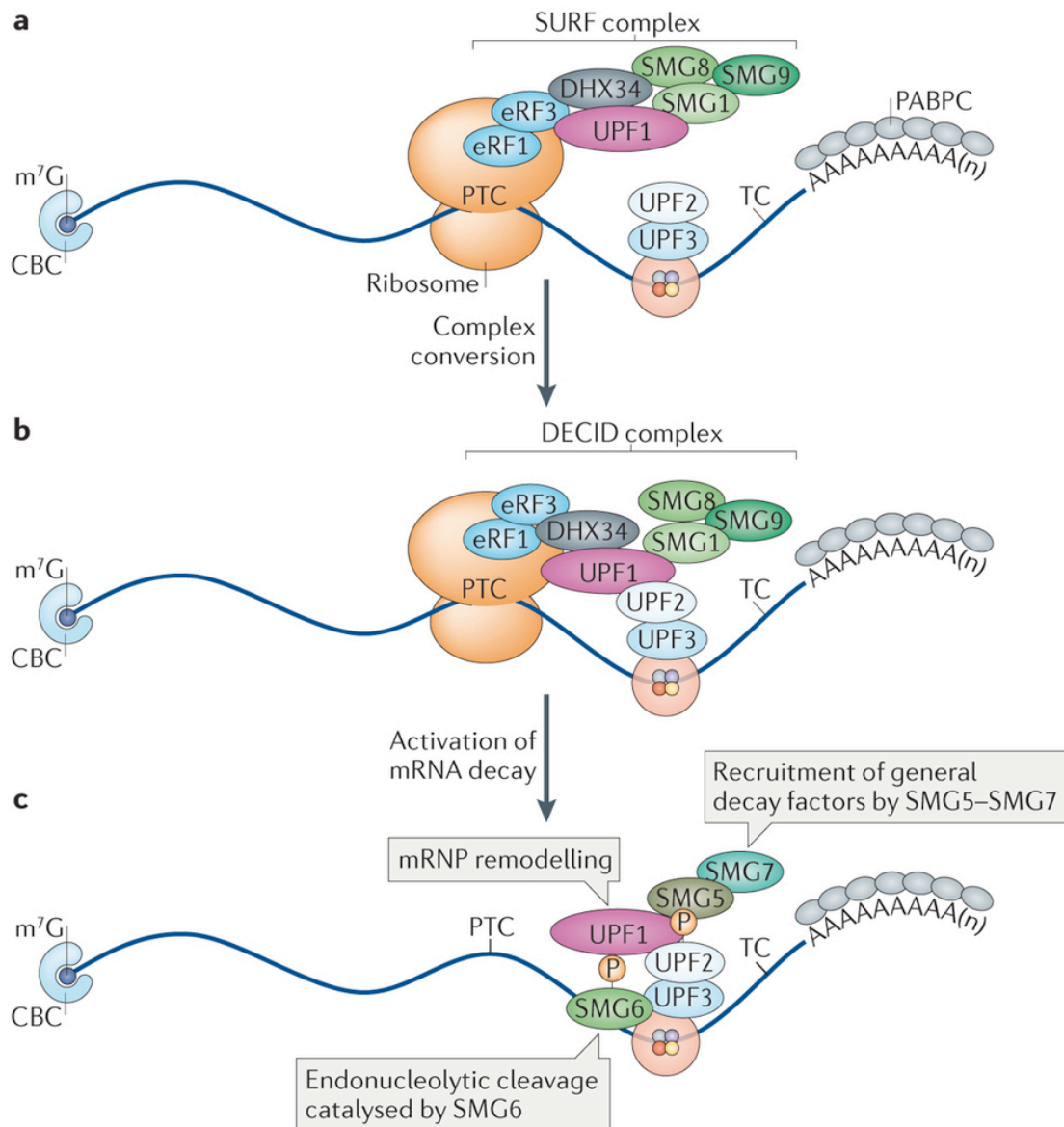
In mammals, NMD is facilitated by a group of proteins, which are conserved in all the organisms known to exhibit NMD. The core group of these factors consists of up-frameshift proteins namely UPF1, UPF2 and UPF3. The central component of the NMD pathway in all organisms studied is the protein UPF1, an ATP-dependent RNA helicase of the SF1 superfamily, which undergoes cycles of phosphorylation and dephosphorylation that are essential for NMD progression (Kurosaki & Maquat 2016). Other factors include the suppressor with morphological effects of genitalia proteins SMG1, SMG5, SMG6, SMG7, SMG8 and SMG9 (Mühlemann & Lykke-Andersen 2010). Another RNA helicase DHX34 also mediates remodeling events during NMD (Hug & Cáceres 2014). The termination of translation involves binding of the eukaryotic release factors (eRF) 1 and 3 which interact with UPF1 to determine whether NMD ensues or not (Schweingruber et al. 2013).

### 4.3.3: NMD Mechanism

In the last few years, there has been a significant increase in our understanding of how the NMD mechanism operates, how the different sub-complexes are assembled and the role of some of the NMD trans-acting factors (Hug et al. 2016; Brogna et al. 2016). In summary, the prematurely terminating ribosome recruits UPF1. Phosphorylation of UPF1 is carried out by SMG1 and by two additional subunits, SMG8 and SMG9 (Yamashita et al. 2001; Grimson et al.

2004). Initially, UPF1 associates with SMG1 and the eukaryotic release factors eRF1 and eRF3 to form the so-called surveillance complex: SURF (SMG1-UPF1-eRF1-eRF3) complex in the vicinity of the PTC (Figure 8) (Kashima et al. 2006; Yamashita et al. 2009). Subsequently, promoted by the RNA helicase DHX34, the SURF complex interacts with UPF2, UPF3b and an EJC downstream of the PTC to form the decay-inducing (DECID) complex that triggers UPF1 phosphorylation and dissociation of eRF1 and eRF3 (Kashima et al. 2006; Hug & Cáceres 2014). The phosphorylation of UPF1 by SMG1 signals the 'point of no return' in the NMD process (Kurosaki et al. 2014). This leads to translation inhibition (Isken et al. 2008) and the recruitment of phospho-binding proteins SMG6 and SMG5-SMG7 heterodimer (Okada-Katsuhata et al. 2012). Recruitment of SMG6 to PTC containing transcript causes endonucleolytic cleavage in the vicinity of PTC (Eberle et al. 2009). This leads to recruitment of decapping enzymes at 5' end and deadenylases and exosome at 3' end that removes crucial modifications at these extremities and allow access to RNA degradation enzymes (Kervestin & Jacobson 2012).

This canonical mammalian NMD pathway is not universal, since alternative NMD branches that are independent of some NMD factors have been described (Gehring et al. 2005; Chan et al. 2007; Ivanov et al. 2008). We do not know to what extent these alternative compositions modulate NMD efficiency.



**Figure 8: Functions of the exon junction complex (EJC) in nonsense-mediated mRNA decay.** **a** | A ribosome stalled on a PTC provokes the formation of the SURF complex. An EJC containing both UPF3 and UPF2 is located downstream of the SURF complex and upstream of the normal termination codon. **b** | The SURF complex is remodelled to form the decay-inducing (DECID) complex, in which UPF1 interacts with UPF2–UPF3, which have been loaded on the EJC core. **c** | UPF1 is activated by UPF2 leading to mRNP remodelling, and the recruitment of SMG6 and of SMG5–SMG7, which in turn recruit general decay factors. (Adapted from Le Hir et al. 2016)

#### 4.3.4: Role of EJC in NMD

The involvement of EJC in NMD is explained by the traditional ‘50nt rule of NMD’, according to which a stop codon must be situated more than 50-55 nucleotides upstream of spliced junction in order to trigger NMD. During the first

round of translation, the scanning ribosome removes all the EJC from the transcript. However, if the ribosome stalls > 50-55 nucleotides upstream of an EJC, due to the presence of a PTC, it is unable to displace the downstream EJC from the mRNA, inducing the NMD. Although it has been clearly established that the presence of an EJC downstream of a PTC promotes NMD in mammalian cells, there is also increasing evidence of an active NMD response in its absence. Therefore, NMD activation can rely on both EJC-dependent and EJC-independent pathways. Indeed extended 3' UTRs, termed faux 3' UTRs, in which there is an abnormal distance between the terminating ribosome and the 3' end of the transcript, can trigger EJC-independent NMD (Kervestin & Jacobson 2012; Schweingruber et al. 2013; Amrani et al. 2004; Brogna & Wen 2009). However, NMD is less efficient when independent of EJC, therefore EJC is considered to be a strong NMD enhancer (Bühler et al. 2006). Both EJC-independent and EJC-dependent NMD pathways are evolutionarily co-existing (Kerényi et al. 2008). Interestingly, EJC-dependent NMD shows distinct variabilities across intron-containing organisms. In the unicellular fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, there is no evidence of EJC assembly or requirement for NMD (Wen & Brogna 2010). The NMD in vertebrates is mostly splicing- and EJC-dependent (Wittkopp et al. 2009), the requirement for the EJC is dispensable in the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans*. Such discrepancies can be attributed to differential assembly of EJC on transcripts. NMD efficiency can vary owing to the absence of canonical EJC (cEJC) on some exons or non-competence of non-canonical EJC (ncEJC), supporting the notion that, in metazoa, the presence of an intron downstream of a stop codon does not necessarily trigger NMD.

#### **4.3.5: Role of EJC in translation control of natural NMD targets**

Apart from PTC containing mRNAs, recent studies show that NMD regulates the expression and abundance of transcripts encoding functional proteins. Genome-wide studies done in *S. cerevisiae* cells, *D. melanogaster* and *H. sapiens* where NMD has been disrupted, reveal that the NMD regulates directly and indirectly the abundance of 3 to 20% of cellular transcripts (Mendell et al. 2004; Rehwinkel et al. 2005; Weischenfeldt et al. 2008; Chan et al. 2009;

Weischenfeldt et al. 2012; Tani et al. 2012). Indeed, NMD can affect the expression of natural transcripts that present features triggering NMD like introns in the 3'-UTR, uORF upstream of the main ORF or a long 3' UTR. In this context, the mRNA is recognized as a target of the NMD and therefore degraded. Arc (activity-regulated cytoskeleton-associated) mRNA takes advantage of this mechanism to be expressed in a spatiotemporal manner in neuronal synapses (Giorgi et al. 2007). Arc pre-mRNA has two introns present in its 3'UTR that potentially lead to deposition of EJCs after splicing. This makes it a natural target for NMD. Arc mRNA is transported through the dendrites in a translational silent state. Its translation is activated in synapses where after few rounds of translation, it is degraded by NMD pathway. By this way, neurons maintain expression of Arc protein in a snapshot of time and at a specific place only. Also, it ensures that the protein is expressed in a limited turnover per molecule of mRNA, thus precisely regulating the total amount of protein based on number of mRNA targeted to the neuronal synapses.

## 4.4: EJC participates to mRNA export

### 4.4.1: General mRNA export adaptors and receptors

mRNPs are exported out of nucleus through the nuclear pore complex (NPC) and further localized to different cellular compartments. The export of mRNP requires their interaction with the general export receptor NXF1-NXT1 via several possible protein adaptors (Köhler & Hurt 2007). The conserved transcription-coupled-export complex (TREX) is an important adaptor coupling transcription to mRNA export (Strässer & Hurt 2001). TREX is found in yeast and higher eukaryotes including *Drosophila melanogaster* and humans (Strässer et al. 2002; Rehwinkel et al. 2004; Masuda et al. 2005). TREX is deposited at the 5' end of mRNA where it interacts with CBC via Protein-Protein Interactions (Cheng et al. 2006). Since long, splicing has been described as an activator of mRNAs export (Le Hir et al. 2003). So far, there are at least two ways by which splicing contributes to NXF1-NXT1 recruitment (Köhler & Hurt 2007). The connection is ensured by EJCs (see below) but also by several members of the SR family. SR proteins play important roles in both constitutive and alternative splicing (Long

and Caceres 2009). Among members of this large family, the SRSF1, SRF3 and SRSF7 that are able to shuttle between the nucleus and the cytoplasm also serve as adaptors for NXF1-NXT1 (Huang & Steitz 2001).

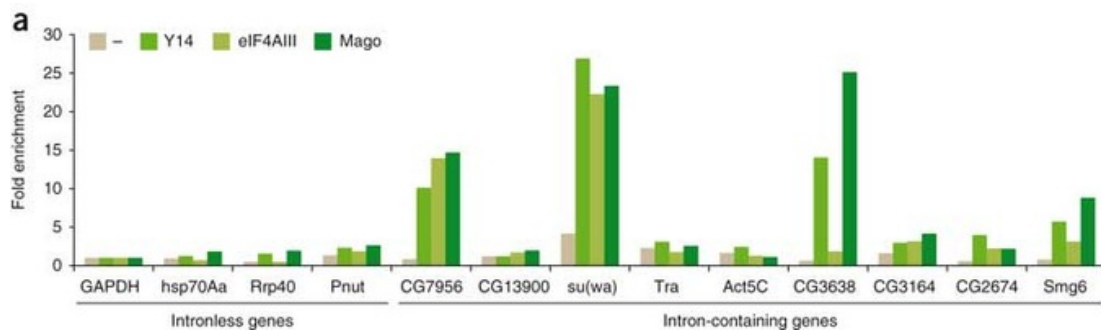
#### **4.4.2: Role of EJC in mRNA transport**

Direct involvement of EJC in mRNA export was first established by testing whether spliced mRNAs, which do not harbor EJC, could be transported to cytoplasm or not in *Xenopus laevis* oocytes (Le Hir et al. 2001). The loading of EJC at the 5' end of the mRNA was shown to enhance the export of short spliced mRNA reporters compared to similar reporters devoid of EJC. Immunoprecipitation experiments revealed that EJCs are associated to several export factors (Aly/Ref, UAP56, SRSF1 and SRSF7) as well as to NXF1- NXT1 (Le Hir et al. 2001; Zhou et al. 2000; Luo et al. 2001; Kim et al. 2001). The EJC could also facilitate export by stabilizing adaptor SR proteins as well as the overall mRNP structure (Singh et al. 2012). So far, only export of short transcripts (of a few hundred nucleotides long) was found to be highly dependent on splicing and the EJC (Le Hir et al. 2001). Since longer mRNAs have more binding sites for different adaptors, EJC is only one adaptor among others, which explains why the overall export of mRNAs only marginally depends on splicing and the EJC (Gatfield & Izaurralde 2002; Nott et al. 2003). Whether EJC is required for the nucleo-cytoplasmic export of specific transcripts remains an open question.

## **5.0: Global view of EJC deposition on mRNAs**

As the EJC is assembled by spliceosome on mRNAs in a sequence independent manner, it was assumed that every splicing reaction leads to deposition of EJC on all exonic junctions with same functional potential. However, several observations made in *D. melanogaster* led to hypothesize that the EJC might be bound to only a subset of spliced junctions. The first notion against the constitutive EJC loading came from the study of the Oskar mRNA in fly. The localization of Oskar mRNA to the posterior pole of drosophila oocytes requires both splicing of its precursor and the four EJC core proteins (Hachet &

Ephrussi 2004); however, among the three introns of Oskar pre-mRNA, only removal of the first one by the splicing machinery is required for the correct localization of the mRNA (Hachet & Ephrussi 2004), suggesting that an EJC is deposited exclusively on the first junction or that the function of EJCs may differ from one splice junction to another. Second, even though EJC components are well conserved in drosophila and human, NMD in drosophila was first proposed to be independent of splicing and EJC (Gatfield et al. 2003), suggesting that EJC might be incompetent for NMD or not stably associated to mRNAs. The first evidence about differential EJC deposition came from the analysis of EJC binding at multiple NMD targets with intron containing 3' UTRs in drosophila (Saulière et al. 2010). The study demonstrated that EJCs are present on certain, but not all, spliced mRNAs (**Figure 9**) and their deposition could trigger NMD. The deposition onto a subset of mRNAs might explain why EJCs are mainly dispensable for NMD in drosophila.



**Figure 9:** EJCs are deposited on specific spliced mRNAs, but not on all. The quantification of RNA content of endogenous RNA-protein complexes immunopurified by Y14, Mago and eIF4AIII are shown. (Adapted from Saulière et al. 2010)

## 5.1: Differential EJC loading on human transcriptome

The first transcriptome-wide mapping of EJC binding sites showed that EJC deposition on mRNAs is more complex than previously imagined. *In vivo*, EJC occupancy on transcripts displays diverse variability in terms of position and magnitude. Two studies independently mapped the transcriptome-wide deposition of EJC in human cells (Singh et al. 2012; Saulière et al. 2012). They applied two different approaches to purify EJC-bound RNA fragments before deep sequencing. While Saulière *et al.* used cross-linking and immunoprecipitation coupled to high-throughput sequencing (CLIP-seq) to

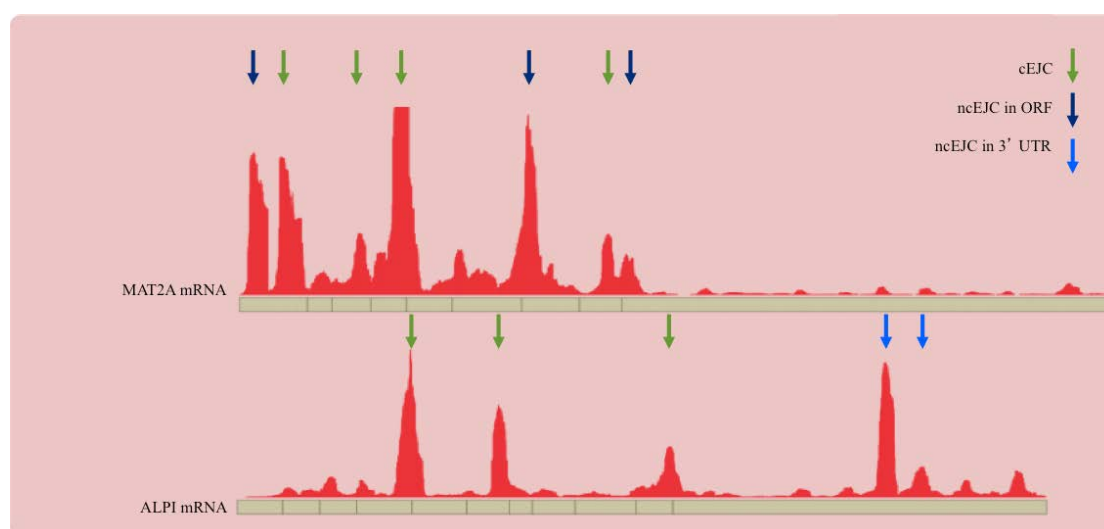


determine eIF4A3-binding sites in the HeLa cell transcriptome, Singh *et al.* purified EJCs together with their tightly bound RNA fragments from HEK cells by RNA immunoprecipitation in tandem (RIPiT) and subsequently identified associated proteins by mass spectrometry and RNase-protected RNA fragments by deep sequencing. Despite using different experimental approaches, both studies revealed remarkably similar findings (Mühlemann 2012). These studies showed that eIF4A3 is associated mainly with coding sequences, which is in agreement with splicing-dependent exonic loading of eIF4A3 as a part of the EJC (Le Hir *et al.* 2000). However, only 80% of exon junctions are bound by EJCs, strongly suggesting that at least 20% of the exons are not associated with an EJC (**Figure 9**: blue arrows). This indicates that even for the most strongly expressed mRNAs, splicing does not always result in EJC deposition. Secondly, the efficiency of EJC occupancy may vary from one exon junction to another between the mRNAs or within the same transcript. Despite the fact that the methods used cannot be considered as highly quantitative, these studies highlighted the notion that EJCs can occupy some junctions more preferably than others. This raises the question of which factors influence the presence of the EJC on spliced junctions. The bioinformatics analysis could not find any strike-through difference in 5' and 3' splice-site strength between EJC-occupied and EJC-free exon junctions except the slight tendency to prefer shorter exons and upstream of long introns. Therefore, it is tempting to speculate that the sequence is not a major player in EJC-recruitment on some exon junctions but, probably other factors that influence EJC binding. Since EJC map in human cells is merely a binding map of eIF4A3 alone, another speculation might be that the EJC-void exons (20% of total exons) could actually be eIF4A3-independent EJCs. In support of this possibility some spliced mRNP-specific proteins can be recruited independently of eIF4A3 (Zhang & Krainer 2007).

## **5.2: The canonical and non-canonical EJC**

EJC mapping showed that eIF4A3 is docked to the canonical EJC position ~24 nucleotides upstream of exonic junctions (cEJC) on thousands of exons. This strict positioning of EJC deposition is maintained for individual spliced exons from both protein-coding and non-coding RNAs, as well as exons upstream of

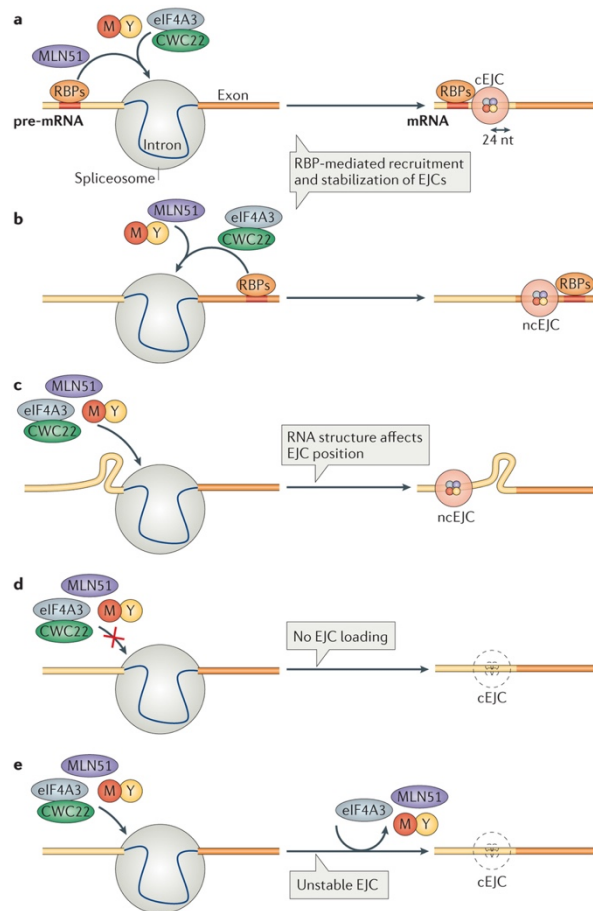
introns removed by spliceosomes (Saulière et al. 2012; Singh et al. 2012). In addition to the cEJC centered at the  $-24$  position, EJCs are also observed at other exonic locations (non-canonical EJC or ncEJC). Surprisingly only half of the EJC on mRNAs is in form of cEJC. The other halves of EJCs are ncEJC, located at variable distances from spliced junctions (**Figure 10**: blue arrows, Figure 10). NcEJC were mostly present in the coding sequence but also present to a low extent in 5' and 3' UTRs. Both cEJC and ncEJC have many characteristics in similar. The ncEJC were not present in intron-less mRNAs suggesting that their assembly on mRNAs is also splicing-dependent. The eIF4A3 binding sequences associated with either of these EJCs are enriched in purines. Both kinds of EJCs show slight enrichment in short exons flanked by long introns and preference for unstructured RNA sequences. This is in agreement with eIF4A3 being incapable of binding double stranded RNA as a part of EJC (Andersen et al. 2006) . However, the mechanism behind the positioning of EJCs on transcripts still remains unclear.



**Figure 10: Differential deposition of EJC in human cells.** Two mRNAs having variable EJC occupancy are shown with cEJC binding prior to exon junctions (denoted by green arrows) and ncEJC in ORF (dark blue arrow) and in 3' UTR (light blue arrow). (Modified from Saulière et al. 2012)

The variability in splicing may alter the deposition of EJC. So far, no correlation has been detected between the strength of splice site and the presence of EJC (Singh et al. 2012; Saulière et al. 2012) but deletion and substitution studies showed that the sequences surrounding this site are crucial

for EJC loading (Ghosh et al. 2012; Saulière et al. 2010). Both cEJCs and ncEJCs are preferentially associated with unstructured motifs (GAA/G triplets) resembling known binding sites for several serine-arginine-rich proteins (SR proteins) (Long & Cáceres 2009). It is corroborated with the fact that SRSF1 and SRSF7 were co-immunoprecipitated with EJCs (Saulière et al. 2012; Singh et al. 2012). SR proteins along with other RBPs bound to specific exonic sequences might not only facilitate the recruitment of EJC and stabilize the complex but also play a role in positioning the ncEJC in case of unfavorable canonical regions such as secondary structures. It was already shown that EJC loading could shift from its canonical positions when it encounters physical obstruction (Mishler et al. 2008). Therefore, at least a portion of ncEJCs may represent the displaced cEJCs whose assembly at canonical positions was precluded by physical constraints. The differential loading of EJCs can also be due to the absence of EJC recruitment by splicing machinery. It is also speculated that deposited EJC might rapidly disassemble in the absence of stabilizing factors (**Figure 11: e**).



**Figure 11: Differential EJC loading in human cells.** **a** | cEJCs are present at 24 nt upstream of the exon junction. RBPs, such as SR proteins, bound to specific sequences (orange box), may facilitate the recruitment of EJC proteins during splicing or stabilize the EJC core complex once deposited. **b** | RBPs may participate in the assembly or the stabilization of ncEJCs. **c** | RNA structures can prevent cEJC loading, thereby redirecting them to non-canonical binding sites. **d** | The absence of protein(s) required for EJC assembly could impair both cEJC and ncEJC assembly. **e** | EJC components are recruited into spliceosomes, but the EJC rapidly disassembles in the absence of stabilizing factors. (Adapted from Le Hir et al. 2016)

The differential EJC loading could potentially explain numerous cellular processes regulated by EJC such as pre-mRNA splicing, mRNA transport, stability and translation. Given that EJCs enhance translation efficiency (Le Hir & Séraphin 2008; Chazal et al. 2013), we can suppose that spliced mRNAs expressed in the same environment but associated with more or less EJCs could be translated with different efficiencies. This may also be true for pre-mRNA splicing or mRNA transport. In case of NMD, for which presence of EJC downstream of the termination codon has been characterized as strong enhancer (Nicholson et al.

2010), deposition of a ncEJC downstream of stop codon may be sufficient to trigger NMD. Hence it could potentially explain some examples of NMD triggered by termination codon located in last exon (Zhang et al. 1998) or less than 50 nucleotides upstream of spliced junctions (Carter et al. 1996; Bühler et al. 2006). In addition, the efficiency of NMD is not constant and can vary between different cellular contexts (Gudikote et al. 2005; Viegas et al. 2007; Zetoune et al. 2008). We can assume that the variability in EJC presence may also contribute to variability in NMD efficiency in different cellular environments.

## **6.0: mRNA localization and local translation**

The regulated intracellular trafficking and localized translation of mRNA molecules represents an important and prevalent mechanism of gene regulation. This process plays a key role in modulating asymmetric protein distribution linked to a wide variety of biological processes in different organisms and cell types. Here we discuss the diverse biological functions, advantages, and mechanisms of mRNA localization with some examples that underlines the critical importance of this gene regulatory mechanism.

### **6.1: mRNA localization**

The mechanisms controlling the asymmetric organization of cells and tissues are important for a variety of biological processes that rely on the polarization of cellular activities, such as embryonic patterning, asymmetric cell division, and cell migration, either during development or in the context of normal tissue homeostasis. Many of these biological functions are controlled by asymmetric translation of specific mRNAs in particular sub-cellular components of the cell. Isolated translation is regulated by localizing a particular mRNA in distinct compartment of the cell, prior to translation. Polarized accumulation of RNA molecules has been observed since a long time in eukaryotes for several transcripts and has been considered as a mechanism to spatially and temporally restrict gene expression to discrete sites within highly polarized, asymmetric cells (Martin & Ephrussi 2009; Medioni et al. 2012; N. a L. Cody et al. 2013).

Earlier studies on localized transcripts assumed that site-specific-targeting of an mRNA might be restricted to genes only having specialized local function, and it is now considered as a general phenomenon in diverse cell types. Indeed more than 70% of mRNAs in *Drosophila* embryo (25% of transcriptome) are strikingly localized in different sub-cellular compartments (Lécuyer et al. 2007). Similarly in mammalian neurons, hundreds of transcripts were present in distinct neuronal processes, modulating diverse functionalities (Darnell 2013).

## 6.2: Importance of mRNA localization

There are several mechanistic and functional benefits of localizing a transcript prior to translation. Local gene expression offers following benefits to the cell:

### 6.2.1: Energy efficiency for the cell

Localization of mRNAs prior to translation reduces the significant energy costs of moving each protein molecule individually (Jansen 2001). Each localized mRNA can facilitate many rounds of translation to generate multiple copies of protein from a single transcript. Since fewer mRNA molecules need to be localized, it makes it thermodynamically more efficient than transporting each protein individually to a distinct site. This process is also energy saving for proteins that are incorporated in same functional complexes or involved in specific biological functions. Functionally related transcripts can be co-targeted at the same site in order to facilitate the efficient co-translational assembly of functional protein complexes (Mingle 2005; Lécuyer et al. 2007; Lécuyer et al. 2009).

### 6.2.2: Spatio-temporal translation

Another obvious function of RNA localization is that it allows gene expression to be spatially restricted within the cytoplasm. Localized translation also offers to control gene expression in specific areas of the cell in response to environmental signals. In neurons, local regulation of protein synthesis has been shown to modulate synaptic plasticity (Liu-Yesucevitz et al. 2011; Jung et al.

2012). The precise localization of transcripts also ensures that the translated protein restricts to specific part of the cell and does not diffuse throughout the cytoplasm that might have deleterious effects otherwise. For example, in oligodendrocytes, the myelin mRNA is targeted specifically to the myelin producing zones and restricts this sticky protein to the same area. Inappropriate transport of the myelin mRNA causes aberrant membrane aggregation (Jung et al. 2012).

### **6.2.3: Storage**

The sub-cellular trafficking of mRNAs can also serve as storage function to rapidly translate bulk of protein when needed. For instance, in mouse, mature Cat2 mRNAs are stored in nucleus when not immediately needed to produce proteins. When the cytoplasmic presence of Cat2 transcript is rapidly required upon physiologic stress or other cellular signals, it is released from the nucleus (Prasanth et al. 2005). Another example of this is retrograde signaling in neurons, in which the localized translation of dormant mRNAs in axons can produce transcription factors that are trafficked back to the nucleus to control gene expression in response to environmental cues (Cox et al. 2008; Ji & Jaffrey 2012).

### **6.2.4: One transcript, multi-functional protein**

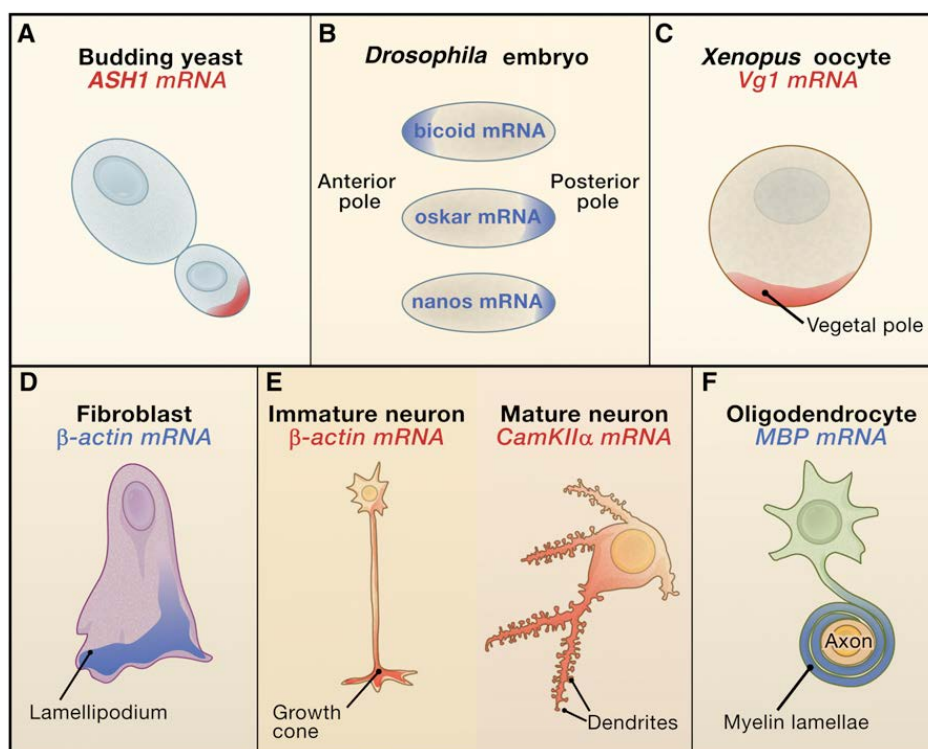
The mRNA localization also modulates the post-translational behavior of a protein with respect to cell territory. Locally synthesized nascent proteins may have properties distinct from pre-existing copies, by virtue of post-translational modifications or through chaperone-aided folding pathways (Lin & Holt 2007; Medioni et al. 2012). For example, in mammalian cells locally translated  $\beta$ -actin can be arginylated, thus preventing actin filament clustering (Karakozova et al. 2006), or glutathionylated, thus restricting actin polymerization (Wang et al. 2001). Thus, single mRNA can serve to produce two distinct proteins in a specific cell region. Together, these examples give an insight on how asymmetric localization of transcripts is functionally beneficial to modulate localized behavior of proteins.





### 6.3: Examples of localized mRNAs

The development of cDNA cloning and gene-specific *in situ* hybridization methodologies greatly contributed to discover specific mRNAs that were asymmetrically localized to different cytoplasmic territories (**Figure 12**). These transcripts encode proteins that play specialized roles within well-defined subcellular compartments. For example, in yeast *ASH1* mRNA is transported to the bud tip of a dividing cell such that it is delivered only to the nucleus of the daughter cell, thereby ensuring that the mother and daughter cells have distinct mating types (Paquin & Chartrand 2008). In flies, *gruhen*, *bicoid*, *oskar* and *nanos* mRNAs are localized precisely in the oocyte in order to regulate their temporal translation during oogenesis for accurate spatial patterning along the dorsoventral and anteroposterior axes of the embryo (Kugler & Lasko 2009).

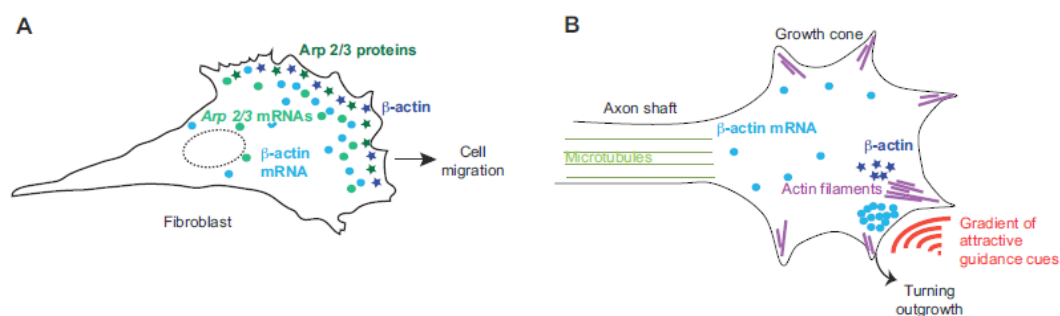


**Figure 12: Classic Examples of Localized mRNAs** (A) In budding yeast, the *ASH1* mRNA localizes to the bud tip. (B) In *Drosophila* embryos, *bicoid* mRNA localizes to the anterior pole; *oskar* and *nanos* mRNAs to the posterior pole. (C) In *Xenopus* oocytes (stage IV), *Vg1* mRNA localizes to the vegetal pole. (D) In chick and mammalian fibroblasts,  $\beta$ -actin mRNA localizes to lamellipodia. (E) In developing, immature mammalian neurons,  $\beta$ -actin mRNA is present in distal growth cones and in mature, fully polarized pyramidal neurons, *CamKII $\alpha$*  mRNA

is present in distal dendrites. **(F)** In mammalian oligodendrocytes, myelin basic protein (MBP) mRNA localizes to myelinating processes that ensheath neuronal axons. (Adapted from Martin and Ephrussi, 2009)

Similarly in *Xenopus* oocytes several transcripts are targeted to the vegetal pole such as *Xcat2/Nanos1*, *Xdazl*, *Vg1* and *VegT* which are crucial for endoderm and mesoderm germ layer specification (Medioni et al. 2012). In higher eukaryotes, migrating embryonic fibroblasts and myoblasts localize  $\beta$ -actin mRNA and Arp2/3 complex at the leading edge (**Figure 13**). This localization is important for the rapid polymerization of actin filaments which directs the cell migration (Kislauskis et al. 1997; Mingle 2005; Leung et al. 2006; Liao et al. 2011). In neurons, a distinct population of mRNAs is localized and translated in a spatially restricted manner (Kindler et al. 2005). Among these,  $\beta$ -actin mRNA, Arc mRNA and CamKII $\alpha$  are some of the extensively studied examples. Localized translation of  $\beta$ -actin mRNA in distal growth cones, Arc mRNA at the synapses and CamKII $\alpha$  at the distal dendrites is shown to be crucial for synaptic plasticity (Martin & Zukin 2006; Liu-Yesucevitz et al. 2011; Plath et al. 2006).

These wide ranging examples demonstrate that mRNA localization is a key biological process in diversity of organisms.

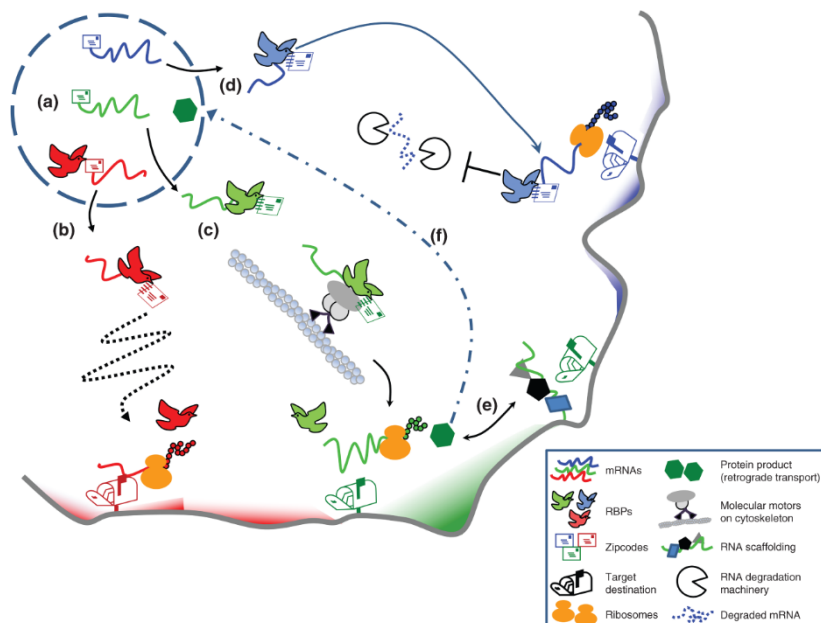


**Figure 13: Targeted mRNAs in migrating cells.** **(A)**  $\beta$ -actin mRNA (light blue) or mRNAs encoding subunits of the Arp2/3 complex (light green) are targeted to the leading edge of migrating fibroblasts. Local synthesis of their corresponding proteins (dark blue and dark green stars, respectively) contributes to directional migration. **(B)**  $\beta$ -actin mRNA (light blue) is transported to the side of the axonal growth cone exposed to an attractive guidance cue (red). Locally translated  $\beta$ -actin protein (dark blue stars) accumulates at the same location, promoting the nucleation of actin filaments (purple) and triggering growth cone turning. (Adapted from Medioni et al. 2012)

## 6.4: Mechanism of mRNA localization

At the molecular level, the trafficking of mRNAs to distinct subcellular destinations is generally dictated by cis-acting elements or 'zipcodes' residing within the RNA, which act as recognition sites for trans-acting RBPs (**Figure 14**). RBPs often serve to coordinately regulate the trafficking, stability, and translation of the target transcript, consistent with the view that localized mRNAs are usually translationally repressed during transit (Besse & Ephrussi 2008). Furthermore, certain effectors assigned in the nucleus modulate the cytoplasmic targeting properties of mRNAs (Hachet & Ephrussi 2004; Horne-Badovinac & Bilder 2008). The effects of nuclear maturation was illustrated in the case of Oskar for which pre-mRNA splicing leads to the formation of a zipcode element required for proper cytoplasmic localization (Ghosh et al. 2012).

To date, three principal mechanisms of transcript localization have been defined, namely, random diffusion combined with localized entrapment, directed transport along the cytoskeleton, and general transcript degradation coupled to localized protection (Cody et al. 2013)(**Figure 14**).



**Figure 14: Different mechanisms of mRNA localization.** The mRNPs can be transported via general diffusion followed by their entrapment in specific components of the cell (b), or direct transport via motor proteins (c) or become localized through degradation protection (d). (Adapted from Cody et al, 2013)

### **6.4.1: Directed transport of mRNA in cytoplasm: Cis-regulatory elements and trans-acting factors**

The most prominent of these mechanisms involves active transport along the cytoskeleton through association of RNPs with specific motor proteins (Holt & Bullock 2009; Bullock 2011). Several *Drosophila* mRNAs have been shown to localize in apical cytoplasm of epithelial cells through the recognition of hairpin zipcode elements by a transport complex containing the RBP Egalitarian, the cargo adaptor Bicaudal-D and the Dynein motor protein (Wilkie & Davis 2001; dos Santos et al. 2008; Dienstbier et al. 2009). Interestingly, The RNA zipcode itself can control the dynein number to manage the speed and directionality of RNA movement via cytoskeleton (Bullock et al. 2006; Amrute-Nayak & Bullock 2012; Ghosh et al. 2012). Although the molecular mechanisms underlying specific recognition of zipcodes by RNA-binding proteins have long been elusive, recent structural studies have revealed requirements for highly specific motifs and/or structures. For example, a 54 nucleotides sequence required for the targeting of  $\beta$ -actin mRNA in fibroblasts has been shown to contain a bipartite element comprising two RNA motifs recognized by the zipcode-binding protein Zbp1 (Chao et al. 2010; Patel et al. 2012). The recognition of  $\beta$ -actin zipcode by ZBP1 occurs through RNA looping mechanism. ZBP1 recognize a bipartite RNA element composed of two sequence motifs separated by a spacer region of precise length (Patel et al. 2012).

Characterizing localization elements (LEs) for localized mRNAs remains a challenging task to date. Despite recent advances in genome-wide analysis of transcriptome, defining common signatures shared by RNA molecules targeted to the same cellular sites still remains decisive.

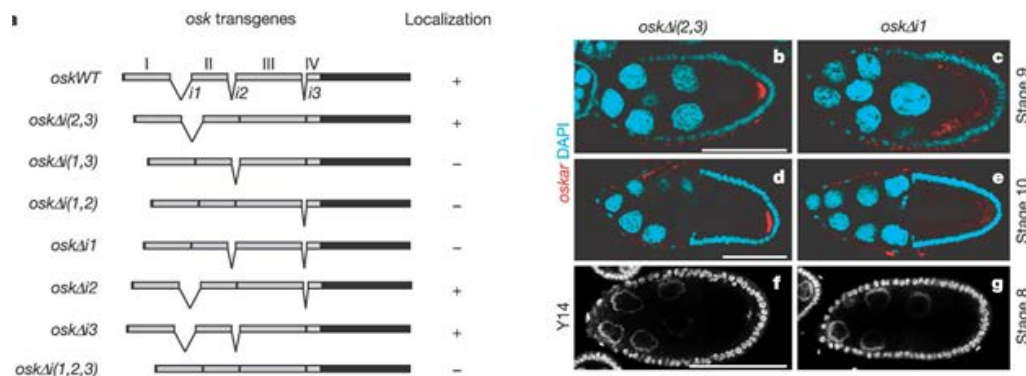
### **6.4.2: Directed transport of mRNA in cytoplasm: Recruitment of molecular motors**

The mRNA targeting to different cytoplasmic regions is majorly dictated by the active molecular motors used for mRNA transport. The nature and number of the motor proteins determine the cytoskeletal tracks (via actin filaments or microtubules), the directionality (uni or bidirectional), and the properties (e.g. speed, processivity) of mRNA motion (Bullock 2011; Gagnon &

Mowry 2011; Marchand et al. 2012). For example, the recruitment of several molecules of the myosin motor Myo4p by multiple localization elements increases the efficiency of ASH1 mRNA transport on actin filaments in yeast (Chung & Takizawa 2010). Furthermore, dendritically transported RNPs exhibit microtubule-dependent bidirectional movement, suggesting the recruitment and the activity of opposite polarity motors (Doyle & Kiebler 2011). Consistent with this view, the RNA-binding protein FMRP has been shown to associate with dendritically localized transcripts, and to bind to KLC (a component of the plus-end motor Kinesin-1) as well as to the dynein-interacting BicD protein (Dictenberg et al. 2008; Bianco et al. 2010). A general trend emerging from live-imaging analyses is that bidirectional transport is commonly used in higher eukaryotes for mRNA targeting (Medioni et al. 2012). This might allow RNPs to navigate around obstacles and ensure a constant reassessment and fine-tuning of directional transport.

### **6.4.3: Role of EJC in sub-cellular localization**

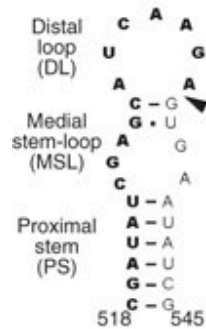
EJC is involved in the localization of Oskar mRNA to the posterior pole of *D. melanogaster* embryos, which is essential for proper patterning and development (Kugler & Lasko 2009). Screening of proteins necessary for the localization of the Oskar transcript has identified eIF4A3, Mago Nashi (MAGOH), Tsunagi (Y14) and Barentsz (MLN51) as components of a conserved protein complex that is essential for mRNA localization (Newmark et al. 1994; Mohr et al. 2001; Van Eeden et al. 2001; Palacios et al. 2004). Protein components of the EJC accompany the Oskar mRNA from the nucleus to its destination at posterior pole of the embryo (Hachet & Ephrussi 2001; Mohr et al. 2001). EJCs reflect the fact that splicing reaction itself may be necessary for Oskar mRNA localization. Localization of selectively expressed different transgene constructs of Oskar pre-mRNA showed that splicing of its first exon is essential for its localization at posterior pole (Hachet & Ephrussi 2004) (**Figure 15**). Thus, the cytoplasmic targeting machinery relies on EJC as a mark of maturation events that occurred in the nucleus.



**Figure 15: The EJC is required for Oskar mRNA localization during *Drosophila* oogenesis.** Left, Schematic representation of the different Oskar transgenes. The capacity of the mRNA to localize is indicated in the right column as + or -. Right, the splicing of the first exon junction, but not both other junctions, is necessary for the localization of the transcript at the posterior pole of the oocyte. Thus, the transcript *oskΔ1* is not properly localized while *oskΔ(2,3)* remains correctly located (adapted from Hachet & Ephrussi, 2004)

Intriguingly, *oskar* mRNA particles not only move less frequently in Mago and Barentsz mutant oocytes, but the directional bias of motile particles is reversed such that the transcript localizes in a shallow gradient in the anterior of the oocyte (Zimyanin et al. 2008). Cumulatively these studies suggested that EJC might recruit cytoplasmic localization factor to the appropriately spliced and exported Oskar transcript. However, it remains unclear why EJC mediated recruitment of these export factors is limited to the *oskar* transcript and not to all mRNAs decorated with an EJC. A recent study using *in vivo* imaging in live oocytes suggested that not only deposition of EJC at first spliced junction, but also an RNA secondary structure is important for the directional mobility of *oskar* transcript (Ghosh et al. 2012). Splicing of *oskar* pre-mRNA most likely leads to EJC deposition on the first exon and creates the spliced *oskar* localization element (SOLE), a secondary structure generated by joining the 3' end of first exon and 5' end of second exon (**Figure 16**). The structural integrity of SOLE is crucial for *oskar* mRNP motility and localization in the oocyte. Splicing thus has a dual role in *oskar* mRNA localization: assembling the SOLE and depositing the EJC required for mRNA transport. The SOLE complements the EJC in formation of

a functional unit that, together with the oskar 3' UTR, maintains proper kinesin-based motility of oskar mRNPs and posterior mRNA targeting.



**Figure 16: Integrity of the SOLE proximal stem of *Oskar* mRNA.** (a) Mfold prediction suggests that the SOLE (nt 518–545) forms a stem-loop structure comprising a PS, an MSL and a DL structures. *Oskar* exon 1 nucleotides (bold) and exon 2 nucleotides flank the first *Oskar* splice junction (arrowhead). (Adapted from Ghosh et al. 2012).

Most localized mRNAs, such as bicoid, gurken and pair-rule transcripts in *Drosophila*, and ASH1 mRNA in *Saccharomyces cerevisiae*, require a single type of motor for localization: dynein or myosin, respectively. In contrast, *Oskar* mRNA, which depends on dynein for its translocation from the nurse cells to the oocyte, switches to Kinesin heavy chain (KHC) within the oocyte for its transport to the posterior pole. After the accomplishment of dynein-dependent mRNA transport, the EJC and the SOLE become important for the KHC-dependent step of *oskar* transport into the oocyte (Ghosh et al. 2012). The regulatory role of EJC or SOLE in KHC recruitment to *oskar* RNPs or in controlling the switch from a dynein to a KHC-dependent mode of transport is yet to uncover.

The structural integrity of SOLE is crucial for ribonucleoprotein motility and localization in the oocyte. Thus, EJC and the SOLE element act as a functional unit to allow *Oskar* localization through the cellular motors (kinesins and microtubule) towards the posterior pole where it will be expressed. To date, *Oskar* is the only mRNA shown to depend on splicing and EJC for its localization. Whether the EJC could regulate the localization of other specific transcripts in *Drosophila* and other cells remains to be seen yet.

## 7.0: EJC in physiological contexts

The proteins of EJC have an important role in regulation of various physiological functions. It is reflected by the discovery of an increasing number of developmental defects and diseases linked to altered expression of EJC proteins. Several studies have linked EJC proteins with developmental defects, brain development disorders and human pathologies. Here we discuss some of the important highlights to show how EJC regulates cell physiology.

### 7.1: EJC in development

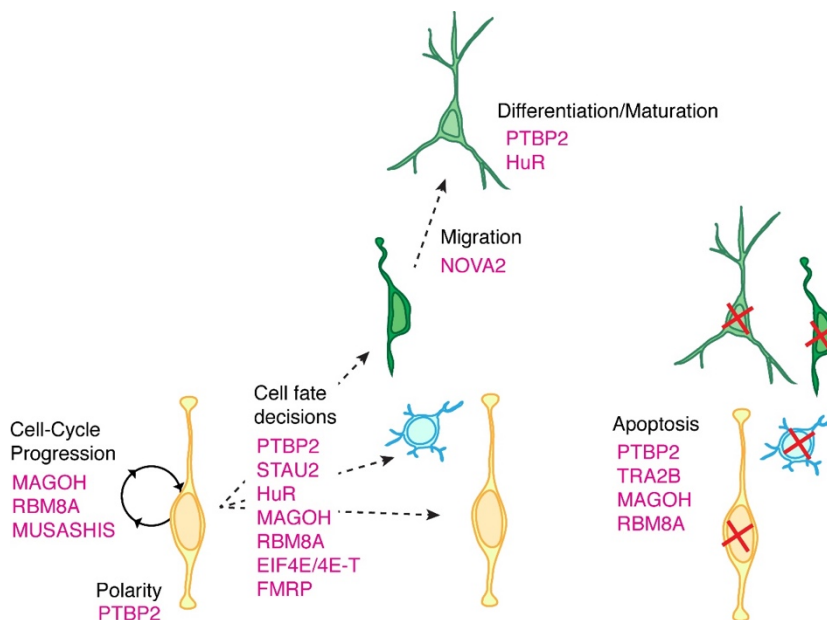
In mammals, the EJC is essential for cellular functions and development, as the phenotype of *Magoh*<sup>-/-</sup> mice is embryonic lethal (Silver et al. 2010). The EJC also plays an essential role in cell fate determination and germ cell formation in *Drosophila*. It was shown in early 90s that the EJC core components are necessary during early development as mutations in *mago nashi* gene disrupt germ cell formation (Boswell et al. 1991). Later studies exhibited that both *mago nashi* and *Tsunagi* (Y14) regulate *Drosophila* germline stem cell differentiation and oocyte specification (Parma et al. 2007; Lewandowski et al. 2010). Also, by regulating the splicing of *mapk* mRNA in *Drosophila*, the EJC has very specific effects on photoreceptor differentiation via the regulation of the epidermal growth factor (EGFR) pathway (Roignant & Treisman 2010). Also in *Marsilea vestita*, *mago nashi* functions at multiple levels to control gametophyte development. Indeed, in the absence of *mago* protein, the normal patterns of stored  $\beta$ -tubulin accumulation and localized centrin translation in the spermatogenous cells are disrupted (van der Weele et al. 2007). In *Caenorhabditis elegans*, EJC components *Ce-Y14* (Y14) and *MAG-1* (MAGOH analog) are required for embryogenesis and germline sexual switching (Kawano et al. 2004). In *Xenopus laevis* *eIF4A3* is required for embryonic movement, and for melanophore and cardiac development (Haremaki et al. 2010). In most of this physiological functions the mechanisms by which EJCs are involved remain mainly unknown. We can suppose that during early phases of development, EJCs



interact with embryonic transport machinery in order to establish the cell polarity required for oocyte differentiation.

## 7.2: EJC in mammalian brain development

Recent studies in model organisms and humans have collectively highlighted roles for post-transcriptional regulation in virtually all steps of corticogenesis. Many RBPs play a causal role in neurodevelopmental pathologies by controlling the production, differentiation and migration of neurons in developing cortex (**Figure 17**). These include factors involved in splicing such as NOVA2, PTB2 and TRA2B; with other RBPs controlling the translation and stability of transcripts such as HuR and FMRP and Eif4E/4E-T complex.

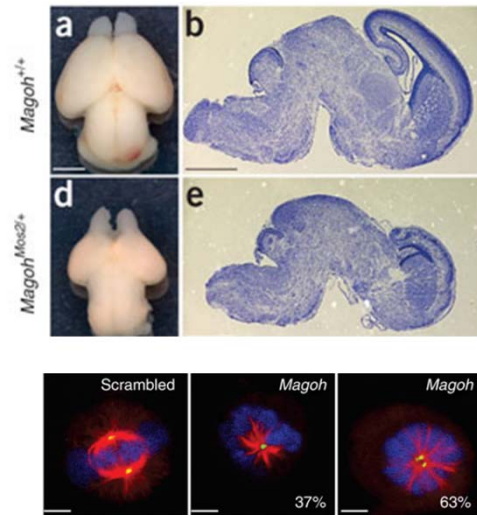


**Figure 17:** Regulation of corticogenesis by RNA-binding proteins. Different aspects of neural progenitor function (cell cycle progression, cell fate decision, apoptosis) and neuronal function (migration, differentiation, maturation, apoptosis) are indicated along with the RBPs. (Adapted from Pilaz and Silver, 2015)

Among the RBPs, EJC proteins play a central role in regulation of neurodevelopmental disorders. In mammals, mutation of Magoh disrupts brain size as a result of defective neural stem cells (NSC) division and neuronal apoptosis (Silver et al. 2010). Magoh-haploinsufficient mice have defects in neuronal stem cell division, disrupted spindle orientation and genome instability, resulting in reduced brain size (Silver et al. 2010) (**Figure 18**). Similarly, conditional haploinsufficiency for Rbm8a induce microcephaly due to depletion of

progenitors and dramatic apoptosis especially of neurons (Mao et al. 2015). Rbm8a mutant embryos show vigorous neuron production and faster cell cycle exit of progenitors. The phenotypic similarities induced by Magoh and Rbm8a haploinsufficiency support the fact that Magoh and Rbm8a exist as a heterodimer as part of the EJC or not. Apart from this, Magoh has been shown to regulate the proliferation and expansion of melanocytes derived from neural crest (Silver et al. 2013). The peripheral EJC component Upf1, essential for NMD, is also expressed in the developing neocortex and promotes a stem cell state in primary cells (Lou et al. 2014). Copy number variations in several EJC components, including UPF3B, eIF4A3, Y14, and Magoh, are found in patients with intellectual disability and neuro-developmental disorders (Nguyen et al. 2013). Thus, the EJC, most likely in part via its role in NMD, is crucial for the proper expression of genes essential for neuronal activities.

Recently, it was shown that mitotic delay in neuronal progenitors alters the fates of radial glia progeny by producing substantially more apoptotic neurons and thus generating brain size disorders such as microcephaly (Pilaz et al. 2016). EJC core components Magoh, Y14 and eIF4A3 regulate mitosis by modulating mitotic spindle integrity, in part by regulating centrosome separation and duplication. Magoh genetically interacts with cell cycle regulators, cdc2 and cks2 in mammals (Inaki et al. 2011) suggesting the role of Magoh in the expression of cell cycle regulators and thus mitosis. Together, these results indicate that EJC is an essential regulator of stem cell maintenance and division, and thus affects brain development.



**Figure 18: Mutation in Magoh gene causes microcephaly and disrupts mitotic spindle orientation.** *Magoh* mutant mice have smaller brain size. The knockdown of *Magoh* disrupts spindle integrity and mitotic pole formation (Silver et al. 2010).

### 7.3: EJC in human diseases

Defects of EJC proteins expression is also linked to several human diseases. The genetic and biological analysis of null mutation in Y14 (RBM8A) gene postulated the hypothesis that Thrombocytopenia with Absent Radii (TAR) syndrome results from an insufficiency of the Y14 protein (Albers et al. 2012a). In the case of TAR syndrome, the combination of a rare deletion and a low frequency regulatory SNP reduces Y14 abundance. This results in decreased levels of Y14 in platelets resulting in a reduce number of platelets in the blood of patients. This was the first human disorder shown to be caused by deficiency in one EJC component. More recently, a noncoding expansion in eIF4A3 gene is at the origin of the Richieri-Costa-Pereira Syndrome, a craniofacial disorder associated with limb defects (Favaro et al. 2014). An expansion of non-coding repeats in the 5' untranslated region (UTR) of the eIF4A3 results in eIF4A3 deficiency which leads to abnormal development of most pharyngeal arches, thus having altered mandible and laryngeal morphogenesis. In addition to this, recent study reported that the heterozygous deletion in eIF4A3 gene is involved in intellectual disability and autism (Nguyen et al. 2013). Using RNA-Seq, the authors showed that the genome-wide consequence of dosage imbalance of

UPF2, UPF3A, SMG6, eIF4A3 and RNPS1 is likely to be the cause of neuro-developmental disorders.

These studies demonstrate a broader than expected involvement of EJC in normal human physiology and function. However, so far no study has investigated the molecular mechanism behind the direct connection of EJC with these physiological functions in specific cell types.

## 8.0: Development of mammalian Brain

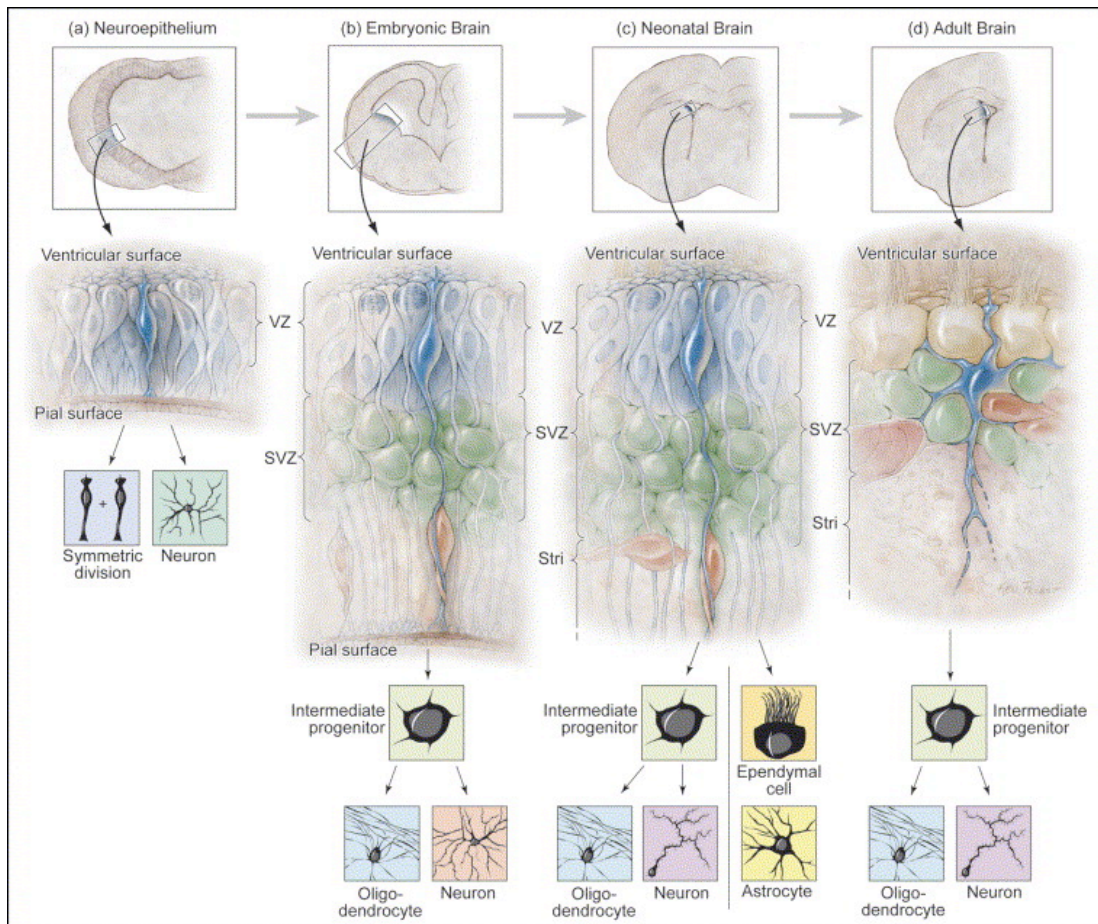
During my thesis, I chose to study the EJC in mouse neural stem cells (NSCs). So, in this last part of my introduction, I will present general aspects of brain development related to my model system: the differentiation of NSC into multiciliated ependymal cells. The central nervous system (CNS) develops from a small number of highly plastic cells that proliferate, acquire regional identities and produce different cell types. These cells have been defined as neural stem cells on the basis of their potential to generate multiple cell types (e.g. neurons and glia) and their ability to self-renew *in vitro*. In the brain, NSC work as a primary progenitor that maintains the potential to generate multiple cell types over long periods of time. In the brain, NSCs are specified in space and time, becoming spatially heterogeneous and generating a progressively restricted repertoire of cell types (Kriegstein & Alvarez-Buylla 2009). In the developing brain, different population of neuro-epithelial cells serve to generate a diversity of cell types. These NSCs serve as primary progenitor cells that give rise to Intermediate Progenitor cells (IPCs) which are transit amplifying cells. The description of these progenitors is as follows:

### 8.1: Primary progenitors

The CNS begins with a sheet of cells which expand and fold their edge to form the neural tube. The cells are primary progenitor cells known as neuroepithelial cells. This layer of neuroepithelium develops systematically from embryonic brain to neonatal brain till adult brain. Neuroepithelial cells are elongated and contact both the surfaces of developing brain: ventricular surface and pial surface (**Figure 19**). They divide at the ventricular surface, forming a

ventricular zone. A series of symmetric and asymmetric divisions take place to maintain the pool of neuroepithelial cells or its differentiation (Haubensak et al. 2004). The symmetric division of Neuroepithelial cells increases the pool of stem cells and later, the asymmetric divisions from this pool generate the intermediate progenitors. In the CNS, radial glia (RG) cells serve as neuronal progenitors in all regions (Anthony et al. 2004). This study showed that RG in all brain regions pass through a neurogenic stage of development and that most neurons are derived from these progenitors. Previous studies showed that RG could be the primary neuronal precursors in the neocortical ventricular zone and already suspected that these cells might serve as the major source of neurons throughout the CNS (Noctor et al. 2002). RG cells share many characteristics with neuroepithelial cells suggesting the direct transformation of neuroepithelial cells into RG cells (Malatesta et al. 2003; Götz & Huttner 2005). In mammals, RG cells disappear from brain soon after birth but a subset of RG cells has been shown to be persistent in adult songbirds (Alvarez-Buylla et al. 1990), lizards (García-Verdugo et al. 2002), turtles (Russo et al. 2004) and fish (Zupanc 2006).

RG cells and astrocytes in subventricular zone share many properties suggesting that they are derived from the same lineage (Tramontin et al. 2003). RG cells of the neonatal lateral ventricular wall occupy the same region as the astrocytic stem cells of adult subventricular zone (**Fig 22 d**). This was confirmed by a study labelling the RG cells and showing that they give rise to neurons, oligodendrocytes, ependymal cells as well as subventricular zone astrocytes that act as NSC both *in vivo* and *in vitro* (Merkle et al. 2004). These works identified RG cells as major intermediates between embryonic stem cells and CNS neurons and established their role as primary progenitors of CNS (Doetsch 2003; Goldman 2003; Götz & Barde 2005).



**Figure 19: Neural stem cells (NSCs) and their progeny in the developing forebrain.** The NSCs (shown in blue) of the lateral ventricular wall change their shape and produce different progeny as the brain develops. They begin as neuroepithelial cells and transform into radial glial cells, which mature into astrocyte-like cells. NSCs maintain contact with the ventricle, into which they project a primary cilium. (Adapted from Merkle and Alvarez-Buylla. 2006)

## 8.2: Intermediate progenitors

In the developing brain, the region above the ventricular zone (VZ) known as sub ventricular zone (SVZ), is appreciated as a major site of neurogenesis (Tarabykin et al. 2001; Nieto et al. 2004; Noctor et al. 2004; Noctor et al. 2007; Martínez-Cerdeño et al. 2006; Pontious et al. 2007). While primary progenitor cells persist in VZ mainly through asymmetric self-renewing divisions, a pool of intermediate progenitors divide symmetrically and amplify the number of cells produced by a given NSC in SVZ (Haubensak et al. 2004; Noctor et al. 2004; Miyata et al. 2004). Imaging the division of precursor cells within the SVZ has directly demonstrated that SVZ cells are derived from RG and subsequently divide to generate neurons (Haubensak et al. 2004; Miyata et al. 2004; Noctor et

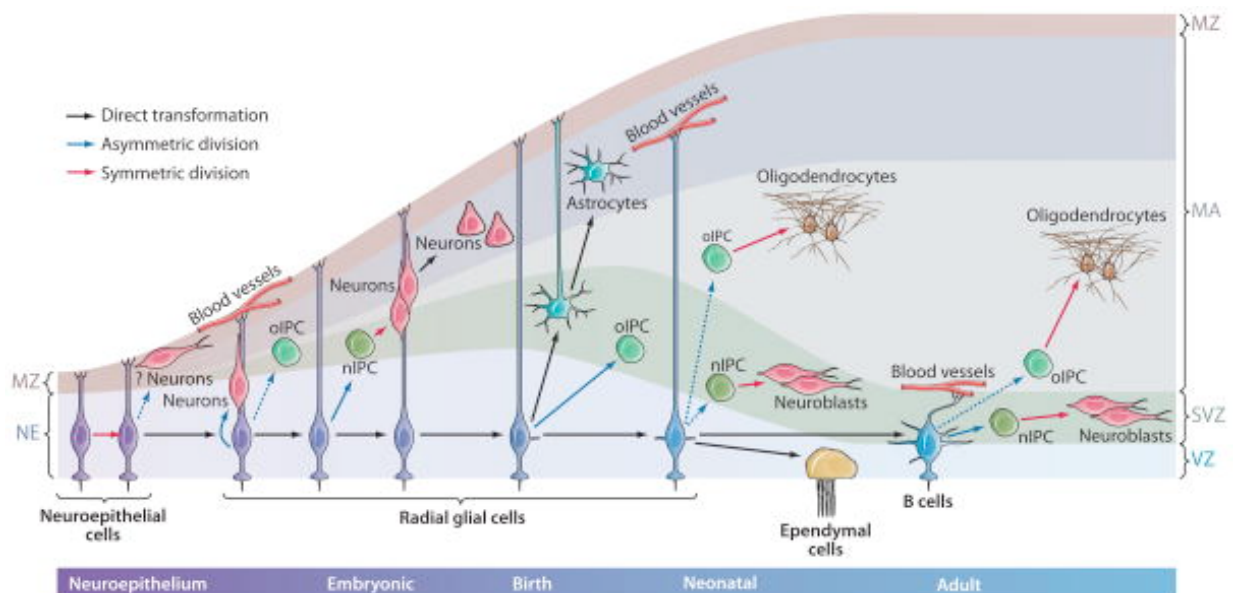
al. 2004). RG cells (or RGs) either generate daughter neurons directly or produce intermediate progenitors that generate neurons (nIPCs) (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004; Noctor et al. 2007). The role of the intermediate progenitor cells can be in determining the size of cerebral cortex. Evolutionarily, the pattern and amount of intermediate progenitor cells are responsible for the presence of the SVZ in the developing cortex and were a critical in the evolution of multilayered and gyrencephalic neocortex (Martínez-Cerdeño et al. 2006). Time-lapse imaging studies have given insight about the dynamic appearance and behavior of NSCs and their progeny. By specifically levelling neurogenic cells, Haubensak and colleagues showed that these cells are present in the ventricular zone and subventricular zone from the beginning of neurogenesis. Along with others, this study demonstrated that intermediate progenitors generate neurons via symmetric divisions in the subventricular zone from early developmental stages. (Haubensak et al. 2004; Noctor et al. 2004; Miyata et al. 2004)

### 8.3: Role of NSC in brain development

Mammalian NSCs produce different cell types at different points in development. Over the course of development, NSCs change their morphology and produce different progeny by changing their expression profile (Abramova et al. 2005). But how the neuroepithelial cells, serving the origin of all NSC, transform at molecular level is not very well understood yet. However, the general principles both of NSC identity and lineage are well characterized for the developing and adult rodent brain (**Figure 20**).

At the time when cortical neurogenesis begins - around embryonic day 9-10 (E9-10) - neuroepithelial cells start to transform into RG cells. RG and a subpopulation of adult astrocytes are characterized as founder cells for most of the neurogenic lineages. RG cells have been shown to function as primary precursors in the adult avian brain (Alvarez-Buylla & García-Verdugo 2002) and in the developing mammalian CNS, giving rise to neurons and/or glia, depending on the age and region of the brain analyzed (Noctor et al. 2002; Merkle et al. 2004).

During early development, neuroepithelial cells maintain their pool by symmetric divisions. As the developing brain epithelium thickens, neuroepithelial cells change their morphology and elongate to transform into RG cells. RG cells generate neurons directly through asymmetric division or indirectly via neuronal intermediate progenitor cells (nIPCs). Asymmetric division of RGs also give rise to oligodendrocytic intermediate progenitors (oIPCs) which generate oligodendrocytes in the cortex. At the end of embryonic development, while most of the RGs detach from the apical site and convert into astrocytes, a subpopulation of RGs retain apical contact and continue to function as NSC in neonatal brain. While many RGs continue to generate neurons and oligodendrocytes via nIPCs and oIPCs, some become committed to becoming or producing ependymal cells (Spassky et al. 2005). Ependymal cells form a layer on adult ventricle lining the interface between the brain parenchyma and the ventricular cavities (Del Bigio 1995). Below we discuss in more details the morphology and functions of ependymal cells in brain development.

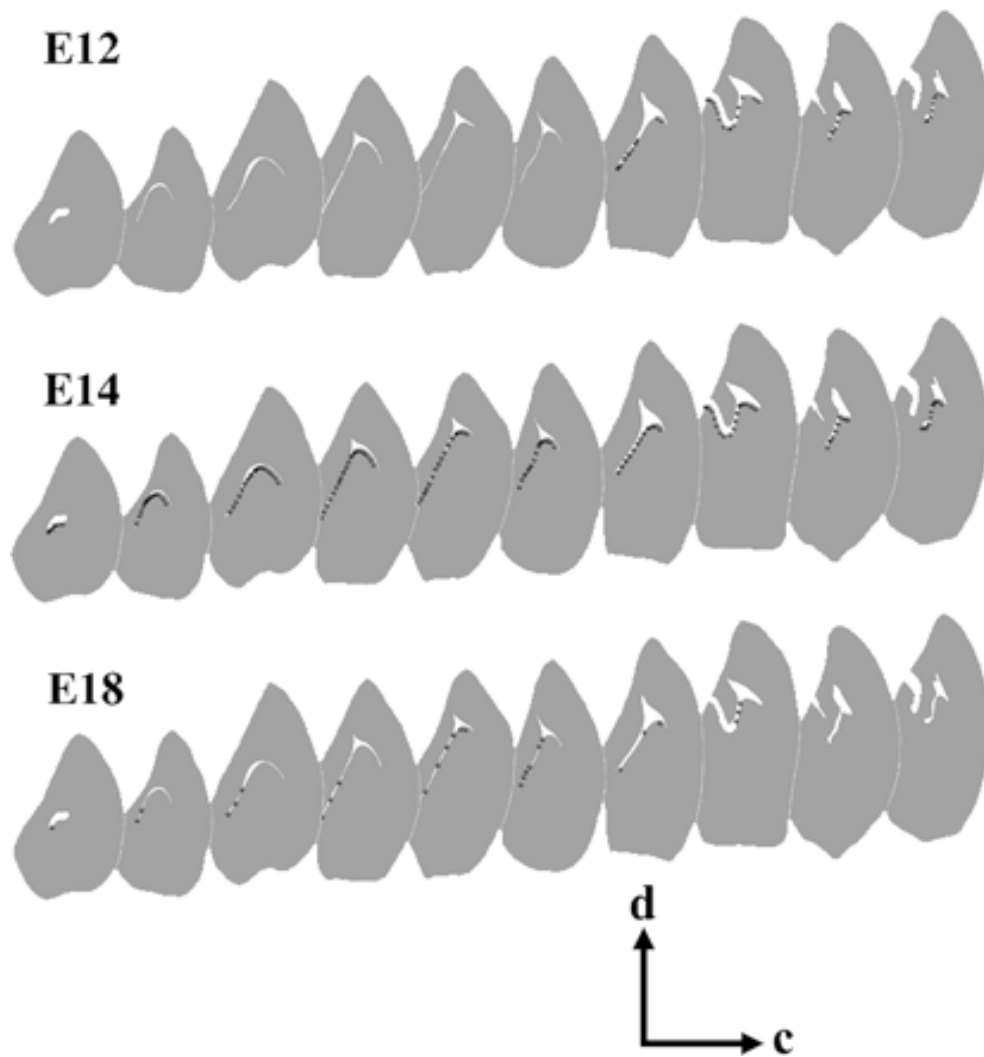


**Figure 20: Neural stem cells (NSCs) in development and in the adult.** Neuroepithelial cells in early development divide symmetrically to generate more neuroepithelial cells. Some neuroepithelial cells likely generate early neurons. As the developing brain epithelium thickens, neuroepithelial cells elongate and convert into radial glial (RG) cells. RG divide asymmetrically to generate neurons directly or indirectly through intermediate progenitor cells (nIPCs). (Adapted from Kriegstein and Alvarez-Buylla. 2009)



## 8.4: Ependymal cells

Ependymal cells are generated in neonatal brain where they form a layer to line the cerebral ventricle in adult brain. These cells are mostly cuboidal and multiciliated (Bleier 1971; Millhouse 1971). It was first presumed that ependymal cells may work as slowly proliferating neural stem cells (Johansson et al. 1999). Early studies tried to find if ependymal cells do have a characteristic of NSC by labelling all the dividing cells in newborn mice. They denoted that at least 2-20% of adult ependymal cells in the anterior lateral ventricle are labeled with [3H]thymidine, suggesting a sub-population of ependymal cells can divide in adult mouse forebrain (Kraus-Ruppert et al. 1975; Chauhan & Lewis 1979; Johansson et al. 1999). However, the identity of these labelled cells as ependymal cells was still a speculation as they were not characterized by high-resolution techniques. Also, later studies could not produce multipotent stem cells from purified ependymal cells (Chiasson et al. 1999; Laywell et al. 2000; Capela & Temple 2002; Doetsch et al. 2002) thus leaving the debate of NSC status of ependymal cells as an open question. To identify the origin of ependymal cells and investigate their proliferative capacity in adult mouse brain, Spassky et al labelled cells with [3H]thymidine and characterized dividing cells close to the walls of the lateral ventricle by light and electron microscopy (EM) (Spassky et al. 2005). This study determined the birth date of ependymal cells and showed that they derive from radial glial cells in the embryonic day 14-16 (E14-16) (**Figure 21**). Continuous labelling with [3H]thymidine for 6 weeks after birth showed no evidence of ependymal cells having any proliferative capacity. Thus they concluded that ependymal cells are post-mitotic and lack any function as NSC in adult brain.



**Figure 21: Birth of ependymal cells along the lateral ventricle.** Camera lucida drawings of BrdU and S100 $\beta$  double-labeled cells in coronal sections through adult lateral ventricular wall. Mice received BrdU as embryos at E12, E14, or E18. Double-labeled cells first arise in caudal regions at E12 and progressively appear in more rostral regions at E14 and E18. Dorsal (d) is up, and caudal (c) is right. (Adapted from Spassky et al. 2005)

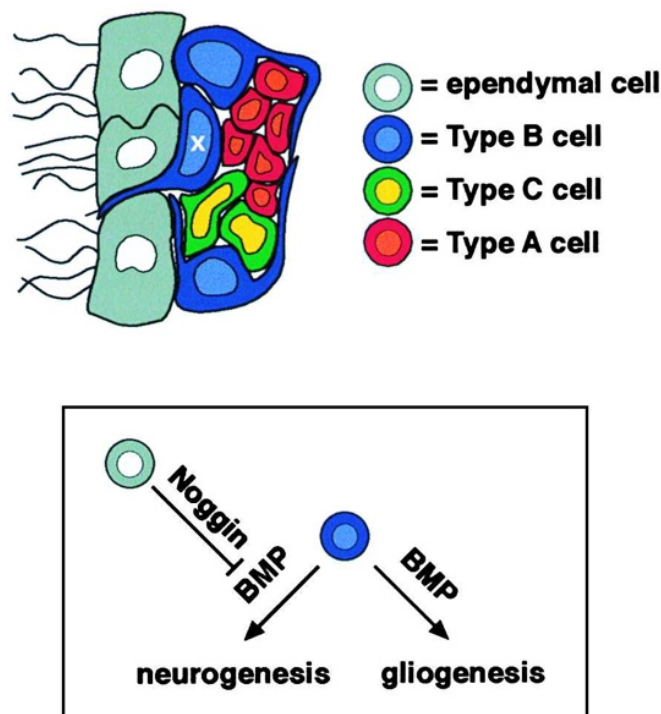
### 8.4.1: Physiological Functions of ependymal cells in brain

The ependyma has been widely considered as a barrier with poorly defined functions. However, the number of investigations that recognize the important roles for the neuroepithelium and mature ependyma in the development and physiology of the CNS is expanding (Jiménez et al. 2014). The knowledge of these roles furthers the understanding of the etiology of developmental and related diseases, such as hydrocephalus, and is useful for the

design of new therapeutic approaches. Here we discuss the known functions of ependymal cells in regulation of physiology of mammalian brain.

### 8.4.1.1: Regulation of neuronal niche

Ependymal cells regulate the SVZ neurogenic niche by promoting neurogenesis (Lim et al. 2000). In brain SVZ, closely associated ependymal cells and type B cells interact with each other to activate the neurogenic lineage of type B cells (Doetsch et al. 1999). Ependymal cells express Noggin which promotes the neuronal lineage of SVZ cells. Generally, type B cells express BMP which blocks the neurogenic pathway and directs type B cells to gliogenesis. Noggin produced by ependymal cells antagonizes type B cell BMP signaling, promoting neurogenesis of SVZ cells (**Figure 22**). Thus, close association of type B cells and ependymal is important for induction of neurogenesis event.



**Figure 22: Proposed role of ependymal cells in promoting the neuronal lineage of SVZ cells.** Type B cell BMP signaling blocks the neurogenic pathway, directing type B cells to gliogenesis (right pathway). Noggin produced by ependymal cells antagonizes type B cell BMP signaling, promoting neurogenesis of SVZ cells (left pathway). (Adapted from Lim et al. 2000)

#### **8.4.1.2: CSF maintenance**

The coordinated beating of cilia in ependymal cells establish a current cerebrospinal fluid (CSF) flow over the ventricular surface (Mirzadeh et al. 2010; Zappaterra & Lehtinen 2012). The ependymal ciliary beating forms chemorepulsive gradients in the SVZ that guide neuroblast migration in the adult brain. Thus by maintaining CSF flow, ependymal cells serve as important conveyors of directional information for neuronal migration (Sawamoto 2006). Several knockout studies selectively proved that ependymal malfunction leads to disturbances of CSF flow and hydrocephaly (Brody et al. 2000; Taulman et al. 2001; Kobayashi et al. 2002). Hydrocephalus is caused by excessive accumulation of CSF in the brain ventricles, which induces lethal compression of the brain parenchyma. In mouse, mutations leading to defects in cilia motility or in the direction of flow always lead to hydrocephalus (Ibañez-Tallon et al. 2004; Lehtreck et al. 2008; Sapiro et al. 2002; Town et al. 2008). Since flow of CSF is maintained by coordinated beating of ependymal cilia, defects in cilia functionality lead to subsequent hydrocephalus in mammalian brain.

#### **8.4.1.3: Metabolic protection of brain**

Adult mature ependyma is considered not only to regulate the transport of ions, small molecules, and water between the CSF and neuropil but also to serve as an important barrier function that protects neural tissue from a variety of potentially harmful substances (Bruni 1998). It was shown long back that ciliary beating of ependymal cells move cellular debris in the direction of bulk CSF flow, and optimize the dispersion of neural messengers in the CSF (Roth et al. 1985). The function of ependymal cells as a selective channel was later observed by feeding the cells with specific neoglycoproteins. In brain and in culture, mannose-containing neoglycoproteins are bound to ependymal cell cilia and penetrate rapidly the brain tissue (Kuchler et al. 1994). However, such phenomenon was not seen with glucose- or galactose-containing neoglycoprotein molecules. Thus ependymal cells selectively pass the CSF components to the brain and form a neuroprotective metabolic barrier at the

brain CSF interface (Del Bigio 2010). The mechanisms regulating these functions are still incompletely understood.

#### **8.4.1.4: Protection of brain from infections**

Ependymal cells also respond to infections and inflammatory conditions in brain. Theiler's virus and La Crosse virus cause ependymal cells to produce interferon alpha and beta (Delhaye et al. 2006). Interferon gamma in the CSF of mice induces ependymal production of the chemokines CXCL10 and CCL5 (Millward et al. 2007). Similarly, interferon administration causes mouse ependymal cells to produce 2',5' -oligoadenylate synthetase, which is capable of degrading viral RNA (Asada-Kubota et al. 1997). Under basal conditions, ependymal cells express caveolin-1 and -2 at the apical surface constitutively (Domínguez-Pinos et al. 2005); these are upregulated in inflammatory conditions (Kim et al. 2007; Shin et al. 2005). Bacterial endotoxin triggers the toll-like receptor 4 in ependymal cells among others (Chakravarty 2005), and ependymal cells upregulate several membrane-bound complement regulators in experimental meningitis (Canova et al. 2006). Together these studies confirm that ependyma play a role in the brain's response to infection, perhaps acting as a line of first defense.

#### **8.4.1.5: Repair of brain after stroke**

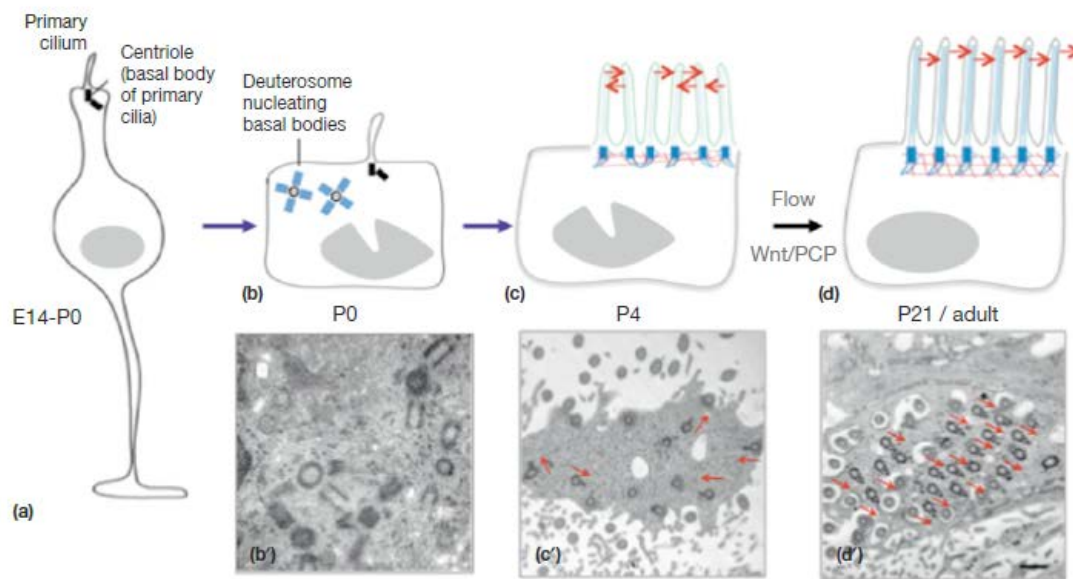
In the adult SVZ, astrocyte-like NSCs that express glial fibrillary acidic protein (GFAP), generate new neurons (Doetsch et al. 1999; Garcia et al. 2004). However, further studies suggest that ependymal cells may also contribute to neurogenesis after stroke (Carlén et al. 2009). In a recent study using mouse model, it was shown how ependymal cells transform their morphology and physiology to aid in neurogenesis post stroke (Young et al. 2013). In mouse experimental stroke model ependymal cells assumed features of reactive astrocytes post stroke. Similar to SVZ astrocytes, they not only robustly express de novo glial fibrillary acidic protein, but also acquire enlarge and extending long processes morphologically. Remarkably, stroke disrupted motile cilia planar cell polarity in ependymal cells similar to astrocytes. These studies confirm that

ependymal cells play a vital role in regulating the physiology of mammalian brain.

### **8.4.2: Ependymal differentiation in mammalian brain**

In lateral ventricle of rodent brain, ependymal cells undergo their final division between E14 and E16 (Spassky et al. 2005). However, the cilia appear on the walls of the ventricles only one week after the final division. During this period, radial glial cells go through intermediate stages as they transform into ependymal cells. (**Figure 23**). In first stage (stage A), bipolar radial glial cells extend long radial processes to both the pial and the ventricular surfaces. A single 9+0 cilium that projects into the ventricular lumen is located at the apical surface where the future patch of ependymal cilia will develop (Mirzadeh et al. 2010). These cells are still expressing RG specific marker RC2 and GLAST (Spassky et al. 2005). In stage B, the nuclei of the radial glial cells invaginate deeply, and multiple deuterosomes appear in the cytoplasm. Deuterosomes are spherical structures that act as nucleation centers for ciliary basal bodies in multiciliated cells. The expression profile of these cells differ from stage A as they start to express the ependymal cell marker S100 $\beta$  while the RG marker GLAST is still expressed. In stage C, future basal bodies migrate from deuterosomes toward the apical surface where they dock to the cell membrane and extend short, randomly oriented cilia. At this stage, the basal bodies are immature and dots of two microtubule triplets and a faint basal foot, which form the appendix of the basal body. This appendix is known to point in the direction of the effective stroke of the cilium. Later on, these electron-dense aggregates disappear, and all the basal bodies rotate to orient their ciliary beating in the direction of flow (Guirao et al. 2010). Although, not much is known about the molecular mechanisms underlying the differentiation of RG cells into multiciliated ependymal cells, the transcription factors FoxJ1 (also known as hepatocyte nuclear factor-3 and forkhead homolog 4) and RFX3 (regulatory factor X) appear to have a role in differentiation process. It was shown by various groups that FoxJ1 mutant mice fail to have differentiated ependymal and also exhibit defective apical migration of basal bodies and defects in the genesis of motile cilia (Brody et al. 2000; Jacquet et al. 2009; Stubbs et al. 2008; Yu et al.

2008; Paez-Gonzalez et al. 2011). RFX3 is actively participating in ependymal differentiation in brain. RFX3 bind FoxJ1 target the promoters of the genes encoding two axonemal dyneins involved in ciliary motility. Analysis of RFX3 mutants in mice suggested that RFX3 plays an important role in ciliary growth and in determining the beat frequency of cilia in developing multiciliated ependymal cells (El Zein et al. 2009). During early postnatal stages, immature ependymal cells extend short, randomly oriented cilia into the cerebral ventricles. As the ependymal cells mature, the cilia increase in length and start beating, producing a fluid flow that orients the basal bodies in the same direction. At mature stages (stage D, **Figure 23**), the basal bodies of ependymal cells show dark basal feet. The cilia get extended with fixed orientations into the ventricles. At this stage, deuterosomes and dots disappear and the planar polarized beating of the cilia directs the flow of CSF through the cerebral ventricles, which is crucial for brain development and function (Ibañez-Tallon et al. 2004; Sawamoto 2006).



**Figure 23: Differentiation of ependymal cells in brain from mono-ciliated radial glia cells to multi-ciliated ependymal cells .** (a) From E14 until birth, a subpopulation of radial glia is specified to enter the ependymal cell lineage. (b) A radial glial cell has entered the differentiation process, which is characterized by the appearance of deuterosomes, which are basal body nucleation centers in the cytoplasm. (b') A sagittal section of a differentiating cell, seen by transmission electron microscopy, showing four deuterosomes in the process of nucleating basal bodies. (c) Young ependymal cells: basal bodies are docked, cilia have not reached their final length and are beating in random directions. (d) Mature ependymal cells: cilia have reached their final length and have oriented their beating. (Adapted from Meunier et al. 2013)

### 8.4.3: Centriole amplification in ependymal differentiation

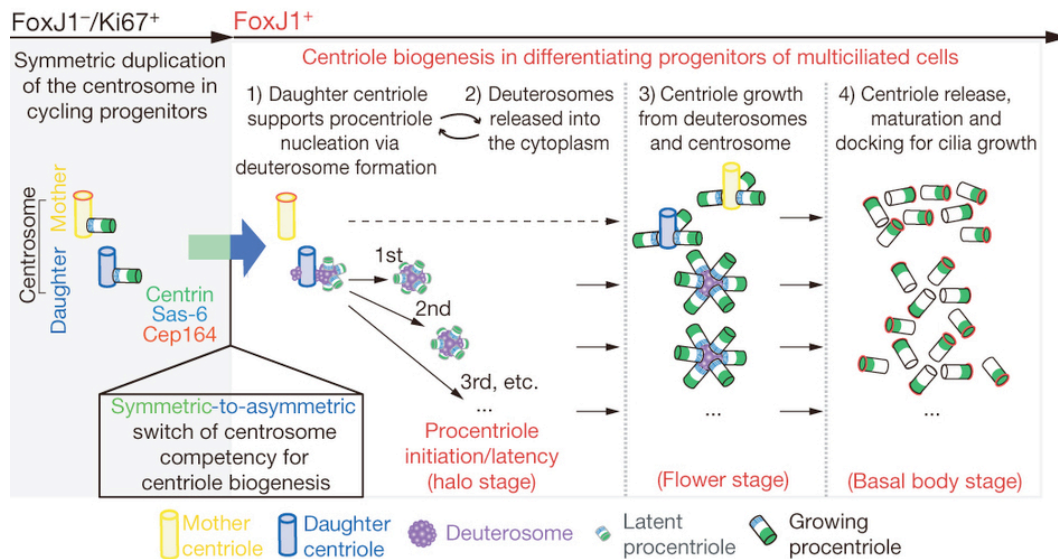
The semi-conservative mode of centrosome duplication in cycling cells gives rise to a centrosome composed of a mother and a newly formed daughter centriole. In this process both centrioles of a centrosome have an equivalent role and this symmetric duplication is critical for maintaining cell-division homeostasis (Marthiens et al. 2013; Ganem et al. 2009; Lingle et al. 2002). However, multiciliated cells do not follow this traditional mode of centrosome duplication. In multiciliated cells, centrosome give rise to more than hundreds of centrioles which form the basal body of motile cilia. A remarkable difference of this centriolar biogenesis from the conventional pathway is that the new centriole arise de novo in the cell around electron-dense structures called



deuterosomes (Klos Dehring et al. 2013; Sorokin 1968; H Zhao et al. 2013). The origin of these newly formed centrioles was unknown until recently. Al-Jord et al. used super-resolution light and electron microscopy to demonstrate how all new centrioles derive from the pre-existing progenitor cell centrosome through multiple rounds of procentriole seeding (Al Jord et al. 2014). They derived ependymal cells from transgenic mice expressing a GFP-tagged version of the distal core centriolar protein centrin2 (Cen2-GFP) and performed live imaging to decipher the centriolar biogenesis from RG cells to mature ependymal.

At the beginning of differentiation, the RG cells have one centrosome configuring one mother and daughter centriole. This centrosome forms the base of primary cilia (a cell antennae nucleated by the mother centriole (Singla & Reiter 2006)). When cells are at cycling stage, they do not express FoxJ1 but are KI67 positive (**Figure 24**). Molecular cues from the environment indicate the RG to leave the cycling stage and become quiescent. At this stage KI67 protein is not expressed and cells are ready to start the differentiation. FoxJ1 is a differentiation marker as it is exclusively expressed when RG cells are amplifying their centriolar number during differentiation. Centriole amplification occurs in the vicinity of this pre-existing centrosome. At the onset of centriole amplification, a cloud is formed around the pre-existing centrosome (**Figure 24**). This cloud later acquires a number of ring like structures in the cytoplasm, termed as 'halo'. Nascent 'halo' bud-out from the wall of the daughter centriole that detaches and accumulates in the nearby cytoplasm. The halos are Sas6 positive which is a procentriolar marker, suggesting that this stage corresponds to the accumulation of centriolar precursors which will eventually form multi-centrioles. These procentrioles are organized around spherical deuterosomes generated from the proximal segment of the daughter centriole. Thus centrosomal daughter centriole greatly amplifies procentrioles by generating intermediate structures, the deuterosomes. Immediately after the formation of the last halo/deuterosome at the daughter centriole, all the halos simultaneously transform into intensely fluorescent flower-like structures (**Figure 24**). At the flower stage, maturing procentrioles grow in a synchronized manner before the simultaneous detachment of procentrioles from both centrosome and deuterosome platforms. Eventually, these dissociated individual centrioles

migrated and dock to the apical membrane where they initiated the extension of the motile ciliary tufts (Meunier & Spassky 2016).



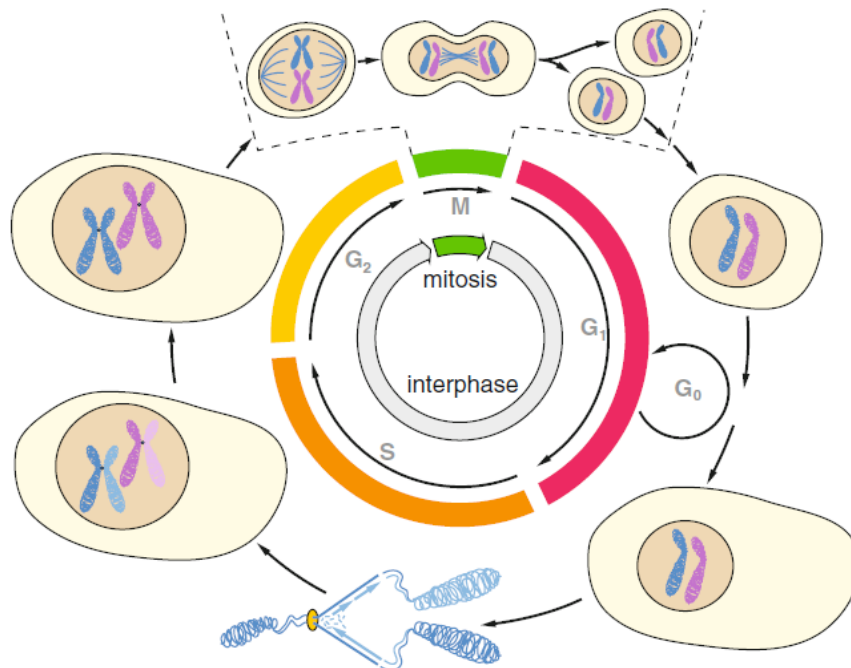
**Figure 24: Model of centriole amplification in multiciliated cells. 1,2)** Procentrioles are formed in the vicinity of daughter centriole. **3)** procentrioles mature in form of flowers **4)** Flowers mature and give rise to basal body stage. (Adapted from Al Jord et al. 2014)

## 9.0: Cell-cycle and cell-quiescence

A cell is the smallest unit of life that can replicate independently. The cell cycle, also called cell division cycle, describes a series of events that occur in a cell leading to its division and duplication.

In eukaryotes, the cell cycle is divided into three major periods: interphase, the mitotic (M) phase, and cytokinesis (**Figure 25**). During interphase, the cell grows, accumulating nutrients needed for mitosis, preparing it for cell division and duplicating its DNA (Bertoli et al. 2013). Typically interphase lasts for at least 90% of the total time required for the cell cycle. Interphase proceeds in three stages, G1, S, and G2, followed by the cycle of mitosis and cytokinesis. During G1 phase, the cell grows in size and synthesizes mRNA and histone proteins that are required for DNA synthesis (Foster et al. 2010). Once the required proteins and growth are complete, the cell enters the next phase of the cell cycle, S phase. The ensuing S phase starts when DNA replication commences; when it is completed, all of the chromosomes have

been replicated, i.e., each chromosome has two (sister) chromatids. During the mitotic phase, the cell splits itself into two distinct daughter cells. During the final stage, cytokinesis, the new cell is completely divided.



**Figure 25: Model of cell cycle progression through different phases.** The division cycle of a cell can be sectioned into different pre (S, G<sub>2</sub>) and postmitotic (G<sub>1</sub>, G<sub>0</sub>) interphases. Chromosome doubling and synthesis of other cell components are necessary before mitosis where the actual separation of a mother cell into two identical daughter cells occurs (M-phase). Cells that temporarily or reversibly escape the division cycle are in G<sub>0</sub> phase (Adapted from Behl and Ziegler, 2014).

Sometimes cells temporarily or reversibly escape the division cycle. These cells rest in quiescent state and the cell cycle stage is called G<sub>0</sub> phase (Pardee 1974). Such non-cycling cells in the G<sub>0</sub> phase can either reversibly re-enter the cell cycle and divide (Cheung & Rando 2013) or remain dormant, losing the potential to cycle and, in some cases, becoming senescent (Campisi 2005). A subset of tissue-specific adult stem cells persists in the quiescent state for prolonged periods of time (Li & Clevers 2010). Nerve cells, muscle cells and red blood cells (erythrocytes) are the most prominent examples of cells in G<sub>0</sub>-phase. Dysregulation and loss of quiescence often results in an imbalance in progenitor cell populations ultimately leading to stem cell depletion (Orford & Scadden 2008). As a result, tissue replenishment is affected during homeostasis and

following damage. Improved methods to analyze quiescence and to identify refined populations of NSCs will deepen our understanding of their intrinsic and extrinsic regulation, thereby providing vital knowledge that can be extrapolated to therapeutics for neurodevelopmental diseases.

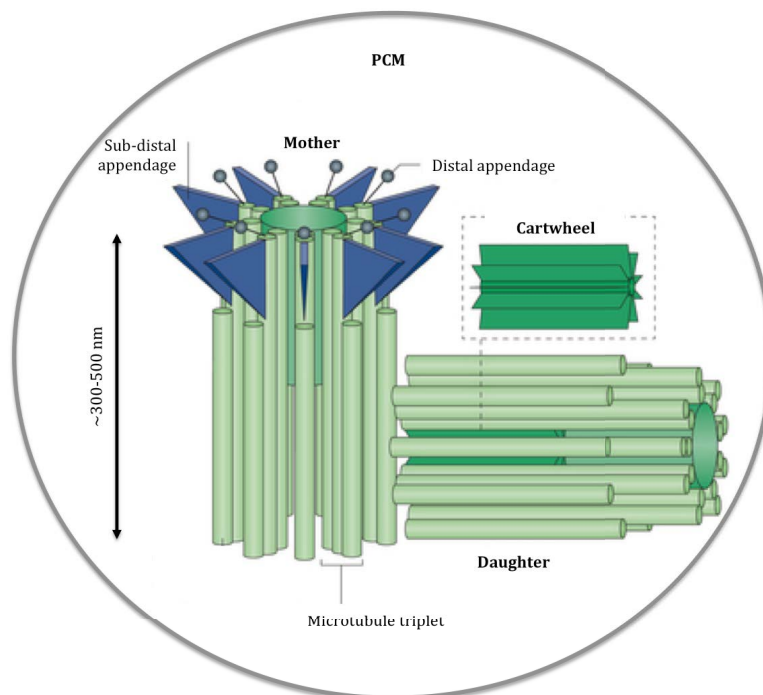
## 10.0: Centrosome

Centrosome is described as the major microtubule-organizing center of animal cells. Through its influence on the cytoskeleton it is involved in cell shape, polarity and motility. It also has a crucial function in cell division because it determines the poles of the mitotic spindle that segregate duplicated chromosomes between dividing cells. Moreover, centrioles have a critical role in assembling the primary cilium, which acts as a focal point for many signaling pathways. Like the nucleus, this organelle grows and replicates autonomously during the cell cycle, and a single copy is then segregated to each new daughter cell during division through its association with the mitotic spindle. Failure to obey this rule can result in disastrous consequences including multipolar mitotic spindles and chromosomal mis-segregation (Boveri, 1914; Boveri, 1929). Indeed, centrosome amplification is thought to be a significant cause of aneuploidy in cancer cells (Lingle et al. 1998; Pihan et al. 1998). The centrosome seems to have evolved only in the metazoan lineage of eukaryotic cells (Bornens and Azimzadeh, 2007). Fungi and plants lack centrosomes and therefore use other MTOC (microtubule-organizing center) structures to organize their microtubules (Schmit 2002; Jaspersen and Winey, 2004). Although the centrosome has a key role in efficient mitosis in animal cells, it is not essential in certain fly and flatworm species (Mahoney et al. 2006; Azimzadeh et al. 2012)). Here we discuss the known composition of centrosome and how centrosome dysfunction impacts on complex physiological processes.

### 10.1: Centrosome Composition

Centrosomes are formed by two major components: a pair of centrioles linked together through their proximal regions by peri-centriolar material (PCM); a matrix consisting in part of large coiled-coil proteins of the pericentrin family. (Azimzadeh & Bornens 2007; Bornens 2012; Bornens & Gönczy 2014). Centrioles are cylindrical structures that are ~450 nm in height and ~250 nm in diameter, and characterized by a radial arrangement of nine peripheral triplets of microtubule. In post-mitotic cells, the centrosome contains a mature centriole called the mother centriole and an immature centriole assembled during the previous cell cycle, the daughter centriole, which is about 80% the length of the mother centriole (Chrétien et al. 1997). Mother centrioles are distinguished by

two sets of nine appendages at their distal ends (Paintrand et al. 1992), which are thought to be required for anchoring microtubules at the centriole and for docking of centrioles at the plasma membrane during ciliogenesis. In most vertebrates, daughter centrioles are assembled around a central cartwheel structure, which sets the ninefold symmetric arrangement of microtubules. The mother centriole usually loses its central cartwheel and is often decorated with the distal (grey) and sub-distal (blue) appendages (**Figure 26**). Centrioles are critical in recruitment of surrounding PCM. Micro-injection of antibodies against poly-glutamylated tubulin, a post-translational modification that is characteristic of centriolar microtubules leads to the disappearance of the whole centrosome structure including centrioles and PCM (Bobinnec et al. 1998) suggesting that the modified tubulin domain immediately proximal, is involved in centriole stability. Related conclusions were reached in *Caenorhabditis elegans* embryos, where partial depletion of components that are essential for centriole assembly results not only in smaller centrioles but also in smaller PCM (Kirkham et al. 2003; Delattre et al. 2004).



**Figure 26:** Schematic illustration of vertebrate centriole structure: Daughter centrioles assembled around a central cartwheel structure (dark green) and mother centriole having its distal and sub-distal appendages is shown. PCM is shown as a circled matrix surrounding the centrioles. (Adapted and modified from Conduit et al. 2015).

Although the PCM appears largely amorphous when observed by electron microscopy, super-resolution light microscopy reveals concentric layers of organization around the centriole (Lawo et al. 2012; Sonnen et al. 2012; Fu &

Glover 2012; Mennella et al. 2012). During interphase, an inner layer of PCM proteins is present next to centriolar microtubules, and notably contains the  $\gamma$ -tubulin ring complex, which is fundamental for microtubule nucleation. PCM architecture changes towards mitosis, with an expansion of the inner layer and the addition of further components, together resulting in a mature centrosome with maximal MTOC activity (Fu and Glover, 2012; Mennella et al. 2012). PCM also reciprocally contributes to centriole biogenesis. Fewer centrioles are generated in *C. elegans* embryos that are depleted of  $\gamma$ -tubulin (Dammermann et al. 2012). Conversely, overexpression of the PCM component pericentrin (also known as kendrin) in Chinese hamster ovary (CHO) cells can increase centriole number (Loncarek et al. 2008). Together these considerations underscore the fact that centrioles and PCM are intimately linked in centrosome.

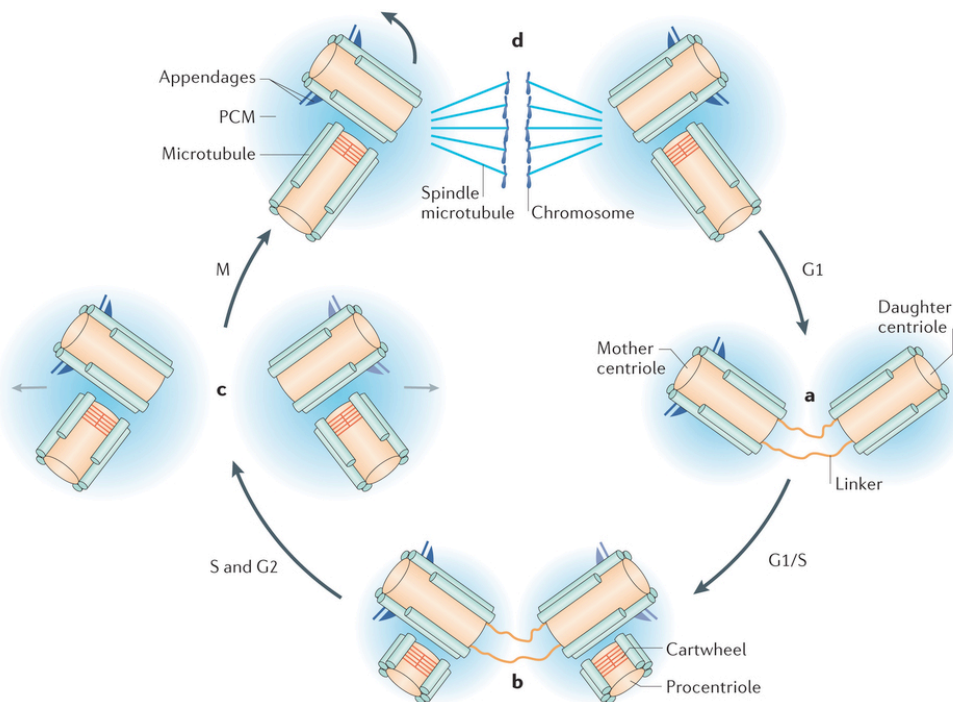
## 10.2: Centrosome duplication in cycling cells

In dividing cell, centrioles of a centrosome duplicate once per cycle, adjacent to a pre-existing centriole. The two centrioles present at G1 stage are distinct from one another with the mother centriole harboring distal and sub-distal appendages. At G1 stage of cell cycle, the two centrioles are close to each other connected through their proximal ends by a proteinaceous linker. The proximity of mother and daughter centriole effectively constitutes a single MTOC by harboring newly nucleated microtubules to the mother centriole (Piel et al. 2000).

The initiation of procentriole assembly appears to take place before or at the onset of S phase. Typically around the G1/S transition, one procentriole begins to assemble orthogonal to the proximal end of the mother centriole and the daughter centriole (**Figure 27b**). The two centrioles then elongate during the remainder of the cell cycle while remaining engaged with their neighbouring centriole. The molecular mechanisms underlying centriole assembly have been best studied in *C. elegans*, in which five proteins essential for centriole duplication have been identified. In human cells, these are the serine/threonine kinase Polo-like kinase 4 (PLK4), as well as the coiled-coil centrosomal protein of 192 kDa (CEP192), the spindle assembly abnormal protein 6 (SAS6), SAS4 and SCL-interrupting locus protein (STIL). PLK4, SAS6 and STIL have a particularly critical role, as their depletion prevents procentriole formation, whereas their overexpression results in supernumerary procentrioles (Gönczy 2012; Brito et al. 2012; Nigg & Stearns 2011).

Additional components that have not been identified in nematodes also contribute to procentriole assembly in human cells, including the interacting coiled-coil proteins CEP152, CEP63 and CEP52, which together form a torus around the proximal end of the centriole (Sonnen et al. 2012; Sonnen et al. 2013; Lukinavičius et al. 2013; Sir et al. 2011; Brown et al. 2013). The kinase PLK4 is recruited through association with CEP192 and CEP152 and triggers

procentriole formation from one location within this torus (Sonnen et al. 2012; Sonnen et al. 2013). Thereafter, nine SAS-6 homodimers assemble into a ‘cartwheel’ structure supposedly acting as a molecular scaffold for procentriole formation (Kitagawa et al. 2011; van Breugel et al. 2011). PLK4 phosphorylates STIL, thereby promoting its association with SAS-6 and thus procentriole formation (Ohta et al. 2014). STIL also interacts with the tubulin-binding protein CPAP, thus potentially bridging the cartwheel with peripheral centriolar microtubules (Hatzopoulos et al. 2013; Cottee et al. 2013; Zheng et al. 2014). Distinct phases of the centrosome cycle have been identified. At the G2/M transition, the proteinaceous linker connecting centrioles is removed following activation of the serine/threonine kinase NEK2, in a step referred to as centrosome disjunction (Agirican et al. 2014) (**Figure 27c**). Second, the two centrosomes separate along the nuclear envelope before nuclear envelope breakdown (NEBD), in a step dubbed centrosome separation (**Figure 27c**). This process is thought to be driven principally by kinesin 5, a tetrameric plus-end-directed motor that pushes apart overlapping microtubules located between the centrosomes (Blangy et al. 1995; Tanenbaum & Medema 2010). Centrosome separation allows the formation of a bipolar spindle, which ensures faithful segregation of the genetic material to daughter cells (**Figure 27d**). During mitosis, the centriole and procentriole disengage from one another within each centrosome, so that each daughter cell inherits two centriolar cylinders, thus completing the duplication cycle. Centriole–procentriole engagement normally prevents further procentriole assembly (M. F. B. Tsou & Stearns 2006; Firat-Karalar & Stearns 2014). Thus centrosome duplication cycle is orchestrated to ensure that the centrosome duplicates once and only once per cell cycle.



**Figure 27: Schematic representation of the centrosome duplication cycle:** **a|** The two centrioles present in G1 phase are distinct from one another, with the mother centriole harboring distal and sub-distal appendages. **b|** Approximately at the G1/S transition, the cartwheel seeds the formation of a procentriole orthogonal to the proximal end of each parental centriole; the procentriole elongates thereafter. **c|** Towards the end of G2 phase, the proteinaceous linker is removed and the two centrosomes separate from one another. **d|** The fully separated centrosomes assemble a bipolar spindle during mitosis. (Adapted from (Gonczy 2015))

## 10.3: Centrosome in post-mitotic cells

### 10.3.1: Formation of primary cilia

When a cell exits the cell cycle, the mother centriole of the centrosome migrates and docks in close proximity to the apical plasma membrane of the cell and converts into a basal body (BB) for primary cilium formation (Vorobjev & Chentsov YuS 1982; Tateishi et al. 2013). The basal body (BB) forms the base of the cilium and arises from the mother centriole of the centrosome (Sorokin 1962; Vorobjev & Chentsov YuS 1982). Primary cilium formation is a dynamic process that can be reverted under mitogenic conditions. Cilia typically begin to form during the G1 or G0 phase of the cell cycle and begin to disassemble as cells re-enter the cell cycle (Tucker et al. 1979a; Tucker et al. 1979b). Two pathways are involved in this process, namely Nek2–Kif24 and AuroraA–HDAC6 (Kim et al. 2015). The mother centriole of the centrosome serves as a physical template for human cilia formation (reviewed in Bornens 2012).

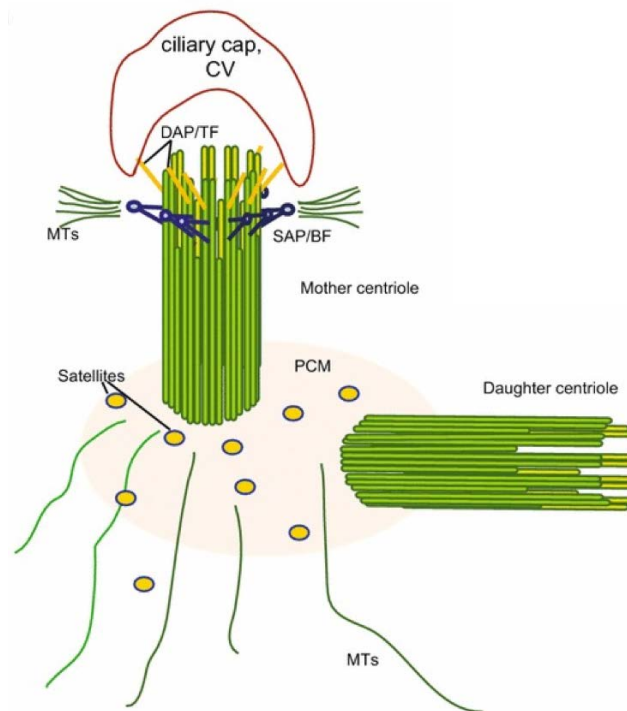
During ciliogenesis, the centriolar appendages mature into transition fibers (Sorokin 1968). Distal appendages of mother centriole (DAPs) dock BBs at the plasma membrane and initiate ciliogenesis (Graser et al. 2007; Tanos et al. 2013; Mikule et al. 2007). DAPs initiate ciliogenesis by mediating the formation of the ciliary vesicle through Rab GTPases (Lu et al. 2015) and IFT20 (**Figure 28**). During DAP assembly, Cep83 is required for recruitment of multiple DAP proteins including Cep89 (Cep123), SCLT2, FBF1, and Cep164 (Tanos et al. 2013). Cep164 is a multifunctional DAP protein that orchestrates several events during early ciliogenesis. Cep164 mediates not only the BB-membrane docking step, but also coordinates ciliogenesis. In addition to Cep164, Cep89 (Cep123) participates in ciliary vesicle formation (Sillibourne et al. 2013). Consistent with a DAP role in ciliogenesis the evidence that mutations in DAP proteins such as C2cd3 (Hoover et al. 2008), Cep83 (Failler et al. 2014), Cep164 (Chaki et al. 2012), and SCLT1 (Adly et al. 2014) result in ciliopathies. Sub-distal appendages (SAPs; also called “basal feet” in cilia, **Figure 28**) are involved in MT anchoring ((Delgehyr et al. 2005)).

Pericentriolar satellites are dynamic dynein and kinesin-driven granules located within and around the pericentriolar material (PCM) (Zimmerman &



Doxsey 2000; Tollenare et al. 2015). Satellites consist of dozens of proteins required for cilia formation (Tollenare et al. 2015). However, the precise role of satellites in modulating the ciliogenesis is still not known. Recent evidences suggest that the satellite proteins, Cep290 and PCM1, are involved in ciliogenesis through modulating Rab8 recruitment to BBs (Kim et al. 2008).

Small ciliary vesicles accumulate at DAPs of the BB before primary cilia formation and later fuse to form a ciliary vesicle “cap” (**Figure 28**). The BB and associated cap is thought to move up to and fuse with the plasma membrane allowing the cilium to grow and extend out into the extracellular space. Once the cilium is fully made, a ciliary pit is created via an interaction between the ciliary vesicle membrane and DAPs (Moser et al. 2009). Upon exit from G0, primary cilia begin to disassemble as cells re-enter the cell cycle (Tucker et al. 1979a; Tucker et al. 1979b). Cilia components and cilia membrane are inherited by the mother centriole at the oldest spindle pole during cell division.



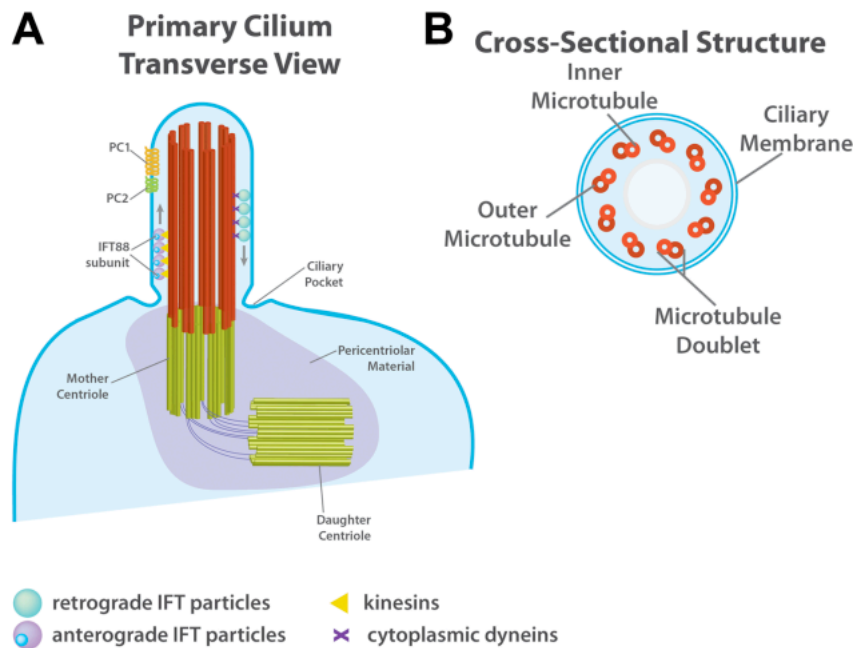
**Figure 28: Structure of the human basal body (BB) at initial step of ciliogenesis:** A Side view of the BB. DAP/TF, distal appendages/transition fibers, SAP/BF, sub-distal appendages/basal feet, CV, ciliary vesicle. (Adapted from (Vertii et al. 2016))

These studies imply that the centrosome-associated ciliary membrane functions in temporal control of ciliogenesis in growth arrested cells. The precise control of assembly of cilia over space and time highlights the complexity of their temporal regulation with fundamental role in corticogenesis. These events might be in regulation with the precise control of post-transcriptional events such as transcriptional silencing/activation at a precise time point of the cell cycle.

Deciphering the role of post-transcriptional control would enable us to characterize more precisely the mechanisms behind cilia biogenesis and ciliopathy.

### **10.3.1.1: Cilia structure and functions**

The primary cilium is an organelle present on most vertebrate cell types at some point during the cell cycle. It is thought to be an important sensory organelle, coordinating a multitude of critical cell processes including cell proliferation, differentiation and cell migration (Singla & Reiter 2006; Christensen et al. 2008; Praetorius & Spring 2005; Zhou 2009). Primary cilia are composed of nine microtubule doublets arranged concentrically in a 9+0 configuration, and they are generally considered non-motile, with the exception of specialized nodal cilia (Nonaka et al. 1998) (**Figure 29A** and **29B**). In contrast, motile cilia structures present in epithelial mucociliary systems such as the airway express a 9+2 microtubule configuration, with a central pair of microtubules in the center of the ciliary axoneme. In addition to structural disparities between the two classes of cilia, nonmotile primary cilia are typically thought to lack the axonemal dynein motor proteins that facilitate the beating motion of motile cilia (Satir 1989). Primary cilia typically localize to the apical cell surface of epithelial cell types and cells grown in monolayer culture. Their structure is contained within a ciliary membrane contiguous with the cell membrane (Farnum & Wilsman 2011). Primary cilia emanate from the mother centriole, anchoring the basal body and docking just below the surface of the cell membrane (Sorokin 1962). As described above, the presence of the primary cilium is intimately associated with the cell cycle, as they are most frequently expressed during the G<sub>0</sub> phase, but they can be observed any time during interphase and are normally assembled during the G<sub>1</sub> phase (Goto et al. 2013).



**Figure 29: Schematic of the primary cilium.** Transverse view of the cilium structure showing the ciliary organization along the ciliary axoneme with the cilium emanating from the mother centriole (A). Cross-sectional view of the cilium showing the microtubule doublet arrangement in the 9 + 0 configuration (B). Abbreviations: IFT, intraflagellar transport; PC1, polycystin-1; PC2, polycystin-2. (Adapted from Bodle and Lobo, 2015)

In addition to their cell cycle link, primary cilium has been identified as a chemo-mechanosensory organelle in a variety of cell types, including those derived from bone, kidney, cardiovascular, and neural tissue (Bodle and Lobo, 2015). The function of the primary cilium is not limited to basic cell physiology, and its dysfunction has been implicated in a number of diseases. Particular cilia-associated genes have been linked to a number of ciliopathies including polycystin-1 (PKD1), polycystin-2 (PKD2), intraflagellar transport protein-88 (IFT88 or Polaris), kinesin like protein (KIF3a), and inversin (INV) (Bodle & Lobo 2016).

### 10.3.1.2: Primary cilia in neurodevelopmental disorders

Defects in the primary cilia have been assigned to a wide array of clinical phenotypes in major body system, including the brain, eyes, liver, kidneys, skeleton and limbs (Hildebrandt et al. 2011). Neurological defects are a common finding in many ciliopathies, highlighting a critical role for primary cilia in brain development. In CNS defects, cilia-associated proteins are shown to be involved in diverse neurological syndromes such as Joubert syndrome, Bardet-Biedl syndrome, Orofaciodigital syndrome and Hydrolethalus syndrome (Valente et al. 2014). Primary cilia have also been implicated in adult neurogenesis, suggesting that ongoing defects in neuronal proliferation and maturation could contribute to the cognitive impairment seen in many patients with ciliopathies. Several

studies have shown that cilia are required for normal progenitor-cell proliferation in the hippocampal dentate gyrus, during a SHH-dependent postnatal growth spurt that expands this structure (Breunig et al. 2008; Han et al. 2008). In addition to deleterious effects on postnatal development, lack of primary cilia in adult progenitor cells results in a reduction in hippocampal neurogenesis and a deficit in spatial learning in mice (Amador-Arjona et al. 2011). Interestingly, primary cilia have also been demonstrated to be critical for synapse formation in adult-born hippocampal neurons from newborn mice (Kumamoto et al. 2012), indicating that these organelles are required for successful integration of adult-born neurons into existing brain circuitry. The deletion of primary cilia is associated with increased Wnt- $\beta$ -catenin signaling (Kumamoto et al. 2012), suggesting that regulation of this pathway by cilia might explain these observations.

These studies have highlighted the central role of primary cilia in a wide spectrum of neurodevelopmental diseases. However, the mechanisms that underlie neurological malformations associated with ciliopathies are partially understood and better understanding of cilia structure and functions would represent promising approaches to the development of effective treatments for ciliopathies.

### **10.3.2: Centrosome amplification in post-mitotic cells**

The canonical model of centrosome duplication accounts for centriole number control in most somatic cycling animal cells. However, specialized cell types such as multi-ciliated epithelial cells form hundred of centrioles near-simultaneously during differentiation. In multiciliated cells of vertebrates, such as mammalian tracheal epithelial cells (Vladar & Stearns 2007), most of the genes for known centriole components and duplication factors are strongly upregulated. Although some centrioles form around the pre-existing centrioles, the majorities are assembled adjacent to deuterosomes, a structure unique to multi-ciliated cells that has no morphological resemblance to a centriole. The molecular pathways underlying centriole duplication in multiciliated vertebrate cells has recently been characterized (Huijie Zhao et al. 2013; Klos Dehring et al. 2013). The centriole amplification is controlled by two duplicated genes, Cep63 and Deup1. Cep63 regulates mother-centriole-dependent centriole duplication. Deup1 governs deuterosome assembly to mediate large-scale *de novo* centriole biogenesis (Zhao et al. 2013). Deup1 localizes to deuterosomes and depletion of Deup1 causes loss of deuterosomes, and greatly reduced centriole number. CCDC78 is another protein that localizes to deuterosomes (Klos Dehring et al. 2013) and is important for centriole assembly. Similarly to Cep63, Deup1 binds to Cep152 and then recruits Plk4 to activate centriole biogenesis. Mother centriole-mediated centriole duplication in cycling cells is generally restricted to S phase. However, deuterosome-mediated centriole amplification occurs in

terminally differentiated multiciliated cells (G1/0), suggesting that even though many of the critical regulators seem conserved, the cell cycle regulation has been modified (Klos Dehring et al. 2013). It is supposed that multi-ciliated cells achieve the ability to form hundreds of centrioles simultaneously by a combination of a transcriptional program that massively upregulates centriole components and the expression of specific proteins that modify the duplication pathway such that it becomes independent of cell cycle progression and of the requirement for a centriole to be the site of duplication (Firat-Karalar & Stearns 2014). The detailed characteristics of centriole amplification in ependymal differentiation is discussed in **section 8.4.3** of this thesis.

## 10.4: Centrosome functions

Centrosomes are referred as MTOCs by their virtue of organize and nucleate microtubules. Features associated with MTOCs include organization of mitotic spindles, formation of primary cilia, progression through cytokinesis, and self-duplication once per cell cycle. MTOCs play new and unexpected roles in several other processes including cell cycle control, cytokinesis, and responses to cellular stress (Kellogg et al. 1994; Arquint et al. 2014; Rieder & Faruki 2001; Doxsey et al. 2005). During cell cycle, centrosome duplicates before mitosis phase and form the poles of the bipolar mitotic spindle. It is known since decades that precise regulation of centrosomal duplication ensures the spindle bipolarity thus ensuring each new daughter cell inherits one set of chromosome and a single centrosome (Rappaport 1961). However, recent studies demonstrate that centrosomes are not the only drivers of spindle assembly and polarity as higher plant cells and oocytes of many animals do not require centrosome for the formation of bipolar mitotic and meiotic spindles (Dumont & Desai 2012; Masoud et al. 2013).

In vertebrates, loss of centrosomes is linked to developmental defects and cell death. Mouse embryos lacking centrosomes show altered growth and a dramatic increase in apoptosis (Bazzi & Anderson 2014; David et al. 2014). Prolonged spindle assembly due to absence of centrosomes is responsible for the previously undescribed p53-dependent cell death pathway in the rapidly dividing cells of the mouse embryo (Bazzi and Anderson, 2014). Mouse embryos lacking centrosome and p53 together do not show microcephaly and alteration in chromosome segregation or cell proliferation suggesting that p53 has a proapoptotic role (Insolera et al. 2014). Similarly, centrosome loss also activates p53 in cultured vertebrate cells (Izquierdo et al. 2014; Wong et al. 2015; Lambrus et al. 2015) in order to protect against genome instability following centriole duplication failure. Inactivation of centrosome duplication by inhibition of polo-like kinase 4 (PLK4) triggers p53-dependent cell cycle arrest in cultured animal cells and growth delay accompanied with increased chromosome mis-segregation in p53 deficient cancer cells. Together these studies indicate that

most vertebrate cells can segregate their chromosomes without centrosome however, the loss of centrosome eventually triggers the specific p53-dependent arrest of cell cycle or cell death. However, the precise mechanism of activation of p53-dependent pathway in the absence of centrosome is still not known. In addition to preventing the proliferation of centrosome-less cells, activation of p53-dependent pathway may also serve physiological functions by acting as a barrier to restrict cell cycle reentry in case of loss of centrosome. Cancer cells continue to proliferate without centrosomes, an aspect which may be implicated for therapeutic purposes. The differential effect of centrosome removal on normal cells and cancer cells suggests the possibility of combining centrosome depletion with other perturbations to selectively target dividing cancer cells.

#### **10.4.1: Centrosome functions in cell fate**

During brain development, neural precursor cells migrate along radial glial (RG) fibers to populate the neocortex. The differential behavior of progenitors and their differentiating progeny is essential for neocortical development. During neurogenesis in mice RG cells predominantly undergo asymmetric division to self-renew while simultaneously giving rise either directly to a neuron, or to an intermediate progenitor cell which subsequently divides symmetrically to produce neurons (Miyata et al. 2004; Noctor et al. 2004; Chenn & McConnell 1995; Noctor et al. 2008). Whereas differentiating progeny progressively migrate away from the VZ to form the cortical plate (CP), renewing RG progenitors remain in the VZ for subsequent divisions. The distinct migratory behavior of RG progenitors and their differentiating progeny is fundamental to the proper development of the mammalian neocortex; however, little is known about the basis of these behavioral differences. Centrosome has been shown to be important in regulation of cell migration during neurogenesis (Xie et al. 2007; Tsai et al. 2007; Solecki et al. 2004). During each cell cycle, the centrosome replicates once in a semi-conservative manner (M.-F. B. Tsou & Stearns 2006) resulting in the formation of two centrosomes: one of which retains the original old mother centriole while the other receives the new mother centriole (Meraldi & Nigg 2002; Delattre & Gönczy 2004). Many studies indicate a critical role for the differential behavior of centrosomes with differently aged mother centrioles in asymmetric division of the progenitor/stem cells (Cabernard & Doe 2007; Spradling & Zheng 2007; Yamashita & Fuller 2008; Gonzalez 2007), although it remained unclear whether centrosome asymmetry affects the behavior and development of the progenitor/stem cells and their differentiating daughter cells. It was shown that asymmetric centrosome inheritance regulates the differential behavior of renewing progenitors and their differentiating progeny in the embryonic mouse neocortex (Wang et al. 2009). During peak phases of neurogenesis, the centrosome retaining the old mother centriole stays in the VZ and is preferentially inherited by RG progenitors, whereas the centrosome containing

the new mother centriole mostly leaves the VZ and is largely associated with differentiating cells. The preferential inheritance of a centrosome containing the mature mother centriole is required for the maintenance of radial glia progenitors in the proliferative VZ of the developing neocortex. These results indicate that preferential inheritance of the centrosome with the mature older mother centriole is required for maintaining radial glia progenitors in the developing mammalian neocortex.

It is worth noting that centrosomes with differently aged mother centrioles differ in their protein composition and thereby in their biophysical properties, such as microtubule anchorage activity (Bornens 2002; Delattre & Gönczy 2004) and the capability to mediate ciliogenesis (Anderson & Stearns 2009; Vorobjev & Chentsov YuS 1982; Preble et al. 2000). The asymmetric inheritance of centrosomes with distinct biophysical properties may thereby differentially regulate the behavior and development of the daughter cells that receive them. For example, given that primary cilia have essential roles in a number of signal transduction pathways, the asynchrony in cilium formation could differentially influence the ability of the two daughter cells to respond to environmental signals and thereby their behavior and fate specification. Furthermore, the strong microtubule anchorage activity associated with the centrosome retaining the older mother centriole would facilitate its anchorage to a specific site (for example, the VZ surface), thereby tethering the cell that inherits it.

#### **10.4.2: Centrosomes in human diseases**

Several studies have highlighted the link between centrosome defects and human diseases. Centrosome abnormalities promote chromosomal instability (CIN) that encourages tumorigenesis in humans, which are common phenomenon related in human cancers (Lingle et al. 1998; Lingle et al. 2002; Pihan et al. 1998; Vitre & Cleveland 2012). However, in mouse models aneuploidy acts both oncogenically and as a tumor suppressor. High levels of CIN potentially suppresses cancer while low levels of CIN potentially promotes cancer (Weaver et al. 2007; Schwartzman et al. 2010). Centrosome amplification generally does not lead to large-scale CIN. Nonetheless it could contribute to low the level of CIN, which may promote cancer more effectively. As described above, centrosomal defects share a close relationship with tumor suppressor gene p53. In human cells, a proportion of p53 localizes at centrosome and p53 loss often leads to centrosome amplification (Fukasawa 2007). It is still not known why do centrosomal defects persist in human cancer cells although in normal vertebrate cells centrosome anomalies activate p53 and block cell proliferation. Recently, it was shown that centrosome amplification can increase the metastatic potential of cancer cells in 3D tissue models (Godinho & Pellman 2014). Therefore, centrosome defects might actively promote tumor progression in humans by

promoting metastasis. However, a strong genetic link between centrosomes and human cancer is still lacking.

Additionally, centrosome defects have been directly associated with primary autosomal recessive microcephaly (MCPH) and primordial dwarfism (Thornton & Woods 2009; Megraw et al. 2011; Barbelanne & Tsang 2014; Chavali et al. 2014). Remarkably, many of the patients with MCPH or primordial dwarfism have mutations in a gene that encodes one of the proteins essential for centrosome assembly. These observations strongly suggest that centrosomes have an important role in human development.

## 11.0: Questions Asked

The interactions of mRNAs with its binding proteins (RBPs) dictate its fate in biological processes. EJC perfectly illustrates the importance of RBPs in determining the future of mRNA. Deposited in the nucleus, this complex conveys the splicing marks to many downstream processes. Owing to its role in mRNA maturation, export, translation but also the degradation of the transcripts, the EJC is distinguished by its ability to link nuclear and cytoplasmic processes. In summary, we know well the EJC from a « fundamental » point of view: structure, assembly, composition thanks to biochemical studies performed *in vitro* and from cellular lysate from basic cell lines. On the other side, accumulating evidences show that EJC play important physiological roles as demonstrated by its direct link to developmental defects and human disorders. However, it exists a real gap between the two. It is now necessary to determine EJC targets and functions in more physiological conditions. We chose to employ several tools that my group has developed to study EJC in primary culture of NSC.

The work carried out during this Ph.D. attempted to better characterize the role of EJC in regulation of gene pool essential of development of NSC by answering following questions.

- What is the subcellular localization of EJC core proteins in NSC? Is there a relation between distinct phases of NSC development and EJC localization? In other cell types having similar physiological state, do EJCs maintain their biological characteristic?
- What is the identity of the EJC bound transcripts in physiological cells, particularly in NSC? Since NSCs differentiation progress with rather



complex but distinct phases, do EJC-bound transcripts relate to specific functions of in conjugation with cell-stage?

- One of the enigmas in EJC functions is whether EJCs participate in mechanized transport of other mRNAs as seen for *Oscar* mRNA in *Drosophila*? Also, do EJCs functionally regulate the localization of some transcripts in higher eukaryotes? If so, what is the functional impact of this localization on the physiology of the cell?

By searching to answer at least some of these questions, I aim to clarify the role of EJC in brain development and other physiological disorders.

# **RESULTS**

## **(Part 1)**

# **ARTICLE**

# **“Enrichment of Exon Junction Complexes at centrosome in quiescent cells over differentiation”**

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## **INTRODUCTION**

Eukaryotic mRNAs are packed into RNP (ribonucleoprotein) particles composed of a myriad of RNA binding proteins (RBPs). Each mRNP is unique and evolves during the successive stages of mRNA life cycle (Müller-McNicoll & Neugebauer 2013). The exon junction complex (EJC) deposited in the vicinity of exonic junctions as a consequence of splicing plays an important role in connecting post-transcriptional steps (Le Hir et al. 2016). The EJC is a multi-protein complex organized around a stable tetrameric core complex made of the proteins eIF4A3, MAGOH, Y14 and MLN51 (Ballut et al. 2005; Tange et al. 2005; Andersen et al. 2006; Bono et al. 2006). This core complex is not pre-assembled and its assembly is tightly coupled to the splicing reaction that brings together the different components (Gehring et al. 2009; Barbosa et al. 2012; Steckelberg et al. 2012; Dagueuet et al. 2012). Recent evidences by transcriptomic approaches revealed that the presence of EJC varies between exonic junctions and that EJCs can be deposited at variable distance from spliced junctions (Saulière et al. 2012; Singh et al. 2012). The EJC accompanies spliced mRNAs that are transported to the cytoplasm (Le Hir et al. 2001) and it is finally disassembled by scanning ribosomes during the primary rounds of translation (Gehring et al. 2009).

The EJC core serves as a binding platform for a dozens of peripherally associated factors in both the nucleus and the cytoplasm conferring multiple functions to EJCs along its travel in the cell (Le Hir et al. 2016). The EJC looks back to pre-mRNA splicing to regulate numerous splicing events (Hayashi et al. 2014b; Malone et al. 2014; Wang et al. 2014). The export of mRNAs to the cytoplasm requires their association with soluble receptors and the EJC is one of the adaptors for these receptors (Kohler and Hurt, 2007).

In *Drosophila*, the localization of *oskar* mRNA to the posterior pole of the embryo that is essential for its patterning and development (Kugler & Lasko 2009) requires the EJC core components (Hachet & Ephrussi 2004; Ghosh et al. 2012). In the cytoplasm, the presence of EJCs enhances the translation efficiency of neo-synthesized transcripts in part by contacting the translation initiation complex eIF3 (Chazal et al. 2013). The best-documented function of the EJC concerns its role in nonsense-mediated mRNA decay (NMD), a quality control process essential for cellular homeostasis that eliminates aberrant mRNA carrying a premature termination codon (PTC) (Kervestin & Jacobson 2012; Fatscher et al. 2015). In this process the presence of an EJC downstream a stop codon is sufficient to trigger mRNA degradation. A mechanism also used to modulate gene expression as exemplified by the translation-dependent decay of neuronal-specific mRNAs such as *Arc* (Giorgi et al. 2007). All these mechanisms involving the EJC show that it plays a central role in the coupling between post-splicing events (Le Hir et al. 2016).

The EJC also has important physiological functions as demonstrated by the increasing number of developmental defects that are linked to altered expression of EJC proteins. Both MAGOH and Y14 are implicated in cell fate determination and germ cell formation in *Drosophila* (Boswell et al. 1991; Parma et al. 2007; Lewandowski et al. 2010), *Marsilea vestita* (van der Weele et al. 2007) and *Caenorhabditis elegans* (Kawano et al. 2004). These phenotypes could be associated to the potential role of EJC in asymmetric mRNA localization, since disruption of MAGO and Y14 often lead to loss of asymmetric cell division and anterior-posterior axis formation, as in the case of *oskar* mRNA (Martin & Ephrussi 2009). In human, the EJC components are associated with neurodevelopmental phenotypes including microcephaly. In mouse, haploinsufficiency of *Magoh* or *RBM8a* (encoding Y14) impacts corticogenesis by misregulation of progenitor cells division and induction of massive cell death (Pilaz & Silver 2015; Pilaz et al. 2016). The implication of EJC in brain functions may explain why the dosage imbalance of several EJC/NMD genes are linked to intellectual disability (Nguyen et al. 2013). Alteration of EJC proteins expression is linked to other human diseases. Null mutation in the *Y14 (RBM8A)* gene causes the TAR (thrombocytopenia with absent radii) syndrome, in which the reduction of Y14 expression notably leads to the reduced number of platelets in blood of patients (Albers et al. 2012b). In addition, an expansion of non-coding repeats in the 5'-untranslated region (UTR) of the *eIF4A3* gene leading to reduction of eIF4A3 expression is associated with the Richieri-Costa-Pereira syndrome characterized by craniofacial abnormalities and limb defects (Favaro et al. 2014).

The EJC undoubtedly plays an essential role in the coordination of post-transcriptional events with important physiological consequences. However, the

analysis of EJC assembly in physiological contexts remain rare since so far, EJC has been studied molecularly almost exclusively in basic cell lines (i.e. HeLa, HEK293) offering obvious experimental advantages. The neurodevelopmental phenotype observed in Magoh and RBM8a mouse mutants prompted us to study the localization of EJC core proteins in primary cultures of mouse fibroblasts (MEFs) and neural stem cells (NSCs). Our study reveals the unexpected concentration of EJCs in the vicinity of centrosomes in quiescent cells and its potential implication for cell proliferation and differentiation.

## RESULTS

### EJC core proteins are present at the centrosome in NSCs

Cells were isolated from the forebrain of newborn transgenic mice expressing a GFP-tagged version of the distal core centriolar protein centrin2 (Higginbotham et al. 2004), Cen2-GFP. Cells were plated at high density in 10% FCS containing medium, and allowed to grow to confluence. Pure confluent astroglial (NSCs) monolayers were plated at high density and maintained in serum-free medium for 2 days. To study the expression of endogenous EJC core proteins, we used affinity-purified polyclonal antibodies specific to eIF4A3, MLN51 (Daguenet et al. 2012), and Y14 (**Figure S1**). Western blotting on total extract of NSCs showed that each antibody displays a signal at the correct size (**Figure S1**). The specificity of the three antibodies was further confirmed by immunoprecipitations (IP) on NSCs lysates. The content of input, supernatant and precipitate were analyzed by western blotting (**Figure S1**). None of the proteins tested were precipitated in the absence of antibodies (lane 5). In contrast, anti-eIF4A3 and anti-Y14 efficiently precipitated the EJC core proteins (lanes 6 and 7) demonstrating that the antibodies efficiently and specifically recognized the corresponding mouse proteins.

We first studied the localization of eIF4A3, Y14 and MLN51 in NSCs by immunostaining (MAGOH was not studied here because no adequate antibodies are available and this protein exist as a stable heterodimer with Y14 (Fribourg et al. 2003)). As previously observed in other cell types (Ferraiuolo et al. 2004; Kataoka et al. 2000; Le Hir et al. 2001; Palacios et al. 2004; Daguenet et al. 2012), eIF4A3 and Y14 showed in quiescent NSCs a characteristic predominant nuclear localization avoiding nucleoli labeled by DAPI staining (**Figures 1A and 1B**). Both proteins showed a punctate labeling due to their concentration in and around nuclear speckles. Nuclear speckles, known as “splicing factor compartments,” are punctuated structures which serve as storage/assembly/modification sites of splicing factors in the close proximity to active

transcription sites (Spector & Lamond 2011). We confirmed this specific localization by showing that Y14 or eIF4A3 colocalized with the splicing factor SRSF2, a nuclear speckles marker (**Figure S2**; (Carmo-Fonseca et al. 1991)). In contrast to MAGOH, Y14 and eIF4A3, MLN51 is a shuttling protein that is almost exclusively detected in the cytoplasm (Degot et al. 2002; Macchi et al. 2003; Daguenet et al. 2012). Immunostaining of MLN51 in NSCs also showed a predominant presence in the cytoplasm (**Figure 1C**).

Interestingly, we noted that Y14, eIF4A3 and MLN51 are also concentrated in the vicinity of centrosome in the cytoplasm (**Figure 1**). All three EJC proteins surround the mother and daughter centrioles labeled by Cen2-GFP in form of a concentrated cloud around centrioles as clearly visible in zoom-in of the centrosomal area (**Figure 1D-F**). This common localization is remarkable because with the exception of MAGOH and Y14 that exist as a stable heterodimer, free eIF4A3, MLN51 and MAGOH/Y14 proteins do not interact together in a pre-assembled complex (Reichert et al. 2002; Daguenet et al. 2012).

#### **EJC core proteins are present at the centrosome in MEF:**

We next wanted to know whether the presence of EJC core proteins at the centrosome was specific to NSCs, or was a general marker of centriolar structures. To answer this question, we first derived MEFs from skeletal muscle cells of Cen2-GFP transgenic mouse embryos. After two days of serum-starvation, cultivated MEFs homogeneously entered in a quiescent state in which the GFP signal from the tagged-centrin 2 marked the two centrioles (**Figure 2**). The three proteins showed a localization very similar to what was observed in NSCs. Y14 and eIF4A3 are mainly nuclear with a punctuated labeling (**Figures 2A** and **2B**) corresponding to nuclear speckles. In contrast, MLN51 is mainly detected in the cytoplasm (**Figure 2C**). As observed in NSCs, all three EJC core proteins were also concentrated around centrioles labeled by GFP-centrin2 (**Figures 2D-F**).

To test the generality of this observation, we also analyzed the localization of Y14 in mouse inner medullary collecting duct (IMCD3) cells. This epithelial cell line is derived from kidney of transgenic SV40 mouse (Rauchman et al. 1993). Under serum starvation, IMCD3 cells are in a quiescent state and immunostaining of Y14 showed that it is mainly present in the nucleus with a characteristic punctuated labeling corresponding most likely to nuclear speckles (**Figure S3**). Interestingly, Y14 was also concentrated in the cytoplasm in the centrosomal region (**Figure S3**).

The detection of EJC core proteins around centrosomes of three different cell types suggest that these proteins are general markers of these structures in quiescent cells.

### **Assembled EJC core are present around centrosomes in MEF.**

We next wanted to determine whether the presence of the three proteins around centrioles is independent of each other or whether it reflects the presence of assembled EJC cores. To distinguish between these two possibilities, we transiently expressed in NSCs, two GFP-tagged versions of Y14 either wild-type (Y14-WT; **Figure 3A**) or carrying the mutations L106R/R108E (Y14-mut; **Figure 3B**). These combined mutations are sufficient to prevent the interaction of Y14 with eIF4A3 and therefore, the incorporation of mutated Y14 into EJC core (**Figure 3B**; Gehring et al. 2005; Andersen et al. 2006; Bono et al. 2006). However, these mutations affect neither the interaction of Y14 with MAGOH (**Figure 3B**; Fribourg et al. 2003; Gehring et al. 2005) nor its nuclear localization (Daguenet et al. 2012). Both wild-type and mutant forms of Y14 were localized in nucleus of NSCs (**Figures 3C and 3D**), however, the mutant form showed a more diffuse nuclear labeling than the WT form that showed small punctuated dots in nucleus (**Figures 3C and 3D**). This difference is explained by the fact that the mutation prevents the incorporation of Y14 into splicing-assembled EJC core that mainly occurs around nuclear speckles (Daguenet et al. 2012). Interestingly, the Y14-WT was also present in the cytoplasm around the centrosome like endogenous Y14 (**Figure 3E**). In contrast, Y14-mut did not show enriched localization at centrosome despite being expressed at similar level to control plasmid (**Figure 3F**).

The transient expression of the WT and mutant versions of GFP-Y14 were also analyzed in MEFs. Exactly like observed in NSCs, GFP-Y14-WT but not GFP-Y14-mut was detected around centrosomes (**Figures 3G-H**). Taken together, we can conclude that fully assembled EJC core complexes concentrate in the vicinity of centrosomes in quiescent NSCs and MEFs.

### **The centrosomal EJC localization disappears during differentiation.**

Upon serum starvation, NSCs have the potential to differentiate into multiciliated ependymal cells (Guirao et al. 2010). Upon serum starvation, NSCs give rise to multiple flower-like centriolar structures in the vicinity of the nucleus (**Figures 4B, 4E, 4H and 4K**). These newly formed centrioles later give rise to multiple basal bodies (**Figures 4C, 4F, 4I and 4L**) from which cilia grow to produce multiciliated ependymal cells (Al Jord et al. 2014). We determined the localization of Y14 and eIF4A3 at three

distinct differentiation stages of Cen2-GFP cells: quiescent NSCs, flower stage and basal body stage (ependymal cells). Interestingly, if Y14 and eIF4A3 were concentrated around centrosomes in NSCs, both disappeared around flowers (**Figures 4E and 4K**) or basal bodies (**Figures 4F and 4L**). At least two main phenomena could explain the disappearance of the proteins, the disassembly of EJCs followed by the rapid dispersion of its components or a down-regulation of EJC proteins over differentiation. We favored the first hypothesis because we did not detect a significant decrease in the expression level of Y14, eIF4A3 and MLN51 over differentiation as observed by western blotting analysis of the proteins over 15 days of differentiation of NSCs *in vitro* (**Figure S4**). Taken together, our data revealed that EJCs mark the peri-centrosomal region when cells are in a quiescent state and that this mRNP mark completely disappear in cells committed to differentiation.

## **DISCUSSION**

Here, we have studied the immunolocalization of the endogenous EJC core components eIF4A3, Y14, MLN51 in different cell types and cellular contexts including the immortalized cell line IMCD, MEF and, NSC over differentiation into multiciliated ependymal cells. We observed that in the quiescent cells tested, all three proteins are concentrated in the vicinity of the centrosome at the base of the primary cilia. This localization reflects the presence of fully assembled EJCs as proved by the analysis of a version of Y14 carrying mutations that prevent EJC core mounting. The enrichment of transiently expressed MAGOH and Y14 also at centrosome in human 549 cells reinforces the general aspect of EJCs at centrosome (Ishigaki et al. 2014). Remarkably, the intense signals of EJC core proteins in the centrosomal region totally disappeared when quiescent NSCs enter in differentiation. As explained below, the presence of EJCs around centrosome strongly argues for the concentration of large population of mRNAs not yet translated that mark in a very distinct manner a determinant cellular state.

The functional outcome of eukaryotic mRNAs including their cellular localization, their translation efficiency and their stability, is specified by the numerous RBPs that composed RNP particles. The identity of RBPs is dictated by mRNA primary sequence but it also echoes the processing history of the mRNA rendering each mRNP unique and dynamic during mRNA life cycle (Müller-McNicoll & Neugebauer 2013). In this context, the peculiarity of EJCs reside in the fact that they mark a precise period of mRNA life. Indeed, EJCs are first assembled in the nucleus by spliceosomes onto spliced mRNAs. Then, EJCs accompany mRNAs to the cytoplasm and remain stably bound to



transcripts until the first round of translation (Dostie & Dreyfuss 2002; Lejeune et al. 2002; Gehring, Lamprinaki, Kulozik, et al. 2009). Concomitantly with translation-dependent EJC disassembly, PYM interacts with the heterodimer MAGOH/Y14 to prevent EJC core reassembly (Gehring, Lamprinaki, Kulozik, et al. 2009; Ghosh et al. 2014). Therefore, fully mounted EJC core complexes present in the cytoplasm distinguishes mRNAs that have not yet experienced translation from the one that are already translated. Taking this specific EJC labeling into account, our data plead for the presence of numerous untranslated transcripts around the centrosome and this, exclusively in quiescent cells. The clear and complete disappearance of EJC signal around the centrosome when cells enter into differentiation is most likely a consequence of the translation of EJC-bound transcripts leading to EJC disassembly. Another scenario that is not mutually exclusive would be that EJC-bound mRNAs are degraded either before or rapidly after their translation. In any case, our results reveal a massive spatio-temporal program of post-transcriptional gene regulation when cells exit the quiescent state toward differentiation.

Several studies reported the presence of specific RNAs in the centrosomal region in different cellular contexts. First, RNA of unknown type were detected in basal bodies of *Tetrahymena pyriformis* and *Paramecium tetraurelia* (Hartman et al. 1974; Dippell 1976) and in centrosomal fraction of *Spisula solidissima* oocytes (Alliegro et al. 2006). Other studies reported the identification of specific mRNAs at centrosomes. During early embryonic development of the mollusc *Ilyanassa obsoleta*, several mRNAs encoding patterning genes are anchored to centrosomes before to be distributed asymmetrically in daughter cells upon cell division (Lambert & Nagy 2002). An exhaustive analysis of mRNA localization in *Drosophila melanogaster* embryos, revealed the presence at centrosome of at least six different mRNAs which function await further characterization (Lécuyer et al. 2007). The only mRNAs which the presence at centrosome is supposedly linked to cell division is the Cyclin B1 mRNA which encodes a critical regulator of mitotic entry. Cyclin B1 mRNAs are slightly enriched near centrosome in *Xenopus laevis* embryos (Groisman et al. 2000) or in centrosomal fractions of rat primary astrocytes (Kim et al. 2011). The translation of this transcript is inhibited by CPEB1 (Cytoplasmic Polyadenylation Element binding Protein 1) until repression is released by CPEB1 phosphorylation. Whether the local translation of Cyclin B1 mRNA in the vicinity of centrosome is required for cell cycle progression remains an open question. An attractive hypothesis would be that the translation of centrosomal transcripts leading to EJC disassembly is coordinated by translation regulators. The identification of centrosomal EJC-bound transcript will constitute an

essential step toward our understanding of the function of these transcripts and the regulation of their expression in coordination with cell fate.

In growth-arrested cells (like the ones used in this study: IMCD, MEF and NSCs), the oldest centriole of the centrosome convert into a basal body which nucleate a non-motile primary cilium. Primary cilia consist of an axoneme of nine doublet microtubules surrounded by the ciliary membrane. The major function of primary cilium is in cell signaling to relay or modulate external signals (such as mechanical stimulations or chemosensations) to facilitate cell-fate decisions like cell cycle re-entry or cell differentiation. In most cases, primary cilia collapse when cell re-enter the cell cycle (Guemez-Gamboa et al. 2014). Interestingly, a variety of receptors as well as some of their downstream effector molecules localize to the cilium or basal body. The enrichment of untranslated EJC-bound mRNAs at the centrosome of quiescent cells might indicate that they are waiting for external signals relayed through the primary cilium that will initiate their translation and/or degradation. To discriminate between these 2 possibilities, it would be very interesting to label nascent proteins in live quiescent cells that are induced to re-enter the cell cycle (or to differentiate). Further work will also attempt to identify which RNAs are specifically localized at centrosome, and which are the molecular mechanisms that maintain them at centrosome in quiescent cells.

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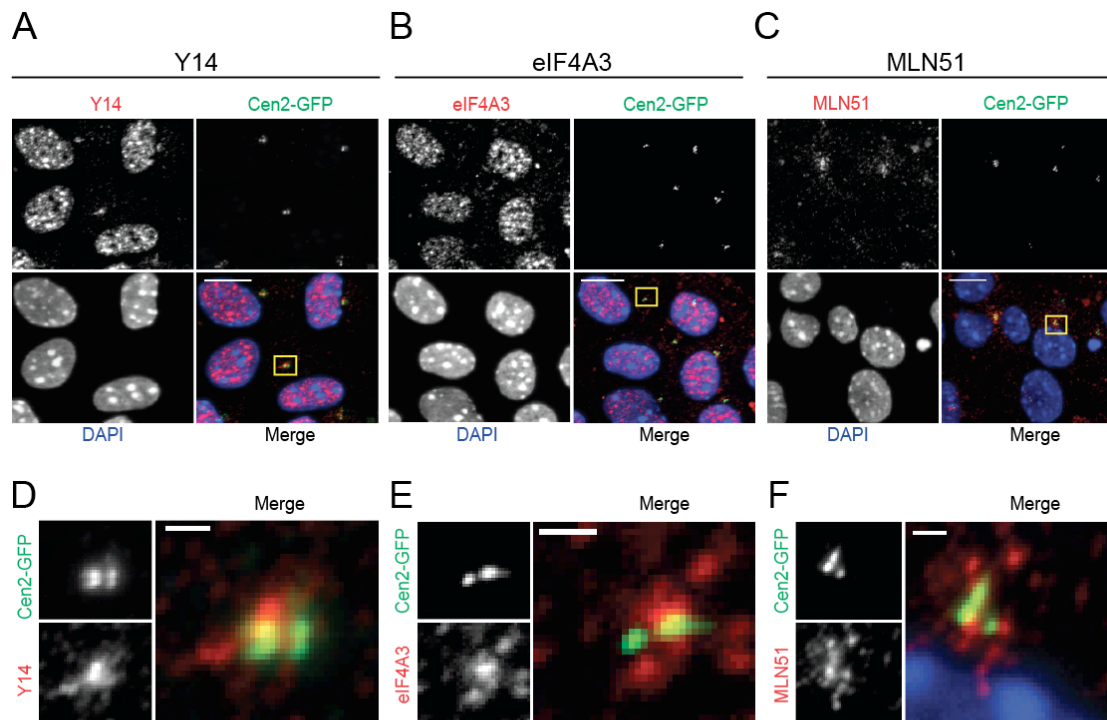
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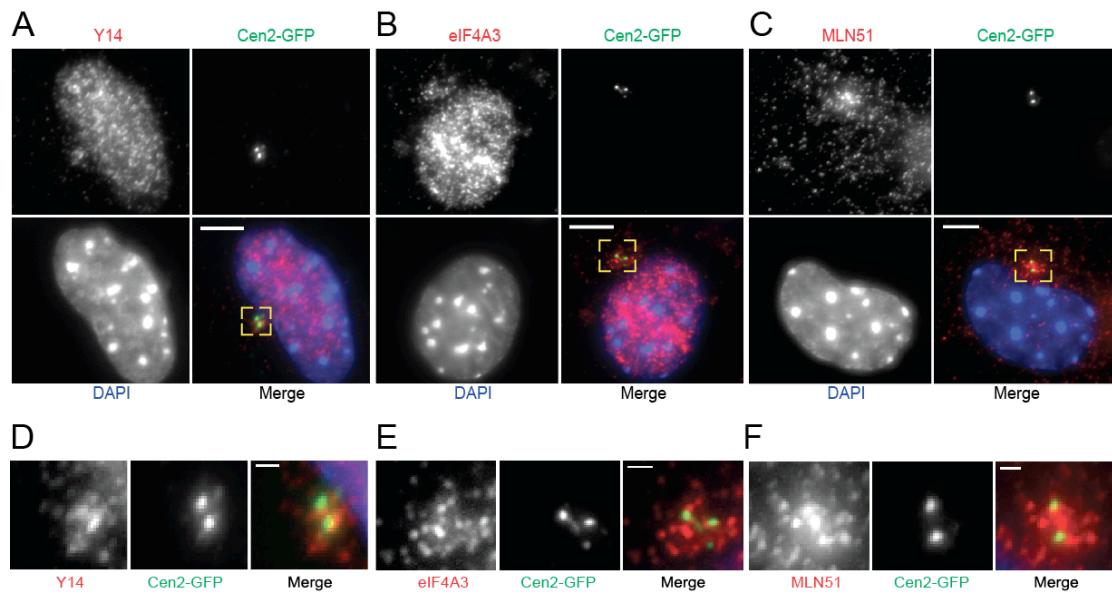


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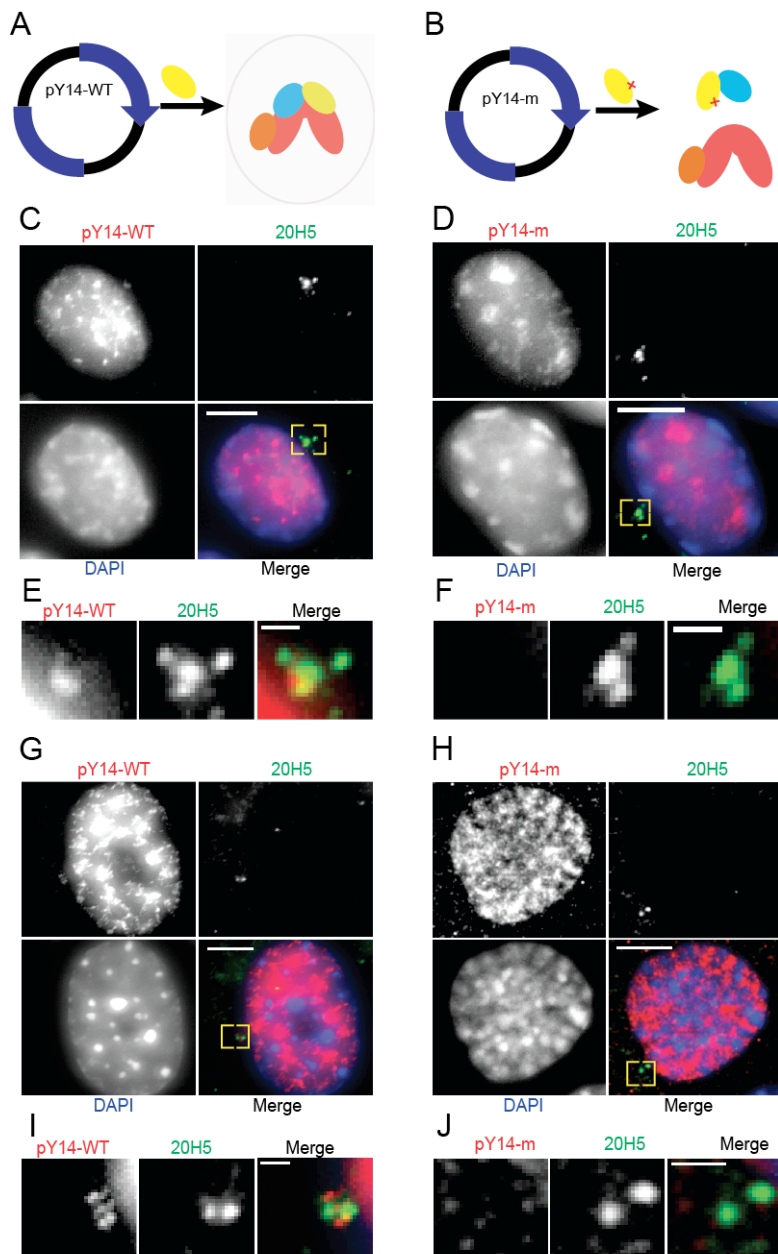
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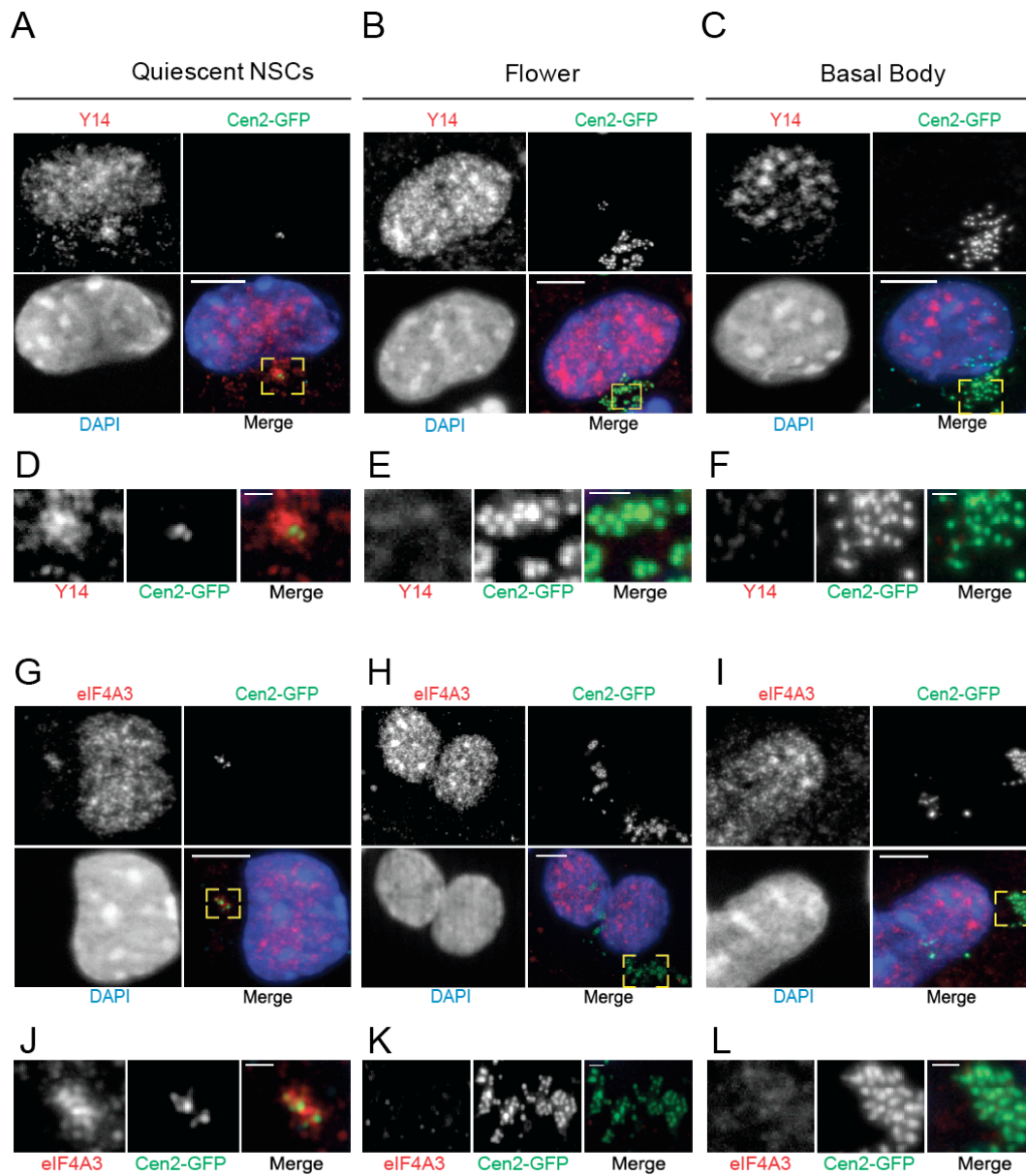
**Figure 1: EJCs are concentrated at centrosome in NSC.** Immunostaining with EJC proteins (red channel) is shown in D2-NSC culture. (A,B,C):Y14, eIF4A3 and MLN51 staining shows their respective localization in NSCs. Green channel shows the centrosome expressing Cen2-GFP endogenously. Centrosomal region from one cell (rectangle) is enlarged for each staining and represented in second row (D, E, F). Scale Bar: (A, B, C): 10 $\mu$ m, (D, E, F): 1 $\mu$ m.



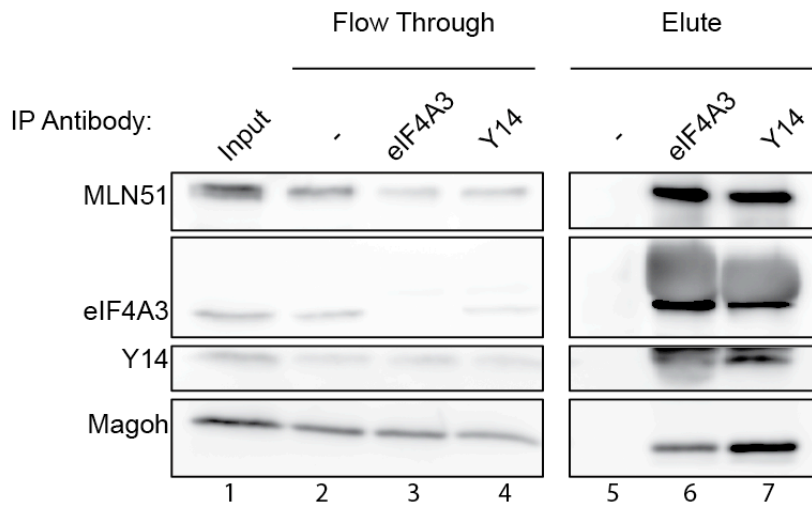
**Figure 2: EJCs concentrate at the centrosome in MEF.** Immunostaining with EJC proteins (red channel) is shown in MEF culture. Green channel shows the centrosome expressing Cen2-GFP endogenously. DAPI is shown in Blue. (A,B,C):Y14, eIF4A3 and MLN51 staining shows their respective localization in MEF cells. Centrosomal region (rectangle) is enlarged and represented in second row (D,E,F). Scale Bar: (A,B): 5 $\mu$ m, (C, D): 1 $\mu$ m



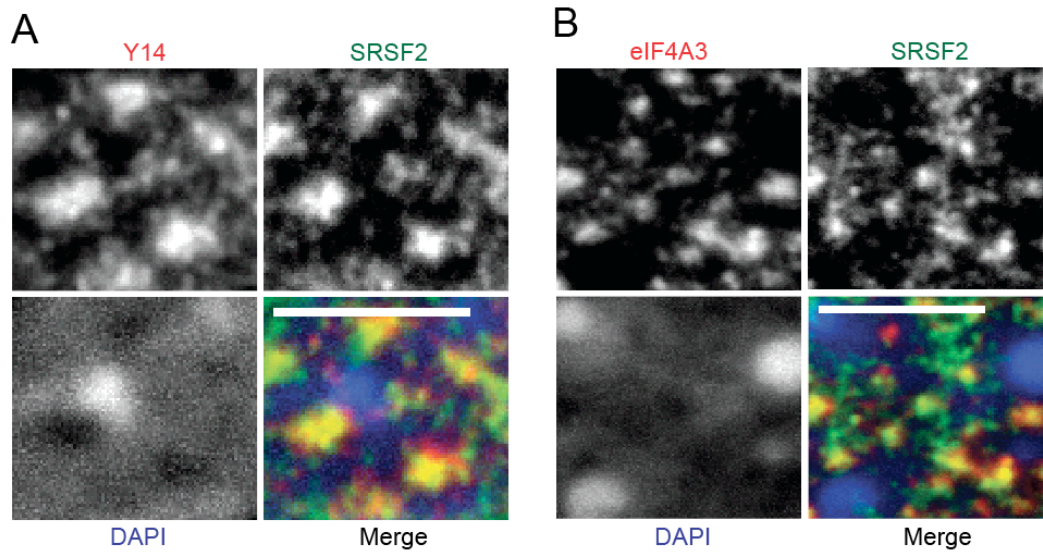
**Figure 3: EJC as a fully assembled core at centrosome:** **A,B:** Schematic representation of the transfected plasmid expressing recombinant protein. The wild type protein expressed by pY14-WT interacts with other EJC partners by getting into EJC core while the mutant Y14 protein (pY14-M) does not get incorporated into EJC core. **C,D:** Immunostaining after transfection in NSC. **E,F:** Centrosomal region of NSC (rectangle) is enlarged. **G,H:** Immunostaining after transfection in MEF. **I,J:** Centrosomal region of MEF (rectangle) is enlarged. While both proteins are expressed in nucleus of NSC and MEF, only wild-type is present at centrosome. The mutant plasmid does not localized at centrosome. Scale Bar: C,D,G,H: 5 $\mu$ m, E,F,I,J: 1 $\mu$ m



**Figure 4: Centrosomal EJs disappear in differentiating NSC.** Centrosomal EJs are only present before the start of differentiation and absent in differentiating or well differentiated NSCs. Immunostaining with EJC proteins (red channel) is shown in NSC culture during ependymal differentiation. Green channel shows centrosomal centrioles expressing Cen2-GFP endogenously at three distinct stages of differentiation. Y14 is concentrated at centrosome in cells at quiescent stage (A, D) but not present at centrosomal centriole in cells at Flower (B,E) and at multi-centrioles in B.B. stage (C, F). Similarly, eIF4A3 is also concentrated at centrosomal centrioles only in RG cells (G,J), its localization diffuses upto cytoplasmic background in cells at Flower (H,K) and B.B. stage (I, L). BC). Scale Bar: (A, B, C): 5 $\mu$ m, (D, E, F): 1 $\mu$ m.

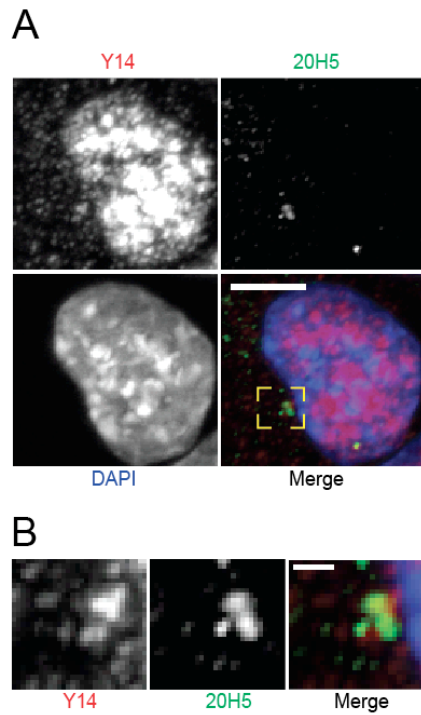


**Supplementary Figure 1: EJC antibodies pull down their core-partners in NSC.** Western blot after immunoprecipitation with both EJC antibodies shows their specificity and efficiency to recognize EJC partners in NSC. Both antibodies could efficiently catch and deplete (Flow Through) the protein and other core partners from the input total cell lysate which is pulled down as Elute. Control IP having no antibodies (lane 5) did not have any elution of EJC.



**Supplementary Figure 2: Splicing mediated assembly of EJC in nucleus of NSC.** In NSC, immunostaining with anti-EJC antibodies (Red) and anti-SRSF2 antibody (green) is shown with DAPI (blue) staining. Both Y14 (A) and eIF4A3 (B) co-localize with splicing protein as punctuated dots inside nucleus. Scale Bar: 5 $\mu$ m.





**Supplementary Figure 3 : EJCs localize at the centrosome in IMCD cells.** In IMCD cells, cytoplasmic EJCs are concentrated at centrosome. Immunostaining with Y14 antibody (red) and centrosomal marker '20H5' antibody (green) is shown with DAPI (blue) to show their localization (A). Y14 is localized inside nucleus and concentrated at the centrosome in the cytoplasm. Centrosomal region is enlarged in (B). Scale Bar: (A): 5 $\mu$ m, (B): 1 $\mu$ m.

# **RESULTS**

## **(Part 2)**

### **ADDITIONAL RESULTS**

## 1.0: Identification of EJC-bound mRNAs in NSC

Our results of immunofluorescence labeling of EJC core proteins in NSC strongly suggest the presence of numerous untranslated mRNAs in the vicinity of centrosomes in quiescent cells (see our manuscript, **Results**). Our next objective was obviously to identify these transcripts or at least some of these transcripts. Two different experimental approaches can be envisaged to isolate RNAs bound to specific RBPs, RIP-seq (RNA ImmunoPrecipitation coupled to high-throughput sequencing) and CLIP-seq (CrossLinking and ImmunoPrecipitation coupled to high-throughput sequencing). Our group was the first one to map EJC binding sites by performing the CLIP-seq of eIF4A3 (Saulière et al. 2012). Briefly the principle of CLIP-seq is the following. Cells are first irradiated to UV at 254 nm to crosslink proteins that are directly bound to RNA and this, in their natural cellular context. Following mild RNA degradation with RNases to isolate RNA-protein complexes from each other, the proteins of interest are immunoprecipitated with their associated RNAs. Then, the co-purified RNAs are reverse-transcribed to prepare cDNA libraries compatible with large-scale sequencing. Hence, CLIP-seq provides like a transcriptome-wide snapshot of RNA binding sites. The first CLIP-seq of eIF4A3 was done in human HeLa cells and provide valuable information about EJC-bound mRNAs and EJC binding sites (Saulière et al. 2012). Members of our group have then tried to perform similar CLIP-seq analysis in both mouse C2C12 myoblast and derived C2C12 myotubes to study EJC assembly during myogenic differentiation (Jérôme Saulière and Hervé Le Hir). Unfortunately, this project did not succeed with the original CLIP-seq protocol and this, mainly because the eIF4A3 polyclonal antibodies immunoprecipitated the mouse protein slightly less efficiently than its human homologue leading to a high background. Indeed, an important caveat of CLIP-seq originates from the fact that UV crosslinking is poorly efficient (1%) and therefore that crosslinked RNP are easily contaminated by uncrosslinked small RNA fragments generated by RNase treatment (interestingly, very recent progresses made in the lab by Rémi Hocq now allow to efficiently EJC RNA

targets by CLIP-seq. The first CLIP-seq data obtained in mouse C2C12 cells are currently analyzed by bioinformatics).

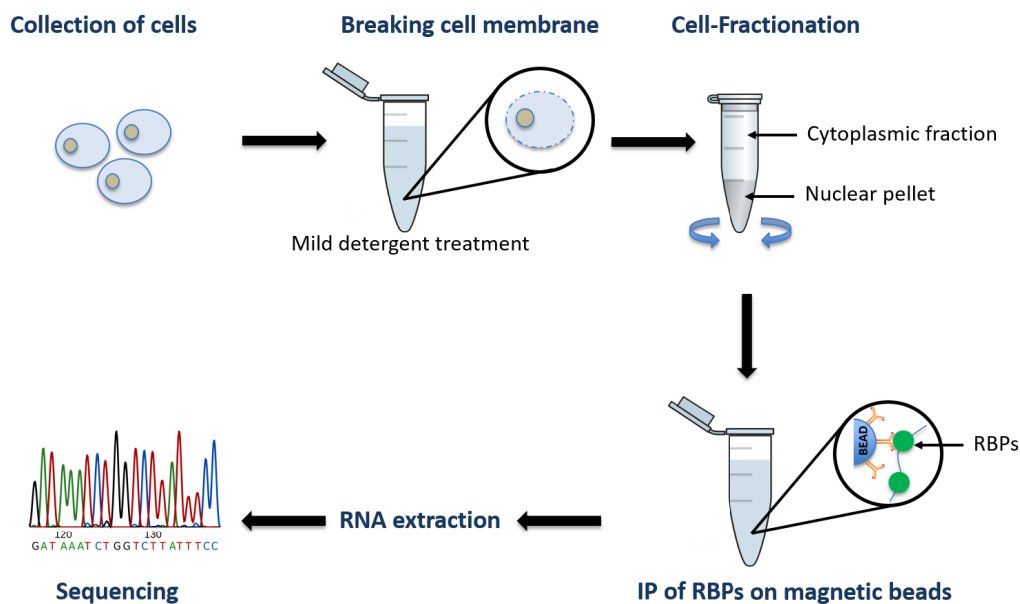
### 1.1: EJC RIP-seq strategy

Therefore, we decided to perform RIP-seq that does not necessitate the crosslinking step and still allow to sequence polyadenylated mRNAs associated to a protein of interest after immunoprecipitation. The main disadvantage of RIP-seq compared to CLIP-seq is that immunopurified RNP complexes cannot wash under stringent conditions that would lead to RNA dissociation. Purification under mild washing conditions moreover do not allow to efficiently eliminate potential RNA contaminants. So, to distinguish the pool of potentially untranslated mRNAs at quiescent cell-stage, we purified EJC-associated mRNAs in Day:2 NSC by doing RNA-IP with EJC antibodies against several EJC core proteins, two against eIF4A3 and one against Y14. Indeed, both eIF4A3 and Y14 have been shown to differentially regulate the expression of divergent neuronal activity dependent genes (Giorgi et al. 2007; Alachkar et al. 2013). Therefore, to get an unbiased view of EJC bound transcripts, we performed IP with two different antibodies of eIF4A3 and one antibody for Y14 in the cytoplasmic fraction. By considering transcripts isolated by all three antibodies we will strengthen the identification of EJC-bound transcripts. As EJC is known to bind transcripts having a vast range of expression level (Saulière et al. 2012, Singh et al. 2012), we performed mRNA sequencing (mRNA-seq) of total RNA and cytoplasmic RNA separately in Day:2 NSC. These mRNA-seq will be necessary to measure the fold enrichment of precipitated mRNA in comparison to cellular mRNA contents.

### 1.2: Cytoplasmic fractionation of NSC

Because we search to identify EJC-bound transcripts located in the cytoplasm, we also developed a cell-fractionation protocol to cleanly separate cytoplasmic fraction of NSC from nucleus before RIP and isolation of mRNAs for large scale sequencing by Illumina method on the IBENS genomic platform (**Figure 30**). Taking into account the life cycle of EJCs (see introduction, section

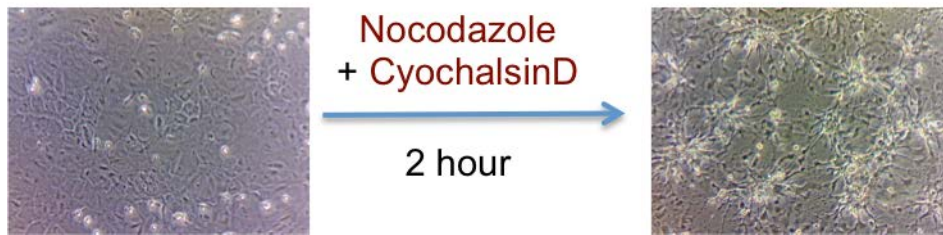
**3.0)** we can suppose that a large proportion of cellular mRNAs associated to EJCs are present in the nucleus because once exported and subjected to translation in the cytoplasm, EJCs are disassembled. Therefore, by eliminating nuclear mRNAs, we may facilitate the identification of cytoplasmic EJC-bound mRNAs that have not yet experience translation. Moreover, three of the four EJC core proteins (eIF4A3, Y14 and MAGOH) are predominantly localized in nucleus. The ratio of these individual proteins vs. those incorporated into fully assembled EJC core would be much higher in nucleus compared to cytoplasm. Therefore, getting rid of the nucleus would help us to avoid this pool of unnecessary RNAs, and would give antibodies more access to catch assembled EJC on mRNAs than just the protein individually. This would help to decrease the background and complexity to our analysis.



**Figure 30:** Schematic representation of cell-fractionation and RIP-seq

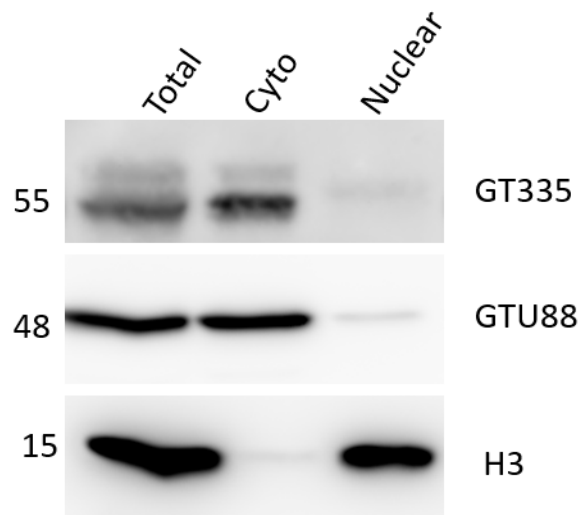
As majority of cells in NSC are in quiescent stage around Day:1 to Day:2 of culture, we decided to fractionate the cells at Day:2. In addition, in order to weaken the link existing between centrosomes and the nuclear membrane via microtubules (Hoebeke et al. 1976; Tassin et al. 1985; Panic et al. 2015), we treated our cells with two microtubule inhibitor drugs, Cytochalasin D and

Nocodazole prior to cell fractionation. After 4 hours of drug treatment, visual confirmation verified that cells have detached from each other (**Figure 31**).



**Figure 31:** NSCs in culture dish before and after the drug treatment.

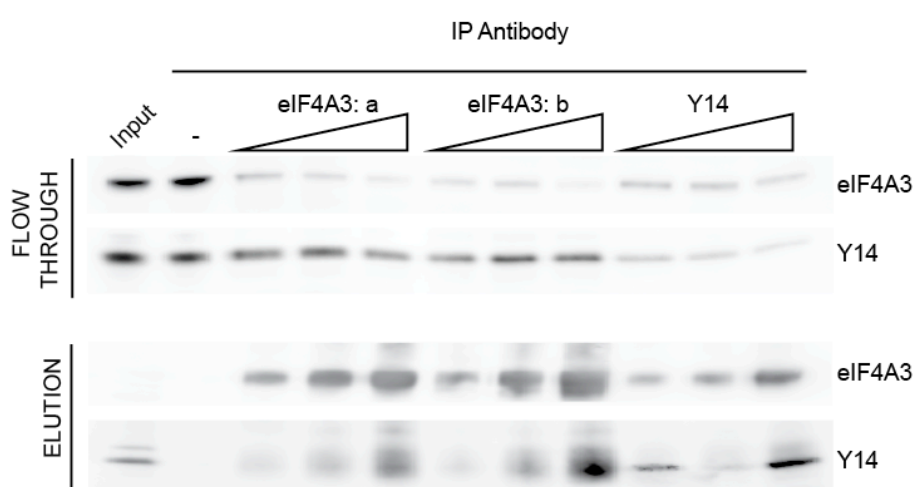
In order to break the cell membrane while keeping the nucleus intact, we treated cells with mild detergent followed by brief centrifugation (Suzuki et al. 2010). This allowed us to collect the cell-lysate containing mainly the cytoplasmic fraction while the nucleus and un-lysed cells were pelleted (**Figure 30**). Western blot analysis of this fraction showed that the centrosomal marker gamma-tubulin (GTU88) and cilia marker (GT335) were mostly present in the cytoplasmic fraction while the nuclear histone (H3) was majorly existent in the nuclear part as expected, confirming the clean separation of both the components (**Figure 32**).



**Figure 32:** Western Blot after cell fractionation showing the clean separation of cytoplasmic and nuclear proteins in their respective cellular fractions.

### 1.3: Tests of immuno-depletion

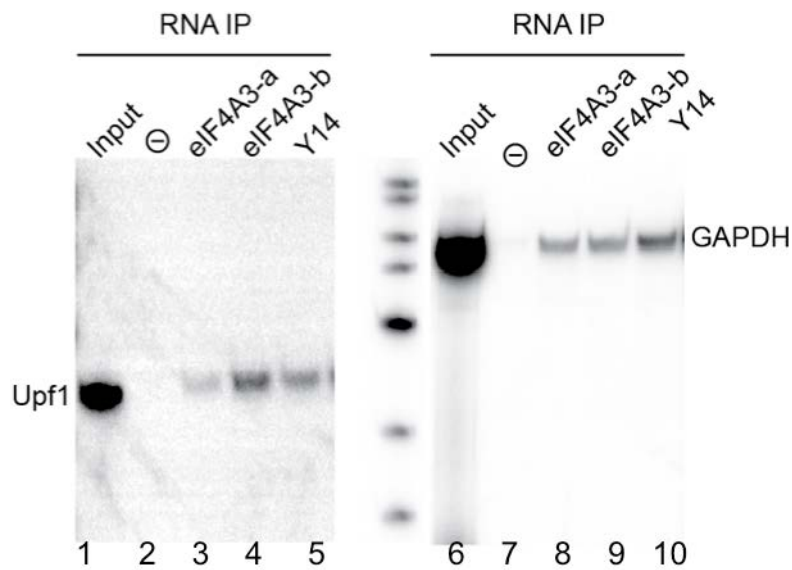
Next, we tested the efficiency and specificity of IP with our antibodies by doing an immuno-depletion tests of cytoplasmic fraction of NSC with increasing amount of antibodies. Western Blot after IP with these antibodies demonstrated their ability to deplete the corresponding protein and respective EJC partners (**Figure 33**). As shown in flow through, all three antibodies were able to deplete the individual protein and to significantly immune-deplete their EJC partner from the lysate. While the analysis of the elution fractions confirmed that these antibodies efficiently precipitated EJCs.



**Figure 33:** Western Blot after IP in cytoplasmic fraction of NSC.

#### 1.4: EJC-RIP and RNA purification

Then, we combined NSC cellular fractionation to immunoprecipitations with eIF4A3 and Y14 antibodies. Owing to variability between the primary cultures, we performed RIP in triplicates to conclude our analysis with more statistical significance and stronger confidence. After IP, RNA was extracted from the eluted material. We tested the presence of some ubiquitous mRNAs by doing a quantitative RT-PCR in an aliquot from this elute. As shown in the figure, Upf1 and GAPDH mRNAs were present in three IP experiments while they were absent in control IP performed in the absence of antibodies (**Figure 34:** lane 2 and 7).



**Figure 34:** Phosphorimage of  $\gamma\text{P}^{32}$ -labeled total RNA extracted after RIP shows the presence of ubiquitous mRNAs (Upf1 and GAPDH) in the IP with all three antibodies but not in control IP.

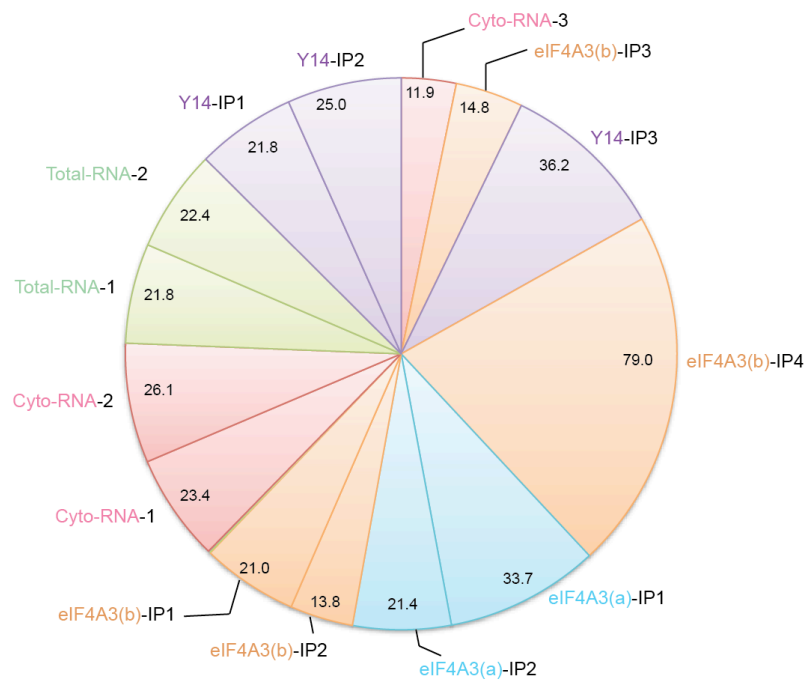
Next, the eluted RNAs were given to genomic platform of IBENS for polyA+ purification (True-seq protocol from Illumina), in order to get only mRNAs identified by high-throughput sequencing. To compare the enrichment of transcripts from the expression level, we also performed mRNA sequencing (mRNA-seq) of total RNA and cytoplasmic RNA prepared from the same pool of cells.

### 1.5: Analysis of sequencing results

After sequencing, the results were analyzed by Leila Bastianelli, engineer in bioinformatics in our group. The reads were analyzed using STAR mapping (Dobin & Gingeras 2015). In total, high-throughput sequencing generated homogenous distribution of raw reads at the end (**Figure 35**). The RNAseq for input RNA (total cytoplasmic RNA) produced 61.4 million of reads. We did not observed ribosomal reads due to prior purification of polyA-plus RNA only. Notably, the fractions of generated reads in all IPs were uniformly distributed and consistent with the input material used for individual IPs. The two IPs performed with first eIF4A3 antibody (eIF4A3-A) generated 55.2 millions of reads, while four IPs with the second eIF4A3 antibody (eIF4A3-B ) gave total



128.7 millions of reads. Similarly, three separate IPs with Y14 antibody generated about 83 millions of reads collectively.

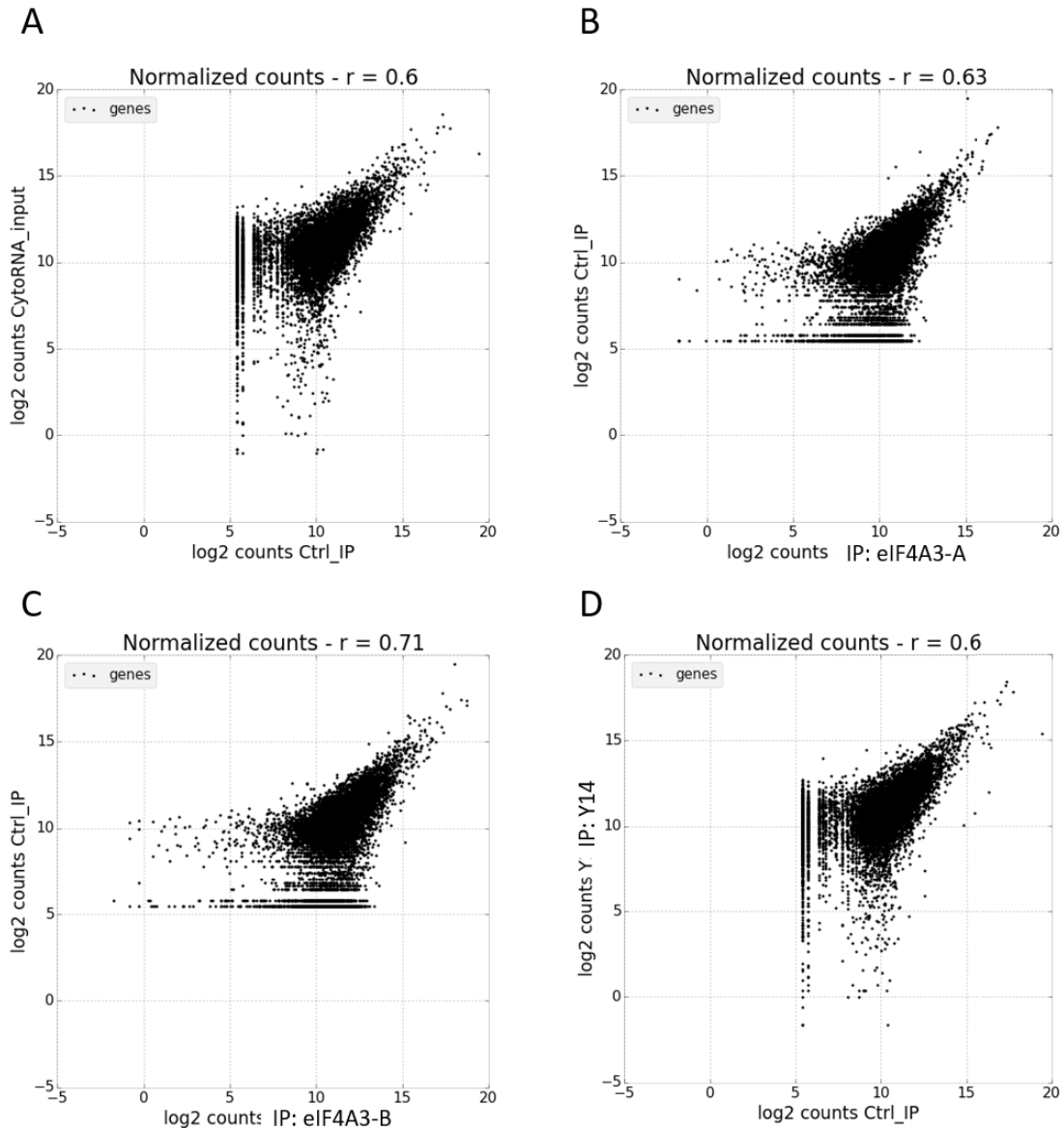


**Figure 35:** Plot showing read-distribution obtained after sequencing from each IP experiment.

## 1.6: Statistical validation of RIPseq

We performed statistical analysis to validate the significance of IP in NSC.

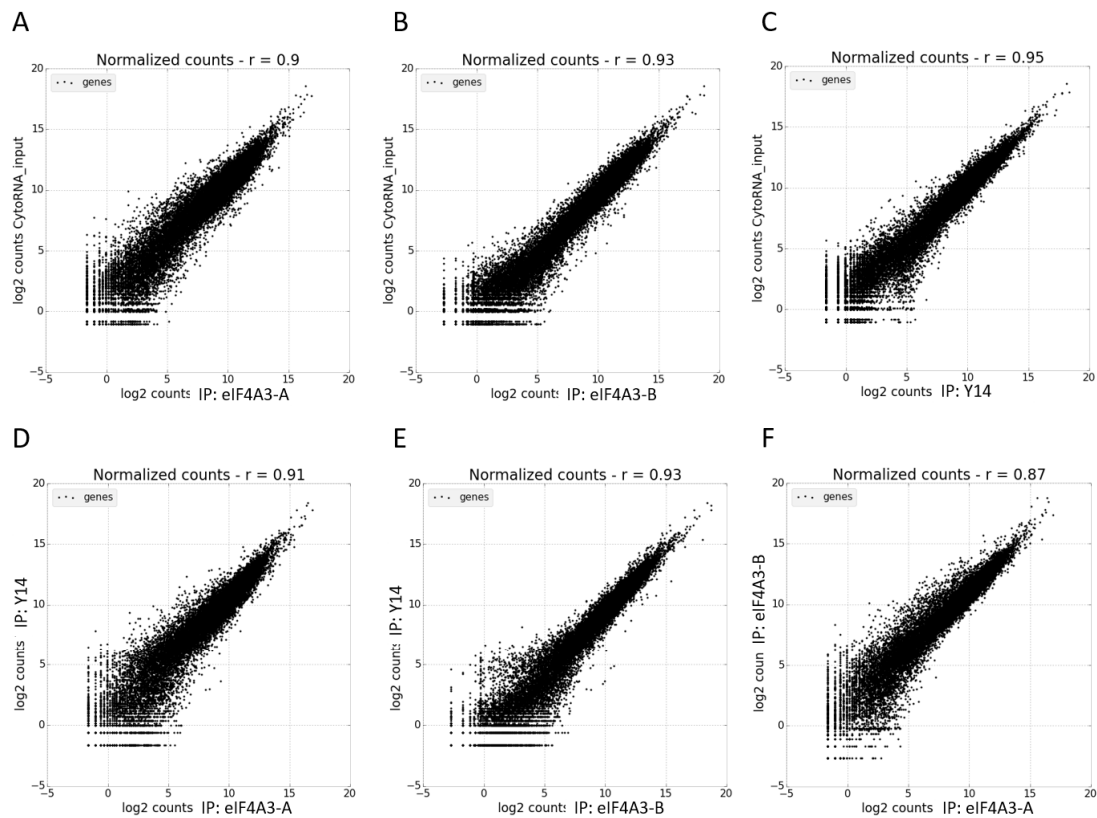
**Figure 36** shows the plots of correlation between replicate IPs vs control IP. Each replicate experiment was normalized and the mean of normalized read counts per condition was treated as one to compare. As shown in **Figure 36** (B, C, D), all three IPs were highly dissimilar from the control IP. This confirms that the IP with the antibodies precipitate the targets discrete from the background.



**Figure 36:** Correlation-plot of individual IP replicates compared to the control IP showing that they are highly dissimilar. Reads mapped to all annotated genes were considered. Each dot represents a gene, value of correlation coefficient ( $r$ ) is indicated for each plot.

Next we compared the grouped results for each batch of experiment. Even when grouping the replicates, we observe the same correlation between eIF4A3/Y14/input mRNA (**Figure 37**). We observed that the pool of replicates for each antibody is highly correlated to the other. Cumulatively, the individual read counts (for each replicate) were more correlating per batch of experiments and not per IP/antibody. This is not surprising, considering that we started each time from variable conditions including the mix of population of cells at different differentiation stage on Day:2. Also, the experiment was done each time with

different mouse, leading to novel cell-cultures. Since the RIP-seq experiment is not very stringent, the validation of significance is highly dependent on the background noise, which is linked to the cell batch.



**Figure 37:** Log plots of the number of uniquely mapped reads per gene between IP triplicates with each antibody compared to each other. Reads mapped to all annotated genes were considered. Each dot represents a gene, value of correlation coefficient ( $r$ ) is indicated for each plot.

### 1.7: Identity of EJC bound transcripts in NSC

Using stringent statistic measures we uniquely identified genes enriched in our IP having fold change  $>1$  compared to the control IP. Individually, we unmasked about 500 unique transcripts captured by eIF4A3-A antibodies and roughly 300 distinct mRNAs with eIF4A3-B antibodies, respectively (pValue: 0.05). Out of these two sets, roughly 200 are common to both. Y14-RIP allowed us to identify 129 individual transcripts significantly enriched compared to the

control IP (pValue: 0.05). Notably 2/3<sup>rd</sup> of these genes is common to either of eIF4A3 IP giving the confidence that in our conditions, the antibodies pull down the true targets i.e. EJC bound transcripts. Finally, grouped analysis of all these replicates together gave a high-confidence list of 45 EJC-bound transcripts enriched in all three IP (**Table 1**). However, on account of stringent statistic measures we applied, it is possible that other potential targets of EJC may have been missed as well.

Interestingly, one third of the genes in our EJC-RIP are proteins involved in different aspects of transcription regulation (**Table 1**). Among the list are many known transcription factors in which some have known functions related to neurogenesis such as inhibitor of DNA binding 1 (ID1), inhibitor of DNA binding 4 (ID4) and CCAAT/enhancer binding protein beta (CEBPB). We also obtained potential transcription regulators with unknown functions in neuronal lineage such as Zinc finger proteins Zfp57, Zfp61, Zfp639 and Zfp 821. Additionally, EJC-RIP brings to light the transcripts regulating cell proliferation such as Bmp4, Samd1, Smad6 and Noggin. Among the negative regulators of cell proliferation we also obtained genes that promote cell-cycle arrest, quiescence and senescence such as Tripartite Motif Containing 8 (Trim8), Cyclin-dependent kinase inhibitor 2C (Cdkn2c) and Erythroid differentiation regulator 1 (Erdr1) respectively. Another interesting candidate in this list is Serine/threonine protein kinase 11 (Stk11), which is a general regulator of cell polarity. In our RIP-seq we also found genes with general functions like splicing related factors such as PRPF39, CWC22, CLK1 and CLK4, the export factor Aly/Ref and the mRNA decay factor Pan3. We also found transcripts coding protein of less or no known functions like the coiled-coil domain containing proteins Ccdc173 and Ccdc101 and the uncharacterized genes Gm17066, RIKEN cDNA 2700099C18 (2700099C18Rik), 6030419C18Rik, Jtb and Fam173a.

Our RIPseq highlights the inter-related regulatory network of transcripts critical for cell decisions related to quiescent stage of the cells. While ID1 and ID4 proteins are positive regulators of cell proliferation and inhibitors of differentiation of adult NSC (Le Belle et al. 2011; Nam & Benezra 2009; Tokuzawa et al. 2010), Cebpb acts as promoter of neuronal differentiation in human NSC (Cheeran et al. 2005) and mouse NSC in the SVZ of brain (Liu et al.

2009). Notably, local translation of CEBP-1 mRNAs regulate synapse formation and axon morphology during development and axon regeneration in adult (Yan et al. 2009). Since majority of the NSCs are quiescent at the time-point of our RIP-seq, translational activation of some of these transcription factors might signal cellular machinery to transcribe other genes important for regulating the commitment of NSCs towards differentiation or proliferation or maintenance of quiescence. Similarly, Bmp4, Samd1, Smad6 and Noggin interact with each other in order to control the cell proliferation and differentiation. BMPs are members of the TGF $\beta$  superfamily that signal through serine/threonine kinase receptors to activate Smad family transcription factors through C-terminal phosphorylation (Shi & Massagué 2003). BMPs can activate a pro-regenerative transcription program in neurons through Smad-mediated signaling pathways. BMP/Smad signaling regulates axon regeneration, astrogliosis and neural progenitor cell differentiation *in vivo* (Zhong & Zou 2014). Interestingly, BMP promotes gliogenesis proliferation and inhibits differentiation by antagonizing NOGGIN thus inhibiting neurogenesis in the SVZ of mouse brain (Lim et al. 2000; Li et al. 2008; Ma et al. 2011). In adult neurogenesis, NOGGIN promotes oligodendrogenesis by redirecting neuroblast differentiation in ventricles (Colak et al. 2008). NOGGIN also allows for self-renewal and multipotential differentiation of hippocampal precursor cells highlighting its function across the brain (Bonaguidi et al. 2008). Indeed, the aspects of NOGGIN as a differentiation regulator in SMAD signaling has been utilized to transform human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells into neural progenitors and functional Neurons (Chambers et al. 2009; Kirkeby et al. 2012). Among the negative regulators of cell proliferation we also identified genes that are responsible for maintenance of quiescence and cell death. The fact that quiescent cells may maintain their resting state or decide to die is highlighted by the enrichment of Cdkn2c, Trim8 and Erdr1 in our RIP-seq. CDKN2C is a cyclin-dependent kinase (cdk) inhibitor that interacts with both Cdk4 and Cdk6 to inhibit their kinase activities, and prevent their interactions with D-type cyclins, thereby negatively regulating cell division and maintaining the quiescence (Broxmeyer et al. 2012). Trim8 is a key functional modulator of cell cycle arrest (Caratozzolo et al. 2012) and Erdr1 functions as a proapoptotic gene (Jung et al.

2011). Functional interplay with these proteins in quiescent NSCs may function as determination of cell fate in terms of quiescence and differentiation. However, Day:2 NSCs utilized in the RIP-seq contain a mix of population of cells in which some have already committed differentiation while some are maintained in quiescent state. Therefore, we cannot precisely sub-group these transcripts based on the population of specific cell state.

Notably, in our RIP-seq we found *Stk11* (also known as *Lkb1*) transcript strongly associated with EJCs. *STK11* kinase plays crucial roles in the establishment and maintenance of cell polarity in different cell types and organisms (Shelly & Poo 2011; Nakano & Takashima 2012). In mammals, *LKB1* functions as axon determinant by developing and maintaining neuronal polarity. Conditional deletions of *LKB1* gene in mouse forebrain (Barnes et al. 2007) or down-regulation of *LKB1* expression in rat cortical progenitors by RNA interference (Shelly et al. 2007) abolish axon formation *in vivo*. The association of EJCs with this master regulator of cell polarity in the cytoplasm of NSC might be critical for determination of cell polarity as observed for EJC mediated patterning of *Drosophila* embryo by *Oscar* mRNA.

Additionally, EJCs also precipitate the transcripts coding for factors involved in post-transcriptional gene expression regulation. Among those we found several factors related to splicing (*PRPF39*, *CWC22*, *CLK1* and *CLK4*), to mRNA export (*Aly/Ref*) and to mRNA degradation (*Pan3*). *PRPF39* and *CWC22* are two splicing factors. If the function of the first one is unknown, *CWC22* is important for spliceosome activation (Yeh et al. 2011). Interestingly, *CWC22* is essential for EJC assembly by the spliceosome (Barbosa et al. 2012; Steckelberg et al. 2012; see introduction). We can suppose that retention of untranslated *CWC22* transcripts allows regulating the amount of protein available and in consequence, the amount of EJC assembled. CDC like kinase 4 (*Clk4*), CDC like kinase 1 (*Clk1*) control of the intranuclear distribution of SR proteins in a cell cycle dependent manner but also play a pivotal role in mobilizing SR proteins from speckles to transcription sites (Ding et al. 2005; Keshwani et al. 2015). These kinases may have a major impact on transcriptome via SR proteins and alternative splicing. Poly(A) Specific Ribonuclease Subunit (*Pan3*) as an

important factor involved in the degradation of mRNAs and miRNA targets could also have large impact on transcriptome.

Two transcripts encoding coiled-coil domain containing proteins: Ccdc173 and Ccdc101 are also enriched in our RIP-seq. Genetic defects in members of some other CCDC proteins have been directly linked to functioning of primary cilia (Knowles et al. 2013; Antony et al. 2013; Zariwala et al. 2011). Since primary cilia plays important functions in regulation of cellular metabolism and cell fate in NSC (Discussed in section **8.4.1.2** of Introduction), association of EJC with these transcripts might be vital for cilia functioning and its specific roles in quiescent cells. However, since the functions of CCDC173 and CCDC101 are not well characterized they might not be related to cilia functions in NSCs.

In summary, this list of cytoplasmic EJC-bound transcripts is clearly enriched in messengers encoding important determinant of cell fate including factors promoting proliferation, quiescence or differentiation suggesting that EJCs may participate to the regulation of the expression of these transcripts. All these transcripts may co-exist in similar cells but we cannot exclude that this diversity also reflects the heterogeneity of cellular states in our cell culture at Day2. It is important to notice that all these transcripts bound to EJCs are most likely cytoplasmic mRNAs that have not yet experienced translation but it does not mean that a certain proportion of them have been already translated.

The major objective of these RIP-seq experiments was to identify cytoplasmic mRNAs bound to EJCs in order to determine whether some of them are enriched in the centrosomal region of quiescent NSCs. Indeed, the association to EJCs does not necessarily mean that these transcripts are located around centrosomes. We can suppose that numerous cytoplasmic mRNAs are present in the cytoplasm without being concentrated in the same location, in other words that the vast majority of EJC-bound mRNAs are diluted in the cytoplasm and that only a small proportion are enriched at the base of the cilia.

S. No.	Name of the gene	IP: eIF4A3-A	IP: eIF4A3-B	IP: Y14	Functions involved
		Foldchange	Foldchange	foldchange	
1	<b>Gm17066</b>	5.27	5.4	2.42	Non Coding?
2	<b>2700099C18 Rik</b>	7.57	4.47	2.55	?
3	<b>Erdr1</b>	7.05	4.42	2.96	Apoptosis
4	<b>Clk1</b>	5.93	4.13	2.23	Splicing
5	<b>Snhg4</b>	3.12	3.64	2.02	snRNA host gene
6	<b>Clk4</b>	4.02	3.03	1.87	Splicing
7	<b>Prpf39</b>	3.02	2.95	1.79	Splicing
8	<b>Zranb1</b>	2.38	2.95	2.13	?
9	<b>Ccdc173</b>	2.45	2.86	2.26	?
10	<b>Supt20</b>	3.66	2.79	1.75	Transcription Factor
11	<b>Nog</b>	2.18	2.73	2.15	Differentiation
12	<b>Bok</b>	3.27	2.71	1.92	Apoptosis
13	<b>Dbp</b>	2.35	2.69	2.06	Transcription Factor
14	<b>Gtpbp6</b>	4.05	2.61	1.97	GTP binding?
15	<b>Cebpb</b>	3.19	2.53	2.63	Transcription Factor, Differentiation
16	<b>Cdkn2c</b>	2.11	2.52	2.25	Cell cycle regulation
17	<b>Trim8</b>	2.18	2.48	2.25	Quiescence
18	<b>Miip</b>	4.47	2.37	1.91	Cell migration, Cell cycle regulation?
19	<b>Ssbp1</b>	3.66	2.37	1.89	DNA binding
20	<b>Zfp821</b>	3.5	2.35	1.99	Transcription Factor?
21	<b>Smad6</b>	2.39	2.33	2.01	Differentiation
22	<b>6030419C18 Rik</b>	2.09	2.32	2	?
23	<b>Ccdc101</b>	2.03	2.32	2.07	Transcription Factor
24	<b>Bmp4</b>	2.66	2.3	2.1	Differentiation
25	<b>Id1</b>	2.61	2.29	1.68	Transcription Factor, Cell proliferation
26	<b>Zfp57</b>	3.18	2.25	2.19	Transcription Factor
27	<b>Rpl27</b>	2.98	2.24	2.36	Ribosomal protein
28	<b>Usf1</b>	2.35	2.14	2.03	Transcription Factor
29	<b>Zfp639</b>	2.44	2.13	1.95	Transcription Factor
30	<b>Pan3</b>	2.19	2.13	1.76	mRNA degradation
31	<b>Pdgfa</b>	2.1	2.13	1.96	Growth Factor
32	<b>Zfp61</b>	3.4	2.12	1.88	Transcription Factor
33	<b>Sdccag3</b>	2.65	2.11	1.85	Cytokinesis
34	<b>Tfpt</b>	2.01	2.07	2.09	Transcription regulation
35	<b>Stk11</b>	1.98	2.07	2.16	Cell polarity



36	<b>Samd1</b>	2.3	2.05	2.1	Differentiation
37	<b>Arglu1</b>	2.91	2.05	1.97	?
38	<b>Nr2f6</b>	2.09	2.05	2.02	Transcription Factor
39	<b>Rnf167</b>	2.01	2.04	1.81	Cell cycle transition?
40	<b>Jtb</b>	2.35	2.02	1.94	Transcription Factor
41	<b>Cwc22</b>	2.44	1.99	1.97	Splicing
42	<b>Fam173a</b>	2.1	1.99	1.86	?
43	<b>Id4</b>	2.35	1.93	1.94	Transcription Factor, Cell proliferation
44	<b>Irf2bp2</b>	2	1.91	1.96	Transcription regulation
45	<b>Alyref</b>	2.1	1.76	2.03	mRNA transport

**Table 1:** List of genes enriched in all three IP in cytoplasmic fraction of Day:2 NSC. Fold change value of genes in each IP compared to the cytoplasmic level is shown in columns

## 2.0: Visualization of expression of mRNAs by smFISH

An important step toward our understanding of the function of centrosomal EJs in quiescent NSC would be to identify the transcripts associated to these EJs. So far, there is absolutely no evidence that fully assembled EJs exist without being associated to splice transcripts but given that « absence of evidence is not evidence of absence! » and that « seeing is believing », we next performed “single molecule Fluorescence *in situ* hybridization” (smFISH) to physically characterize the localization and the expression behavior of mRNAs in the RIPseq results, and to potentially isolate the ones localized at/around centrosome. This method enables to visualize the presence of even a single molecule of endogenously expressed mRNA.

### 2.1: smFISH model

Conventional RNA *in situ* hybridization methods using hapten- (biotin or digoxigenin) labeled RNA probes rely on antibody binding for visualization, and are thus only semi-quantitative at best (Raap et al. 1995; Levsky & Singer 2003). Additionally, hapten-labeled probes are prone to diffuse localization (when conjugated with alkaline phosphatase), low sensitivity (when conjugated with fluorescent molecules), and non-specific probe binding. The smFISH method differs from conventional approaches by using many short (about 20-30 base

pairs long) oligonucleotide probes to target different regions of the same mRNA transcript (Raj & Tyagi 2010; Xu et al. 2009). Each oligonucleotide is conjugated with two fluorophores and thus faintly visible by itself. Binding of multiple oligonucleotides to the same transcript yields a bright spot, indicative of a single mRNA transcript. Since mis-bound probes are unlikely to co-localize, this method effectively reduces false-positive signal from non-specific probe binding. The small oligonucleotide size allows the probes to efficiently penetrate through target cells, yielding robust detection of even low-abundant transcripts. Subsequently, the total number of fluorescent spots within a single cell or region can be unambiguously counted and compared across different differentiation stages and cellular backgrounds.

Given its many advantages, the smFISH method is a powerful tool to study transcriptional regulation. Its high sensitivity allows accurate characterization of the spatio-temporal patterns of endogenous gene expression. Its single-molecule resolution enables precise quantification of gene expression levels. Such quantitative information can in turn be used to assess, for example: 1) differentiation stage specific correlations in gene expression, 2) similarity and difference in gene expression across different cell types, 3) variability in gene expression, and 4) cell-stage specific signaling dynamics. To date, similar smFISH methods have been successfully applied to study a variety of questions in mammalian cells, tissues and other organisms (Raj et al. 2010; Harterink et al. 2011; Middelkoop et al. 2012; Lyubimova et al. 2013).

## **2.2: Design and Synthesis of Fluorescent Oligonucleotide Probe Sets**

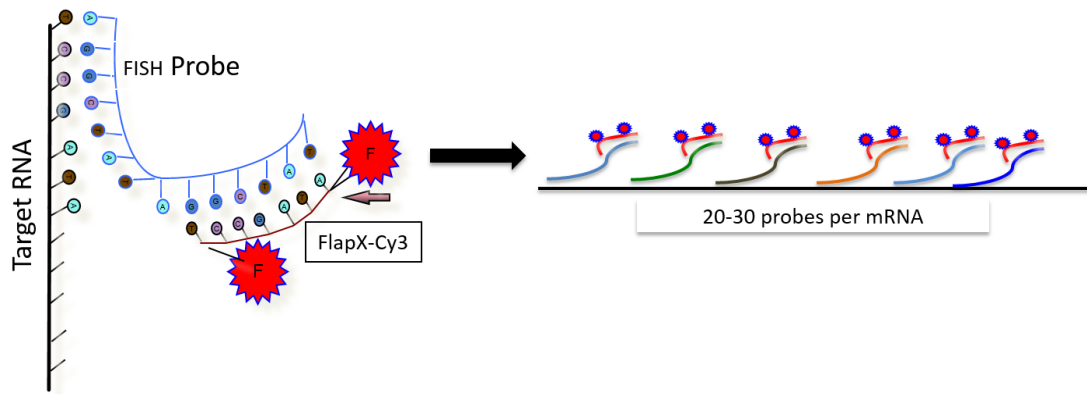
We collaborated with the group of Edouard Bertrand in IGMM at Montpellier in order to benefit from their great expertise in RNA-FISH. The unique method developed by their team allows to visualize single molecules of endogenous mRNAs in variety of cell types. I spent several weeks in Edouard's laboratory to get trained in optimization of FISH conditions for primary cells. It turned out that general FISH conditions were not suitable for FISH in NSC and important aspects of cell permeabilization, probe concentration, hybridization and washing conditions needed to modify in order to reduce the background and

auto-fluorescent aggregates generally seen with primary cells. After much learning and optimizations, we were successful in finding optimum conditions suitable for smFISH in NSCs. Next; we designed probes to perform smFISH for some candidates enriched in our EJC-RIPseq.

### **2.2.1. Design**

The designing of smFISH probes involves the synthesis of a set of fluorescently labeled oligonucleotides (which we call the 'probe set') that will hybridize along the length of target RNA molecule. There are a few general guidelines we typically followed when designing these oligonucleotides. Firstly, the probe sets typically consist of anywhere between 20 and 48 (typically 30) different 26-32 mer DNA oligonucleotides, each complementary to a different region of the target RNA. The lab of E. Bertrand tested and confirmed that this number of probes appear to be sufficient to generate a robust signal in most instances. The GC content of probes is kept ideally around 45% to ensure uniform binding efficiency. The probe length and number is adjusted depending on target transcript length. All of these probes have an additional overhang of 28mer which is complementary to a conserved sequence (which we call the 'FLAP') (**Figure 38**). The FLAP sequence is unique and has no complementarity with the genomic sequences. On both sides, the FLAP is hybridized with two Cyanine dyes.

To select the probes with highest specificity, we tested them at 'RepeatMasker' web tool. This tool allows us to estimate the repetitive DNA content and low complexity regions of individual probes. We synthesized our probes with high stringency of 5-10% binding repeats allowed against the mouse genome. This ensures that as many probes as possible will bind at a given hybridization stringency, allowing maximum numbers of probes from the set to recognize unique site. Following these parameters allowed us to design FISH-probe sets that, given a target RNA sequence, a desired number of probes and a target GC percentage, will generate a set of oligonucleotides whose GC contents are as uniform as possible.



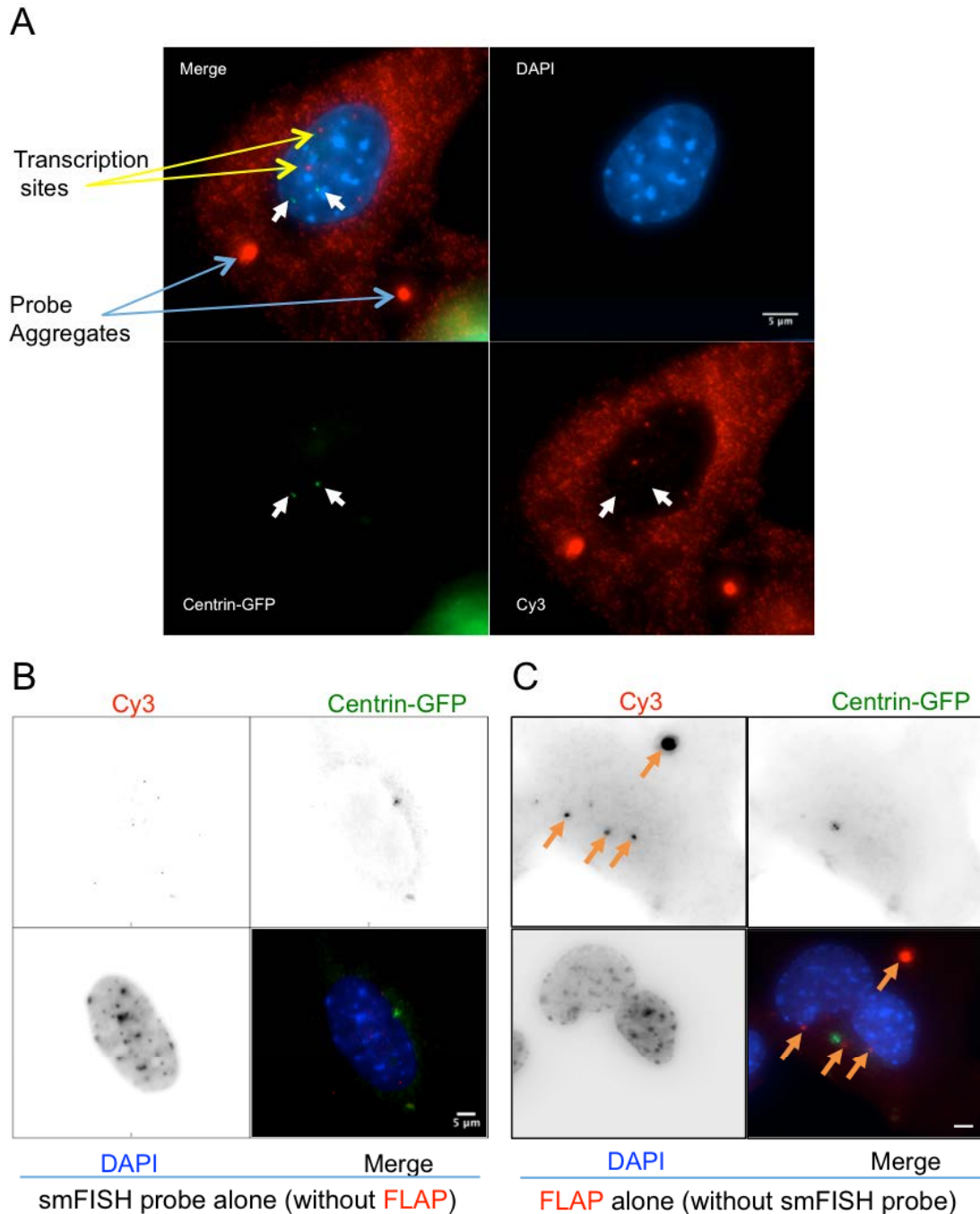
**Figure 38:** Scheme showing the model of individual smFISH probe and the set of multiple probes per mRNA.

## 2.3: Results obtained

### 2.3.1: smFISH in cycling MEF

To start with, we first set out to test if the technique is working for primary cell cultures. For this we tested the intracellular distribution of  $\beta$ -actin mRNA as a positive control. Since  $\beta$ -actin mRNA is an abundantly expressed mRNA, its detection by FISH must be easy and robust. As MEF cells are easy to cultivate and have relatively simple physiology compared to NSC, we chose cycling MEF cells to optimize conditions and test the efficiency of FISH. The results showed that single molecules of  $\beta$ -actin mRNA could be seen in abundance in the cytoplasm of cycling MEF cells (Cy3 channel) (**Figure 39 A**). Also, the bright dots of transcription sites were clearly visible in the nucleus (yellow arrows). There were some probe aggregates observed in the cytoplasm too (blue arrows) but they did not influence the signal of mRNA molecules. In parallel, we performed control experiments to test the background signal and specificity of the probe mix. We performed smFISH experiments separately without the FLAP sequence from the hybridization mix (**Figure 39 B**). As shown, smFISH experiment without the FLAP gave almost no signal in the Cy3 channel. Similarly, parallel experiment done with FLAP alone in the reaction mix gave only some background signal that are not punctuated dots in the cytoplasm as observed for mRNA signals (**Figure 39 C**). This confirms that the FLAP does not hybridize with any mRNA in the cell. However, there were bright spots found in the cytoplasm which were most likely the aggregates of FLAP. Together these

results confirmed the specificity and efficiency of smFISH in visualizing single mRNA molecules in MEF cells.

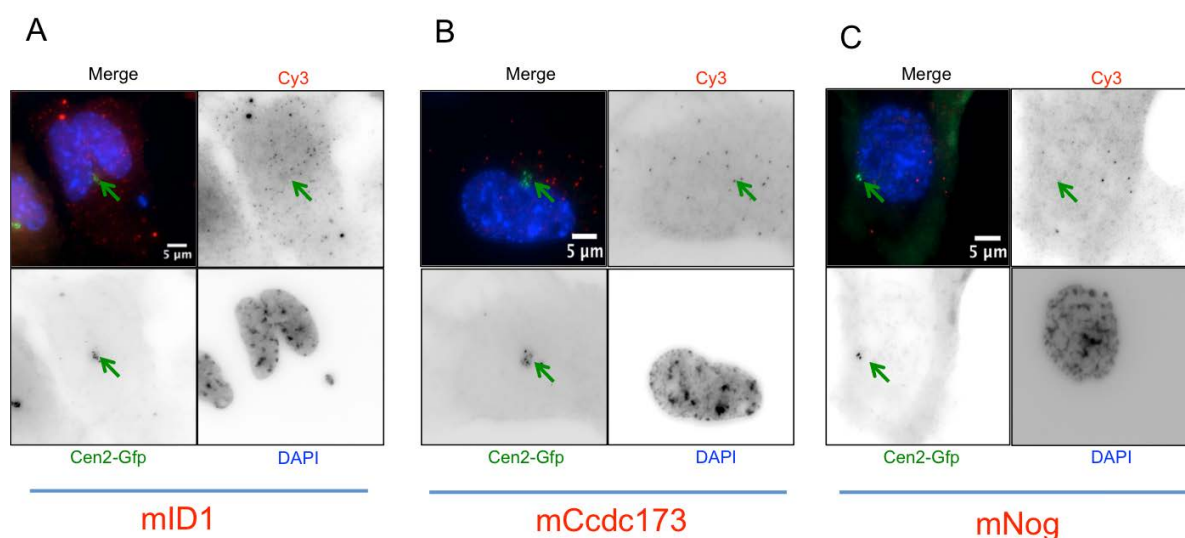


**Figure 39: (A)** FISH for mouse  $\beta$ -actin mRNA in cycling MEF shows clean and bright signal in the cytoplasm. Centrioles of the centrosome (white arrows) are shown with centrin labeling in GFP channel and nucleus in DAPI. **(B)** Control experiment without hybridizing the FLAP to the probe mix, shows no signal in Cy3 channel. **(C)** FISH experiment using just the FLAP alone shows no punctuated dots but aggregates of FLAP only (orange arrows).

### 2.3.2: smFISH in quiescent NSC

After satisfactory result with our control experiment, we next performed FISH with some of the candidates found in the RIP-seq in NSC. As synthesis of multiple probes per mRNA is required for smFISH, synthesis of several probe set turns out to be expensive. Therefore, we first set out to test four genes from our RIPseq for their visualization namely: Id1, Id4, Ccdc173 and Nog. Our mRNAseq indicated that Id1 and Id4 are highly expressed in NSCs while Ccdc173 and Nog are poorly expressed. Id1 and Id4 are one of the most abundant mRNAs expressed in developing and adult mammalian brain (Allen Mouse Brain Atlas: <http://mouse.brain-map.org>). Since single molecules of mRNAs expressed at very low endogenous level can be hard to distinguish from the background, Id1 and Id4 mRNAs would serve as positive control for our FISH experiments. We assumed Ccdc173 and Nog to be locally translated at centrosome owing to their specific functions related to the cilia functioning and differentiation of NSCs in quiescent NSCs. FISH for Id4 mRNA resulted in non-specific signal in nucleus and other places therefore it is omitted in the results. The other three mRNAs could be efficiently visualized with specific signals in the cytoplasm. The single dots of mRNAs were clearly seen in all three cases. The optimization of smFISH conditions for primary cells has been a major bottle-neck on which I spent most my time during the last year. As indicated by the mRNA-seq, Id1 is one of the better-expressed mRNA in NSCs and has abundant red-dots denoting single mRNA molecules (**Figure 40: A**). Ccdc173 and Nog are expressed at very low level and very few dots of mRNA signal can be seen for these mRNAs (**Figure 40: B, C**). All the experiments were done together in same conditions and the relative total fluorescence of all three probe-sets is theoretically the same as they were designed to have similar number of individual probes attached to identical fluorochroms. Therefore the signal generated for these mRNAs is reflection of their endogenous expression level. Though we cannot exclude the possibility of their differential access to the concerned mRNAs or having inequality in their binding efficiency. In all three cases, no clear evidence of centrosomal localization could be seen. Though, smFISH for these three mRNAs did not result in any striking discovery especially in terms of centrosomal localization.

However, it is important to notice that the transcripts that we localize by smFISH are not necessarily associated to EJC in the cells observed. These results demonstrated that the visualization of mRNAs is feasible in NSC even for very low expressed mRNAs. These optimized conditions for primary cells will be used in the future to test localization of more candidates and potentially over the differentiation of NSC. Unfortunately, due to time constraints I could not analyze additional candidates so far.

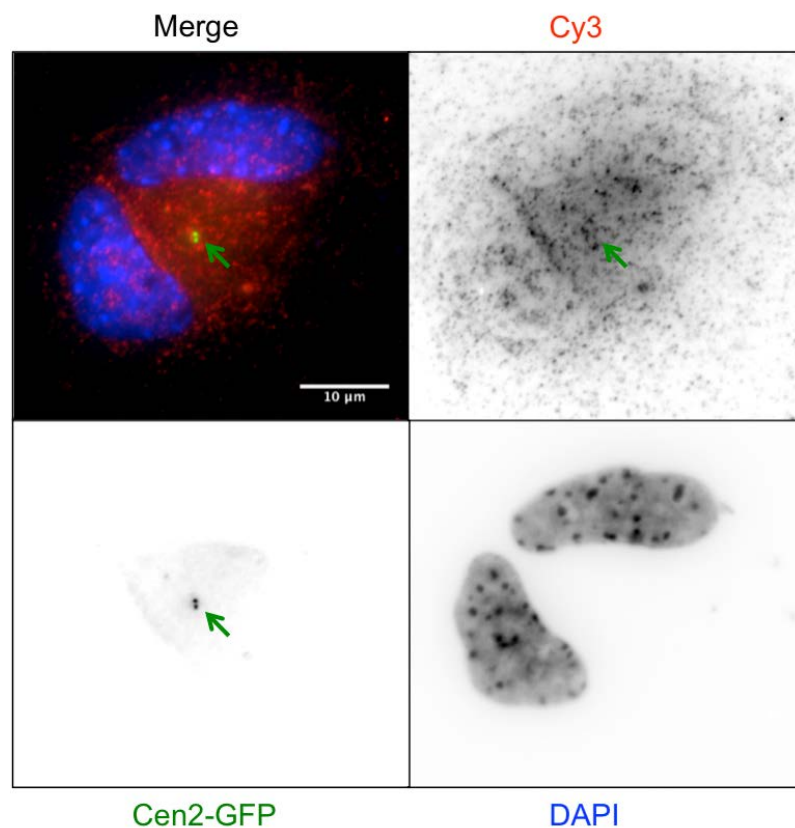


**Figure 40: Visualization of single mRNAs in quiescent NSC:** Pointed dots in the Cy3 channel represent the single molecules of mouse mRNAs (A):ID1, (B): Ccdc173 and (C): Nog. Centrosome is shown in GFP channel and nucleus in DAPI.

### 2.3.3: Distribution of total mRNAs in quiescent NSC

Additionally, we tested the intracellular distribution of poly-adenylated (poly-A) mRNA in quiescent NSCs. For this, we performed FISH with oligo-dT probes (43mer), coupled to Cy3 fluorochrome. This would potentially enable us to determine whether some unidentified mRNAs concentrated around centrosome, and if yes are they significantly enriched than total distributed population? Robust FISH signals were observed, with the characteristic poly(A) RNA localization pattern (**Figure 41**). While most of poly-A RNA population are abundant in the cytoplasm, a minority of them are present in nucleus with punctuated concentrations most likely in perispeckles. Apart from most of the mRNAs distributed across cytoplasm, there are some small aggregates at various places including centrosome (green arrow). However, as EJCs were highly

enriched at centrosome compared to cytoplasm, this is not the case with poly-A RNA. Strikingly, only a fraction of poly-A RNAs are concentrated around centrosome. This aggregation of mRNAs could be a large pool of few identical mRNAs, or a mix population of diverse mRNAs, accumulated around centrosome for local translation. Since other patches of concentrated mRNAs are also visible in the cytoplasm, it might be possible that these are just randomly aggregated mRNAs in the cytoplasm. However, this experiment was done only once due to limitation of time. To confirm this localization pattern, several more experiments are needed. It would also be very interesting to test whether this accumulation around centrosome in quiescent cells remain stationary during differentiation or changes, as observed for EJCs.

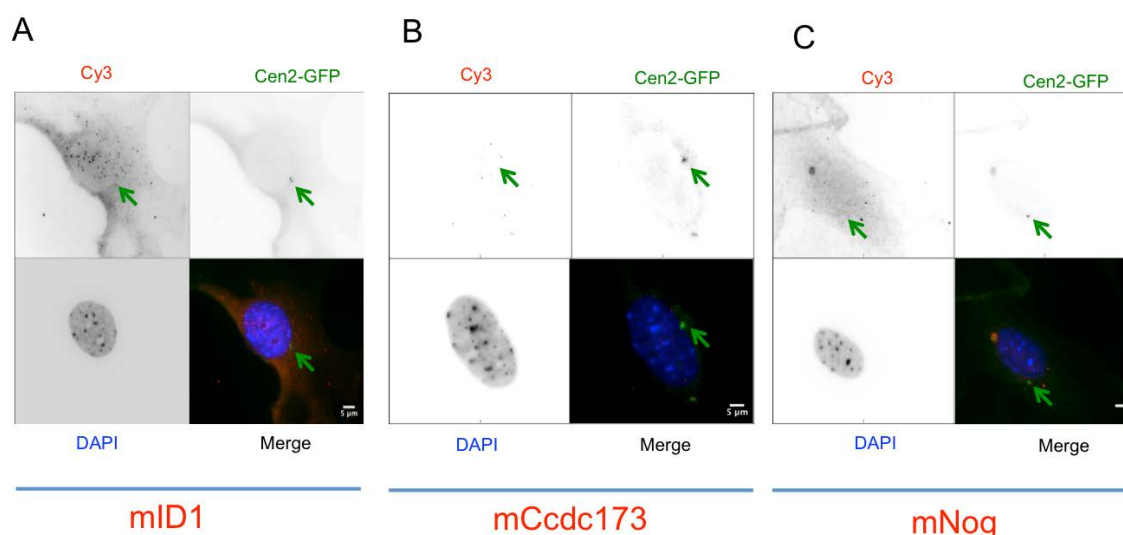


**Figure 41: Distribution of total mRNAs in the NSC:** Pointed dots in the Cy3 channel represent the molecules of polyA+ RNAs in quiescent NSCs. Centrosome is shown in GFP channel and nucleus in DAPI.

#### 2.3.4: smFISH in quiescent MEF



As the localization pattern of EJCs is identical in variety of quiescent cells, we wanted to know if the EJC bound mRNAs in NSC also localize in the same manner in other cell types. Therefore, as done in quiescent NSC, we tested the localization of the above-mentioned three mRNAs in quiescent MEF also. The results demonstrated a strikingly similar localization pattern of these mRNAs in quiescent MEF (**Figure 42**). Id1 mRNA is the most expressed mRNA among all (**Figure 42: A**), while Ccdc173 and Nog are very low expressed (**Figure 42: B, C**). Only few dots of Ccdc173 and Nog mRNA could be seen in quiescent MEF. Apparently, none of these mRNAs showed any clear enrichment at centrosome (green arrows). These results demonstrate that similar to EJC localization, these mRNAs have a resemblance in expression and localization pattern in two quiescent cell-types.



**Figure 42: Visualization of single mRNAs in quiescent MEF:** Pointed dots in the Cy3 channel represent the single molecules of mouse mRNAs (A):ID1, (B): Ccdc173 and (C): Nog. Centrosome is shown in GFP channel and nucleus in DAPI.

Given the fact that EJCs are differentially loaded on mRNAs, one mRNA can potentially be differently decorated with EJC in different cellular environments. Thus variation in EJC occupancy may lead to alternative mRNA levels and localization pattern. An investigation of other candidates would lead to draw a general idea about EJC-mediated localization of transcripts with regard to quiescence stage of cells.

# **DISCUSSION AND PERSPECTIVES**

Regulation of gene expression has long been considered in terms of coding sequence and genetics. In recent years, this very linear vision has been greatly altered by the discovery of new mechanisms such as epigenetics and interfering RNAs that activate or repress gene expression and their transcripts. Although mRNAs include the nucleotide sequence serving as a template for protein synthesis, they also carry the information necessary for their export, their location, their stability but also their translation. This information is largely taken up by the presence of RBPs. These RBPs play the role of adapters that communicate to different cellular machineries to determine the fate of mRNAs. In this context, the attribute of EJCs to mark the mRNAs for a specific period of time gains much interest. Our study gives a practical exhibition of EJC behavior in physiological context. Following, we discuss the possible explanation of our results and future perspectives to better determine the coveted molecular mechanisms by which EJCs contribute to gene expression regulation.

## **1.0: Resume of our results**

My project has attempted to study the EJC in primary cell cultures recapitulating physiological conditions and in different cell states. In my thesis, I have observed the localization of endogenous EJC core components eIF4A3, Y14 and MLN51 by immunofluorescence in different cell types and contexts including immortalized cell lines IMCD, MEF and, NSC over the differentiation into multiciliated ependymal cells. In all these cellular types, we observed a characteristic and unexpected behavior of EJCs at quiescent cell stage. While all three proteins are mainly present in their respective expected cellular localization (eIF4A3 and Y14 around nuclear perispeckles and MLN51 in the cytoplasm), they show a marked localization at the centrosome. The centrosomal EJCs are not localized as free proteins but in form of a fully assembled core as proved by the analysis of our transfection experiments with EJC-deficient Y14 mutant. These data are supported by the recent observation that transiently expressed MAGOH and Y14 in human 549 cells are also visualized around centrosomes but this without notion of EJC assembly and quiescence status (Ishigaki et al. 2014). Remarkably, the intense signals of EJC core proteins in the

centrosomal region totally disappeared when quiescent NSCs enter in differentiation. Taken together, our data revealed that EJCs mark the peri-centrosomal region when cells are in a quiescent state and that this mRNP mark completely disappears in cells committed to differentiation. As explained below, the presence of EJCs around centrosome at quiescent cell stage specifically, may support the centrosome maturation and can relate to polarization through the centrosome regulation shown by MAGOH- deficient mice (Silver et al. 2010). The presence of fully assembled EJCs strongly supports the presence of untranslated transcripts because so far EJC are supposed to be exclusively loaded on spliced mRNAs. This discovery prompted us to identify EJC-bound transcripts in the cytoplasm of NSCs. Thus, we performed several RIP-seq to isolate and sequence cytoplasmic EJC-bound transcripts in NSC. Our data brings out a high-confidence list of genes which might regulate the cell physiology of NSCs by regulating the quiescent stage and potentially differentiation in an EJC-dependent manner. Finally, we optimized smFISH protocol to study the localization of specific transcripts in different primary cell culture including NSC. So far, the coupled of transcripts targeted did not reveal a particular peri-centrosomal concentration but additional targets must be tested in the future.

## **2.0: Presence of mRNAs at centrosome in variety of cell types**

Several studies reported the presence of specific RNAs in the centrosomal region in different cellular contexts. In the beginning, studies claimed to have found RNA in centrosomes or centrosome-related material (Heidemann et al. 1977). First, RNA of unknown type were detected in basal bodies of *Tetrahymena pyriformis* and *Paramecium tetraurelia* (Hartman et al. 1974; Dippell 1976), but proof of them being centrosomal RNA was lacking because of the low sensitivity of the methods applied during that period. Many interesting experiments have since been undertaken in this direction; in particular, a study of *Ilyanassa obsoleta* embryos showed that mRNAs encoding embryonic patterning proteins are transported to the centrosomes by microtubules, ensuring their assymmetric distribution in cytoplasm and eventually bringing about differential gene activity (Lambert and Nagy 2002). The investigation of centrosomes in *Spisula*

*solidissima* mollusk oocytes has revealed a specific type of RNA, subsequently called cnRNA (centrosomal RNAs) (Alliegro et al. 2006). Later more cnRNAs were isolated to create a library of 120 cnRNAs (Alliegro & Alliegro 2008). These RNAs were unique transcripts and shared the characteristic of being the only RNAs that were exclusively associated with the centrosome. They do not match to known nucleotide, translated nucleotide, or predicted protein sequences suggesting that they might be an exclusive class of RNAs. It is possible that these RNAs may not be metabolically active but rather serve to scaffold other macromolecular assemblages at centrosome. An exhaustive analysis of mRNA localization in *Drosophila melanogaster* embryos, revealed the presence at centrosome of at least six different mRNAs which function await further characterization (Lécuyer et al. 2007). Based on these findings, it is evident that the centrosomally localized mRNAs is a general phenomenon across various cell types.

Our study reveals the presence of highly concentrated EJCs at the centrosome in contrast to the cytoplasm. The remarkable accumulation of fully assembled EJC at centrosome means the presence of numerous mRNAs. However, we do not know the exact number and identity of these mRNAs. It is intriguing to know if several mRNAs of different identity contribute to this localization, or is it a massive accumulation of only few related transcripts. Purification of the centrosomal fraction and profiling of the related transcriptome would be an important perspective to answer this question. However, isolating centrosomes from NSCs by density gradient centrifugation has been a challenging task so far and optimizations to establish a clean centrosomal purification are underway.

### **3.0: Accumulation of untranslated mRNAs at centrosome**

EJCs are assembled on transcripts during splicing in nucleus and remain stably bound during their travel to the cytoplasm before being finally ripped off by translating ribosomes (Dostie & Dreyfuss 2002; Lejeune et al. 2002; Gehring, et al. 2009). Concomitantly with translation-dependent EJC disassembly, PYM interacts with the heterodimer MAGOH/Y14 to prevent EJC core reassembly (Gehring et

al. 2009; Ghosh et al. 2014). Therefore, EJC mounted mRNAs in the cytoplasm are representative of mature but untranslated mRNPs. In this regard, presence of bright EJC labelling in quiescent cells depicts the existence of numerous untranslated mRNAs accumulated around centrosomes. The fact that EJCs disappear from the centrosome in cells committed to differentiation raises the possibility of two models (**Figure 43**):

### 3.1: Hypothesis 1: Translation dependent disassembly of EJCs

The complete disappearance of substantial amount of EJCs from the centrosome suggests the occurrence of massive translation of mRNAs. When quiescent NSCs commit for differentiation, centrosome generate new centrioles in their vicinity. This *de-novo* synthesis of centrioles requires a large pile of proteins in a short time frame (Al Jord et al. 2014). It remains undiscovered whether local translation at centrosome in quiescent stage generates some of these proteins or elevates signaling events on the onset of differentiation (**Figure 43**). Recent studies have begun to reveal the role of EJC in cellular signaling pathways. Under nutrient-sufficient conditions, the mTOR-activated kinase S6K1 is recruited to the EJC bound to newly synthesized mRNAs to promote their pioneer round of translation (Ma et al. 2008). Similarly, EJCs may be a target of cellular signaling kinases during the differentiation of NSCs as seen for cyclin B1 mRNA, which is concentrated on spindles and centrosomes in dividing *Xenopus* eggs (Groisman et al. 2000) and in centrosomal fractions of rat primary astrocytes (Kim et al. 2011). The translation of cyclin B1 transcript is inhibited by CPEB1 (Cytoplasmic Polyadenylation Element binding Protein 1) and release from this repression is obtained by the phosphorylation of CPEB1. The programmed translation of numerous transcripts could be orchestrated by common RBPs involved in either inhibiting or activating their translation. Interestingly, post-transcriptional regulation has emerged as a critical mechanism for driving corticogenesis (Pilaz & Silver 2015). For example, alternative splicing of ROBO1 is involved in axon guidance and neural progenitor proliferation and is implicated in various neurodevelopmental disorders (Borrell et al. 2012; López-Bendito et al. 2007). In addition to spatial differences,

temporal differences in splicing between embryonic to adult stages are also responsible for development of progenitors and neurons (Dillman et al. 2013). Indeed more than 1000 genes have been discovered with shifts in isoform expression during corticogenesis (van de Leemput et al. 2014). Interestingly, many of these alternatively spliced genes are directly linked to cancer and nervous system diseases. The spatio-temporal regulation of splicing relies on the differential expression and function of splicing factors including RBPs. Several of these RBPs have been experimentally shown to be critical for cortical development (McKee et al. 2005; Ayoub et al. 2011). For example NOVA2 and PTBP2 bind thousands of RNAs in the developing mouse brain and their downregulation give rise to significant number of splicing anomalies and defects in proliferation and neuronal differentiation (Yano et al. 2010; Licatalosi et al. 2012). Other RBPs that influence multiple aspects of RNA metabolism including RNA stability and translation have also been shown to play critical role in brain development. In neuroepithelial cells, when progenitor cells undergo primarily proliferative divisions, HuR interacts with Dll1 mRNA and HuR depletion in neural precursors leads to reduced Dll mRNA levels and less differentiation (García-Domínguez et al. 2011). Later in development when neuroepithelial cells have been replaced by radial glial progenitors, HuR is expressed in radial glia, IPs, and newborn neurons (Kraushar et al. 2014). By coordinating the translation of a network of mRNAs coding for functionally similar proteins, HuR regulates the position, identity and maturation of post-mitotic glutamatergic neurons (Kraushar et al. 2014). Similarly, FMRP has been shown to regulate the prenatal cortical development. Postnatally, FMRP localizes at the synapses between neurons, where it inhibits the translation of a sub- set of localized mRNAs encoding proteins involved in synaptic plasticity (Bassell & Warren 2008; Darnell et al. 2011). FMRP is required for generation of intermediate progenitor cells during neocortical development where it regulates the transition from radial glial cells to intermediate progenitor cells (Saffary & Xie 2011). FMRP also regulates the neuronal migration in the cortex. Fmr1 knockout brains showed defective neuronal migration (La Fata et al. 2014). Interestingly, FMRP expression enhances the translation of NOS1, an important regulator of synapse formation and spine maintenance (Kwan et al. 2012; Nikonenko et al. 2008)

highlighting potentially interesting evolutionary differences in FMRP function. A role for translational regulators in corticogenesis is recently highlighted by the study on eIF4E protein family. Once bound to mRNAs this complex can either promote or inhibit translation, depending on its composition and interactions with additional translation factors. For example, eIF4E1 association with eIF4G initiates translation whereas eIF4E1 binding to 4E-T blocks translation or promotes mRNA decay by targeting mRNAs to P bodies (Ferraiuolo et al. 2005). *In utero* knockdown of eIF4E1 or 4E-T in embryonic brains results in fewer neural progenitors and more neurons in either cases (Yang et al. 2014). In neural progenitors, eIF4E1 binds to Neurog1 and Neurog2 and NeuroD1 mRNAs suggesting that eIF4E1/4E-T complex may repress translation of key neurogenic transcripts.

Altogether these discoveries highlight the complexity of posttranscriptional regulation with fundamental role in corticogenesis. These differences in RBPs functioning over space and time during brain development may have direct links with EJCs. Since EJCs function as an enhancer of splicing, the differential presence of EJCs on some RNAs but not on other at one time point in brain development could recruit these RBPs in order to communicate with the transcription machinery and thus regulating their expression level. Similarly, RNA stability and translational control of these proteins might be regulated by the EJC in coordination with the specific identity and position of the cell in developing brain. In the future, the identification of the transcripts will eventually allow to determine whether those mRNAs share common features including binding sites of specific translation regulators. Therefore, identifying RNA targets for EJCs by CLIP-seq is critical to gain a mechanistic understanding of how these EJCs help shape the developing brain. Additionally, molecular profiling of translationally active mRNAs will give a comparative map of when and how these mRNAs get engaged in translation. We are tempting to address these points by doing a RIP with phosphorylated ribosomes in NSC. Phosphorylation of the ribosome can be used as a molecular tag to selectively retrieve translating RNAs (Knight et al. 2012). This may enable the discovery of the genes that are uniquely expressed in a functional population of quiescent or differentiating NSCs. The experiment in triplicate is already done but the results



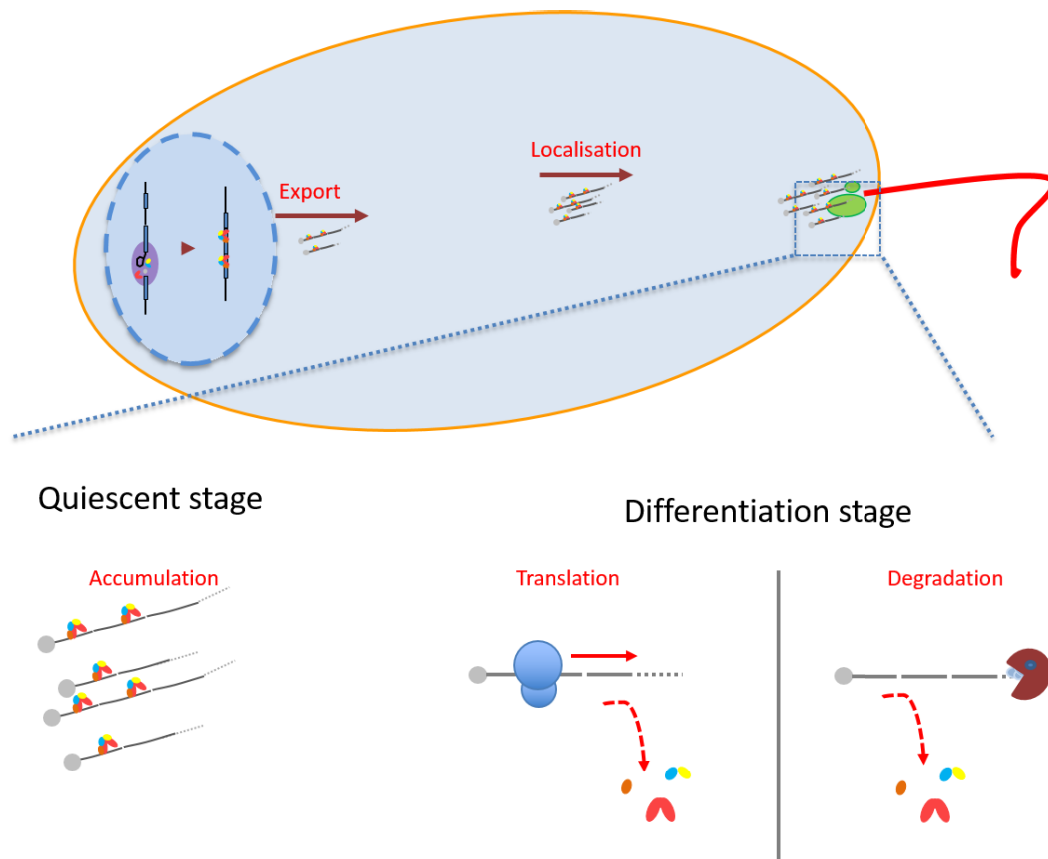
are yet to be analyzed. By characterizing enrichment of transcripts in phosphor-ribosome capture, we will assess the activation or inhibition of numerous genes at a specific stage of differentiation, revealing their coordinated regulation in response to an external stimulus. To access the possibility of local translation at centrosome, we are also planning to employ the “Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay” to label the nascent protein in cycling, quiescent and differentiating cells. Click-iT® AHA is an amino acid analog of methionine containing an azido moiety. Similar to 35S-methionine, Click-iT® AHA is fed to cultured cells and incorporated into proteins during active protein synthesis. Detection of the incorporated amine acid utilizes a “click” reaction between an azide and an alkyne, where the azido modified protein is detected with the green-fluorescent Alexa Fluor® 488 alkyne. In parallel, we are trying to validate the status of untranslated EJC-bound mRNAs in quiescent cells by doing immunostaining with antibodies specific to the nuclear cap binding protein CBP80. Messenger RNAs are acquired the cap binding complex (CBC) very early after transcription starts. The nuclear CBC made of CBP80 and CBP20, accompanies mRNA into the cytoplasm before to be replaced by the translation initiation factor eIF4E (Singh et al. 2015). Even if translation can occur on mRNAs still bound to CBC, CBP largely mark untranslated mRNAs. So, we plan to determine whether anti-CBP80 antibodies could mark centrosomes in quiescent NSC but not centrosomes in differentiated ependymal cells.

### 3.2: Hypothesis 2: Degradation or diffusion of EJC bound mRNAs

Another non-mutually exclusive phenomenon is that once cells start the differentiation, these transcripts might be degraded before or after the translation (**Figure 43**). *Arc* mRNA utilizes the mechanism of EJC mediated NMD to be translated in a spatiotemporal manner in neuronal synapses (Giorgi et al. 2007). *Arc* mRNA, having EJCs in its 3' UTR, is a natural target for NMD. *Arc* mRNA is transported through the dendrites in a translational silent state. Its translation is activated in synapses where after few round of translation, it is degraded by NMD pathway. Similarly, many of the translationally silent mRNAs might be transported and accumulated at centrosomes in quiescent cells and get

degraded before or right after the translation. The EJC mediated degradation of these mRNAs might be a key component for the cells to express regulatory proteins in a snapshot time-frame in order to just kick-start the cellular metabolism resulting in progression of cell state towards division or differentiation.

However, it is also possible centrosomal RNAs are not degraded but diffused in the cytoplasm before or after their translation. For instance, in *Drosophila* embryogenesis, Nanos mRNA can be found throughout the oocyte cytoplasm in a translationally repressed state, but concentrates at the posterior pole by diffusion coupled with a local anchoring mechanism (Forrest & Gavis 2003). Similarly, EJCs can function as an anchor to hold the mRNAs at centrosome and disassembly of EJC by translation machinery may release the transcripts leading to their diffusion in cytoplasm.



**Figure 43: Model showing the possible hypothesis for the disappearance of EJC during differentiation of NSC.** EJCs assembled on mRNAs accumulate at centrosome in quiescent stage. These mRNAs lose EJCs either due to translation or degradation of transcripts prior to differentiation.

In either of the above two possibilities, our results reveal a massive spatio-temporal program of post-transcriptional gene regulation when cells exit the quiescent state toward differentiation. A better view of the RNAs concerned will greatly help to determine their regulation: transport, translation and decay or delocalization during differentiation. Therefore, we aim to apply recent methods to visualize the expression of single mRNAs. The efforts spent by me on optimizing the smFISH conditions for primary cells (NSC, MEF) will greatly help us to advance in our findings. As observed, alteration in EJC dosage have a direct link with neurodevelopmental disorders, including microcephaly (Silver et al. 2010; Albers et al. 2012; Nguyen et al. 2013). Continuing studies with down-regulation of EJCs in physiological cells like MEF/NSCs or rather simple cells like HeLa/HEK will help us to explore the underlying mechanisms and establish if roles for these EJC components in corticogenesis are the root causes for these neurodevelopmental disorders. So far, conditions for down-regulating EJCs by siRNAs in primary culture of NSC and MEF still have to be optimized before to determine the impact of EJC dosage in functioning of these cells.

To our knowledge this is the first study that uses EJC proteins to label cytoplasmic untranslated mRNAs. Given that EJC proteins are ubiquitously expressed, antibodies against these proteins can potentially be used in every cell types. Given the importance of RNA localization and local translation in assymetric cellular establishment, efficient targeting and purification of cytoplasmic EJC-bound transcripts will undoubtedly be a useful strategy. In this study we have used RIP-seq to isolate EJC-bound transcripts. However, this approach presents some disadvantages. Indeed, RBP-binding to mRNA can be too weak to sustain purification conditions and it is known that purification of RNP complexes after cell lysate preparation is associate to the creation of unspecific interaction (Mili & Steitz 2004). To circumvent these negative aspects, the CLIP-seq strategy is in theory perfectly adapted. As I explained earlier, the CLIP-seq of eIF4A3 with our purified polyclonal anti-eIF4A3 antibodies turned out to be very inefficient and noisy in mouse cells. However, Rémy Hocq in our group has recently made tremendous progresses in that sense. We are now able to isolate RNA targets of eIF4A3 by CLIP starting with a much smaller number of cells and more importantly with much less background. These improvements

mainly concerned the library preparation. Therefore, we can now envisage to perform CLIP-seq in NSC. Another large-scale analysis that we can plan to perform is Ribosome Profiling (RP). RP provides a quantitative method to study the amount of translation *in vivo* in a genome-wide manner (Ingolia et al. 2011). This method utilises the fact that translating ribosomes can protect mRNA fragmenton which they sit from nuclease degradation, and the protected fragments can therefore be isolated and sequenced by high-throughput sequencing (Ingolia et al. 2011). Jan Wang recently applied this strategy in the lab to study the impact of EJC on translation (Wang and Le Hir, Manuscript in preparation). Like CLIP-seq, we now can envisage to apply RP to NSC. However, an important parameter to take into account is the homogeneity of the cell lysate to which this large-scale approaches will be applied. Given that EJCs mark centrosome of quiescent cells in different cell types, we can envisage to employ cell culture that could be homogeneously synchronized in different states (quiescence, proliferation, differentiation) in order to increase our chances to identify the mRNAs present in the centrosomal region and to dissect their expression regulation and functions.

#### **4.0: Functions of untranslated mRNAs at centrosome**

An important argument concerning the accumulation of EJCs at centrosome in quiescent cells is if they simply mark untranslated mRNAs present there or do they have a specific function related to the centrosome and/or cilia. The assembly of primary cilia is a dynamic process initiated once cells enter quiescence. In growth arrested cells the centrosome form the base of primary cilia by the fact that mother centriole of the centrosome serving as basal-body (Singla & Reiter 2006). The primary cilia plays an important role in signaling by interplaying with external signals (such as mechanical stimulations of chemeosensations) to facilitate the cell-fate decisions like cell cycle entry and differentiation. This function is modulated by the localization of a variety of receptors and their downstream effectors at the basal body of the cilium. The enrichment of EJCs at the centrosome in quiescent stage might have a relation with exchange of external signals by the primary cilium. Emerging evidences

indicate that cilia functions are regulated at post-transcriptional level. RNA binding protein Bicaudal C (BicC) controls cilia orientation and flow in mouse (Maisonneuve et al. 2009). Inactivation of BicC disrupts the planar alignment of motile cilia required for cilia-driven fluid flow. In an interesting functional similarity to EJC, BicC confines *Oskar* mRNA expression to the posterior pole of *Drosophila* oocyte (Saffman et al. 1998; Mahone et al. 1995). In human cells Bicc1 polymerization regulates the localization and translational silencing of bound mRNAs (Rothé et al. 2015). In a similar manner, the EJCs accumulated at the base of cilia in the quiescent cells might be waiting for the external signals through the primary cilia in order to initiate the translation or degradation of concerned mRNAs. In addition to this, EJCs may also have an impact on the cell fates mediated by centrosome in a “RNA dependent manner”. Centrosome duplication in dividing radial glia progenitors generates a pair of centrosomes with differently aged mother centrioles. (Singla & Reiter 2006). During peak phases of neurogenesis, the centrosome retaining the old mother centriole stays in the VZ and is preferentially inherited by radial glia progenitors, whereas the centrosome containing the new mother centriole mostly leaves the VZ and is largely associated with differentiating cells (Wang et al. 2009). Asymmetric behavior of centrosomes has been observed during asymmetric division of *Drosophila* male germline stem cells and neuroblasts (Yamashita et al. 2007; Rebollo et al. 2007; Rusan & Peifer 2007). Centrosomes with differently aged mother centrioles differ in their protein composition and thereby in their biophysical properties, such as microtubule anchorage activity (Bornens 2002; Delattre & Gönczy 2004). In this context, EJCs may regulate the asymmetric centrosome separation by regulating these proteins. The asymmetric anchoring of EJCs with one of the two centrioles may bring distinct biophysical properties and thereby differentially regulate the behavior and development of the daughter cells that receive them. Furthermore, the strong association of EJCs with cytoskeleton regulating gene such as *Bmp4* may associated with the microtubule anchorage activity of the centrosome thereby ensuring the daughter cell retaining the older mother centriole would facilitate its anchorage to a specific site (for example, the VZ surface) or vice-versa, thereby tethering the cell that inherits it.

Aside from their possible participation in microtubule organization and ciliogenesis, EJCs can associate with mRNAs encoding proteins necessary for proper cell division and differentiation. As shown in mouse, Y14 is essential for embryonic neurogenesis and proper brain size (Mao et al. 2015). Y14 haploinsufficiency causes microcephaly, due to progenitor depletion and massive neuronal apoptosis. Y14 regulates radial glia proliferation, preventing premature cell-cycle exit and neuronal differentiation hence controlling the neurogenesis and maintaining the progenitor pool. Similarly, mutation in MAGOH gene or knockout of *Lis1* causes similar phenotypes (Silver et al. 2010) such as microcephaly, altered NSC mitotic cleavage planes, precocious neurogenesis, an increase in apoptosis and a reduction in neural progenitors (Pawlisz et al. 2008; Gambello et al. 2003; Yingling et al. 2008) which are reversible in both cases by restoring the MAGOH level (Silver et al. 2010). Hence EJC components are critical regulator for neural progenitor maintenance, differentiation, and brain size. Similar to *Lis1* mRNA, EJCs may impact the expression of other mRNAs in developing brain as shown by our RIPseq in NSCs. Though our RIPseq experiment has yielded a concise list of genes associated with EJC, the bigger picture of complete EJC-interactome in physiological cells still remains unexposed. With the recent promising advancements in CLIPseq, it is most tempting to know the transcriptome-wide map of EJC in physiological conditions. A comparison of maps in different physiological cells or different physiological-stages of the same cell will open up the differences and similarities in behavior of EJC in diverse cellular environments. With a comprehensive view of EJC-targets we will test some interesting transcripts in isolation to know the impact of EJC on their regulation.

# **MATERIALS AND METHODS**

## **1.0: CELL CULTURE**

### **1.1: Neural Stem Cell culture**

P0-P5 mouse were dissected to take out lateral ventricular wall from the brain. Culture dishes were coated with Poly-L-Lysine (PLL; 40 $\mu$ g/ml) one 25-cm<sup>2</sup> flask per newborn mice. Dissected telencephala was chopped into little pieces and digested with Enzymatic digestion solution to detach cells. Cells were cultured in DMEM (10%FBS 1%P/S) at 37°C with 5% CO<sub>2</sub>. Upon reaching confluence, cells were shaken overnight at 250rpm. Next day, for RNA and proteins experiments, the medium of the flask was changed to DMEM (1%FBS 1%P/S) to start the differentiation. For immunostaining experiments, cells were trypsinized and seeded on cover slips at the density of 1.5x10<sup>5</sup>-2x10<sup>5</sup> in 20 $\mu$ l drops in DMEM (10%FBS 1%P/S). Cells were allowed to adhere for one hour and then supplemented with 500  $\mu$ l of of DMEM (0%FBS 1%P/S) per well (24 well dish). Next day, the medium was changed to DMEM (1%FBS 1%P/S) to start the differentiation. Cells were used at desired day of differentiation.

### **1.2: Mouse Embryonic Fibroblast culture**

For MEF isolation, uteri isolated from 13.5-day-pregnant mice were washed with PBS. The head and visceral tissues were removed from isolated embryos. The remaining bodies were washed in fresh PBS, minced using sterile blade and transferred into a 0.1 mM trypsin/1 mM EDTA solution (3 ml per embryo), and incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml per embryo DMEM containing 10% FBS) was added and passed through 18G and 20G syringe respectively to help with tissue dissociation. The lysate was passed through cell sorting screen on a 50 ml tube containing 10 to 20 ml of DMEM containing 10% FBS. Cells were counted and 3 × 10<sup>6</sup> cells were seeded in 10cm culture dish (passage 0) at 37°C with 5% CO<sub>2</sub>. Cells were cryopreserved for further use at passage 1. To make cells quiescent, cells were grown in culture dish or on coverslips upto the confluence and then the medium was replaced with DMEM containing 0% FBS. Cells were incubated for two days to achieve quiescent state.



## 2.0: Transfection of plasmids

NSCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics, at 37 °C in a humidified atmosphere containing 5.0% CO<sub>2</sub>. Transfections were conducted using JetPRIME Transfection Reagent (Polyplus-transfection® SA). 50-70% confluent cells from one T25 flask were used for each transfection. For each transfection, 1.5µg of plasmid was incubated for 5 min in 100 µl final volume of JetPRIME buffer. The mixture was vortexed and 3 µl of JetPRIME reagent was added. The JetPRIME reagent/DNA mixture was incubated at room temperature for 10 min and added directly to the cells in a drop-wise manner before agitation to mix. After incubation for 4 hours to allow DNA uptake, the medium was replaced with fresh DMEM containing 10% FBS and 1% antibiotics. After two days the cells were shaken in the flask at 250 RPM overnight. Next day, cells were trypsinized and seeded on coverslips in form of 20 µl drops containing 1.5x10<sup>5</sup>-2x10<sup>5</sup> cells. The medium was replaced next day with DMEM containing 0% FBS. Cells were incubated for two days with serum starvation to make them quiescent. Immunostaining was performed afterwards. Each experiment was repeated in triplicate.

## 3.0: Immunofluorescence and Microscopy

For immunofluorescence, cells on coverslips were washed two times with PBS. Next, cells were permeabilized in PBS-Triton X-100 0.1% for 2 minutes. For fixation, cells were incubated in fresh 4% paraformaldehyde (Electron Microscopy Sciences®) for 5 minutes and further washed 3 times with PBS. Free antigens were blocked by incubation with blocking buffer (BB: PBS containing 10% v/v FBS and 0.1% Triton X-100) for 15-30 minutes at RT. Cells were again washed three times with PBS and the primary antibody was incubated for 2 h at RT and cells were further washed 3 times with PBS. All incubations with antibodies were carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading. The secondary antibody was then incubated for 1 h and cells were further washed 3 times with PBS. The secondary antibodies were conjugated with Alexa Fluor 488 or Alexa Fluor 546 or Alexa Fluor 647 fluorochrome. Nuclei were stained by incubating 1 minute in PBS containing 1 µg/ml DAPI. Finally coverslips were

mounted in 10  $\mu$ l of Fluoromount-G (Southern Biotech®) mounting medium. Coverslips were incubated in closed boxes at 4°C for at least 12 h for hardening of mounting medium. Overlapping microscopic pictures were taken at a magnification of 63x or 100x on Zeiss microscope using imaging software (AxioVision 3.1, Carl Zeiss). Multiple images were taken at the interval of 0.25  $\mu$ m distance on Z plane.

### **3.1: Image analysis.**

The images were processed and analysed with Fiji software (Schindelin et al. 2012). High-resolution images were made by summing the stacks of 0.25  $\mu$ m Z-plane intervals. Colocalization was evaluated by visual inspection of signal overlap on merged images.

## **4.0: PROTEIN ANALYSIS**

### **4.1: Protein extraction**

100mm dish of cultured cells was washed twice with ice-cold PBS. Cells were collected with a cell scraper and pelleted in an Eppendorf tube. The cells were lysed by adding ice-cold RIPA buffer supplemented with a 1:50 volume of RQ1 DNase (Promega), a 1:100 volume of protease inhibitor (Calbiochem), a 1:100 volume of RNasin (Promega) and RNase T1 (Fermentas, 1–10 U). The solution was pipetted up and down several times and incubated on ice for 15 minutes for complete cell lysis. Cell extracts were centrifuged for 5 min at 14,000 RPM at 4°C to pellet all the cell debris. The supernatant was transferred to a new tube and kept on ice for further experiments.

### **4.2: Immunoprecipitation**

For each immunoprecipitation, 50  $\mu$ l of lysate was conserved as input, and the remainder was incubated for 2 h at 4 °C with 60  $\mu$ L Protein-A Dynabeads (ThermoFisher Scientific) conjugated with antibodies (15  $\mu$ g of purified anti-Y14 or anti-eIF4A3 or anti-MLN51). After IP, beads were put on a magnet and flowthrough samples were taken in 50  $\mu$ l aliquots. Beads were washed three times for 5 min with 1 mL IP150 buffer; then proteins were eluted by adding 20  $\mu$ L of 1x loading dye [50 mM Tris·HCl (pH 6.8), 2% SDS, 10% (wt/vol) glycerol,

1.4 M  $\beta$ -mercaptoethanol, 0.05% bromophenol blue] and heating at 60 °C for 10 min. Input, supernatants, and precipitated proteins were resolved onto SDS/PAGE and blotted as described below.

### **4.3: SDS-PAGE**

Samples to be analysed on protein gel were mixed with final concentration of 1x loading dye [50 mM Tris·HCl (pH 6.8), 2% SDS, 10% (wt/vol) glycerol, 1.4 M  $\beta$ -mercaptoethanol, 0.05% bromophenol blue]. Before loading on gel, they were heated at 99 °C for 5 minutes. Samples were run in 8.5 cm x 6 cm SDS polyacrylamide gells (SDS-PAGE) in Laemmli buffer on 120 volts.

### **4.4: Western Blot Analysis**

Proteins were transferred from gel to a 0.2  $\mu$ m nitrocellulose membrane (Protran-BA83; GE Healthcare) in a wet/tank electroblotting system in transfer buffer at 100 V during 1 hour at RT. Membrane was blocked in PBS with 5% (wt/vol) milk and 0.1% Tween-20 (Euromedex). It was then incubated with primary antibody in 5% milk-PBS-Tween for 1 hour at RT or over night at 4°C. Purified polyclonal anti-Y14, anti-eIF4A3 and anti-MLN51 were used in 1:1,000 dilution. Following 4 washes (1 quick, 3x 10 minutes) in PBS-Twen, the membrane was incubated with a secondary antibody for 1 hour at RT. After 4 additional washes as above, the membrane was incubated with SuperSignal West Femto (Thermo Scientific) chemiluminescent reagent. Signals were visualized using ImageQuant LAS 4000 (GE Healthcare Life Sciences).

## **5: RNA analysis**

### **5.1: Cellular fractionation**

NSCs in 100mm culture dish were washed twice with icecold 1x PBS. Cells were scraped and collected in a microcentrifuge tube on ice. Cells were spun for 5 minutes at 1200 RPM at 4 °C on a benchtop centrifuge. The supernatant was discarded and the pellete was smoothly suspended in 1000  $\mu$ L ice-cold PBS containing 0.1% NP40 supplemented with a 1:100 volume of protease inhibitor (Calbiochem) and 1:100 volume of RNasin (Promega). The solution was kept on ice for 5 minutes to allow cell lysis. 100  $\mu$ L of this mix was aliquoted as a whole-

cell sample and kept on ice. Remaining 900  $\mu$ L sample was centrifuged at 10000 RPM for 10 seconds. The supernatant was taken as cytoplasmic fraction. The nuclear pellet was dissolved in 500ul of RIPA buffer. Both nuclear and cytoplasmic fractions were supplemented with 1:50 volume of RQ1 DNase (Promega). Finally, the fractions were centrifuged at 14000 RPM for 5 minutes to pellet cell debris. The supernatant containing clean cytoplasmic fraction was transferred to a new tube for further experiments.

## **5.2: RNA Immuno Precipitation**

Cytoplasmic fraction of NSCs were subjected to immunoprecipitation with Protein-A Dynabeads (ThermoFisher Scientific) alone, purified polyclonal antibodies for anti-eIF4AIII and anti-Y14(gift from C. Tomasetto) for 2 h at 4 °C. Ten percent of the supernatant was taken as input. After immunoprecipitation, the beads were washed 4 times with NET2 buffer (50 mM Tris, 200mM NaCl, 0.11% NP-40) containing 1:200 volume of RNasin (Promega). Beads were dissolved in 150  $\mu$ l 'Splicing Dilution Buffer' at RT. RNA was isolated by Phenol-Chloroform extraction method and precipitated as described bellow.

## **5.3: RNA isolation**

### **5.3.1: Isolation of immunoprecipitated RNA**

150  $\mu$ l Acid Phenol-Chloroform (Ambion) was added to the beads. The samples were vortexed briefly to mix and centrifuged at 14000 RPM for 1 minute at 4 °C. The upper aqueous phase to a new tube.

#### **5.3.1.1: RNA precipitation**

For precipitation of isolated RNAs, 1 ml of GlycoBlue®(Ambion) and 450ml (3 volume) 100% Ethanol were added to the samples. After mixing briefly, samples were kept at -20°C overnight to allow efficient precipitation. Next day, tubes were centrifuged at full speed at 4°C for 20 minutes. Precipitated blue pellet of RNA was washed twice with 70% Ethanol by adding 1 ml of ice-cold ethanol, inverting the tube twice and centrifuging at maximum rpm for 5' at 4°C. Tubes were air-dried for 10 minutes at RT and RNA was dissolved in 20  $\mu$ l of DEPC treated water. To facilitate suspension, samples were shaken for 10

minutes at 1000 rpm at 30 °C. 1 µl aliquot was used for NanoDrop 2000 (Thermo Fisher Scientific) quantification and rest was stored at -20 °C.

### **5.3.2: Total RNA isolation**

Total RNA from whole cell lysate or cytoplasmic fraction was isolated using TRI® Reagent (Ambion). 4 volumes of TRI® Reagent was added to cell lysate/fraction, mixed by inverting the tubes and wait for 5 minutes on RT. 300 µl of Chloroform was added, shaken vigorously and kept for 10 minutes at RT. Samples were centrifuged at maximum speed for 10 minutes at 4°C in Eppendorf benchtop centrifuge. The upper aqueous phase (approx. 1 ml) was carefully transferred to a new tube avoiding contamination with middle organic phase. 0.7 volume of isopropanol was added, mixed gently and kept for 10 minutes at RT. The tubes were centrifuged at maximum G for 15-20 minutes at 4°C. The supernatant was discarded and the pellet was washed twice with ice-cold 70% Ethanol. Tubes were air-dried for 10 minutes at RT and RNA was dissolved in 20 µl of DEPC treated water. 1 µl aliquot was used for Nanodrop quantification and rest was stored at -20 °C.

### **5.4: Quantitative RT-PCR**

In order to remove any contaminating DNA, the RNAs from immunopurifications were incubated with 5 units of DNase RQ1 in the manufacturer buffer (Promega) at 37°C for 30 min. The RNA was phenolchloroform extracted and ethanol precipitated, then resuspended in 20 µl of RNase-free water. The RNA quality and concentration was assessed using NanoDrop 2000 (Thermo Fisher Scientific). 2.5 µg of random hexamer (Thermo Fisher Scientific) was used to reverse transcribe 5 µl of the immunopurified RNA samples or 5 µg of total RNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer recommendations. The cDNA samples were cleaned up using the QIAquick PCR purification kit (QIAGEN). Purified cDNA samples were radio-labeled by doing PCR with 10mM mix of dCTP, dTTP, dGTP and 0.1 mM dATP supplemented with 0.1mM dATP32- $\alpha$  using DreamTaq DNA polymerase (Thermo Fisher Scientific). 5 µl PCR product was mixed with equal amount of 2x loading dye (Thermo Fisher Scientific) and run on 8% urea denaturing gel in 1x TBE buffer. After run, the gel was placed

on a phosphor-screen and kept overnight in dark at -80°C. Next day, the screen was imaged at Typhoon FLA 9500 phosphorimager (GE Healthcare). The relative abundance of RNAs was analysed by quantifying the signal intensity by ImageQuant software (GE Healthcare).

## 6.0: smFISH analysis

### 6.1: Probe synthesis

The probes were designed according to the method of (Xu et al. 2009). The sequence of different probes for a mRNA is mentioned in **Table 2**. Each set of probes was ordered for synthesis from IDT (Integrated DNA Technology). Commercially synthesized probes were received in 96 well plates in a lyophilized form. The probes were dissolved in desired volume to achieve 100µM concentration for each probe. Equal volume of probes were mixed manually in one tube to form an equimolar mixture of probes. This mix was further diluted 5x to achieve the working concentration before annealing with Flap. The FlapY-Cy3 oligos containing a conserved sequence /5Cy3/AATGCATGTCGACGAGGTCCGAGTGTA/3Cy3Sp/ were also synthesized by IDT.

### 6.2: Hybridization of probes with FlapY-Cy3

The hybridization of the probeset with the FLAP was performed in a PCR machine. 40 pmol of total DNA from probe-set was mixed with 100 pmol of Flap in final volume of 10µl containing 1x NEB3 buffer (New England Biolabs). The mixture was denatured at 85°C for 3 minutes. The annealing was performed at 65°C for 3 minutes and then samples were cool down to RT slowly. The hybridized probe mix was kept on ice and used the same day or stored at -20°C for not more than 7 days.

### 6.3: Cell fixation

Cells grown on coverslips were fixed for 20min at RT with 4% paraformaldehyde (Electron Microscopy Sciences) freshly prepared in PBS. Cells were rinsed twice with PBS and permeabilized in 70% Ethanol overnight at 4°C,

with a Parafilm sheet wrapped around the plate. Cells were stored for not more than two weeks in 70% Ethanol at 4°C.

#### 6.4: *In situ* hybridization

Next day, cells were washed once with PBS and incubated in 15% formamide freshly prepared in 1X SSC buffer for 15 min at RT. 2 µl of hybridized probe set (for individual mRNA) was mixed with 26.5 µl of 40% dextran sulphate and 34 µg of *E. coli* tRNA in 1X SSC buffer containing 15% formamide and 2mM VRC in a final volume of 100 µl. 50 µl of this mix was dropped on a 10cm Petri dish and the coverslip was laid inverted on the drop avoiding air bubbles. The surface of the coverslip containing the cells was facing the drop. A 3.5cm Petri dish containing 1mL of 15% formamide/1X SSC solution was kept inside the 10cm Petri dish to provide humidification. The 10cm Petri dish was covered and wrapped with a Parafilm sheet around and incubate at 37°C overnight. Next day, cells were washed twice for 30min in freshly prepared 15% formamide/1X SSC at 37°C. Cells were further rinsed twice with PBS. Coverslips were mounted on slides by laying down the coverslip on a drop of 10 µl VECTASHIELD Antifade Mounting Medium (Vector Laboratories). The slides were analysed on a fluorescence microscope or stored at - 20°C upto a few weeks.

Probe ID	Sequence (5'-3')
Id1_01	GGA GGC TGA AAG GTG GAG AGG GTG AGG CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_02	GTT GAT CAA ACC CTC TAC CCA CTG GAC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
Id1_03	GAT GTA GTC GAT TAC ATG CTG CAG GAT CTC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
Id1_04	TAG CAG CCG TTC ATG TCG TAG AGC AGG ATT ACA CTC GGA CCT CGT CGA CAT GCA TT
Id1_05	TCC GAC AGA CCA AGT ACC ACC TCG CCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_06	GGT TGA TTA ACC CCC TCC CCA AAG TCT TTA CAC TCG GAC CTC GTC GAC ATG CAT T
Id1_07	CCG AGA GCA CTT TTT TCC TCT TGC CTC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
Id1_08	GAA GGG CTG GAG TCC ATC TGG TCC CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_09	GTG CGC CGC CTC AGC GAC ACA AGA TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_10	ATC GTC GGC TGG AAC ACA TGC CGC CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_11	CCG AGT TCA GCT CCA GCT GCA GGT CCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_12	CCT TGC TCA CTT TGC GGT TCT GGG GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
Id1_13	GGT GGG CAC CAG CTC CTT GAG GCG TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT

<b>Id1_14</b>	TTC ACC TGC TGC TCG TCC AGC AAG GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_15</b>	AGC GCA GCG CGA GAT GGC CAC GCT TTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_16</b>	GGC ACT GCC ACT GGC GAC CTT CAT GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_17</b>	TTT CAG CCA GTG ATC ATT GTA ATA TAC AAT ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_18</b>	AAT ATT TCC TCA GAA ATC CGA GAA GCA CGA AAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_19</b>	ATC CGC CAA GAG TCC GGT GGC TGC GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_20</b>	GTG TCT TTC CCA GAG ATC CCC TGG GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_21</b>	CCG CCA AGG CAC TGA TCT CGC CGT TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_22</b>	CGG CCT CCG GTG GTC CCG ACT TCA GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_23</b>	CTG CCC GCC TTC AGC GAA CAG CTA GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_24</b>	CTG AGA ACA GGC GGA GGG GAG CGG AGG ATT ACA CTC GGA CCT CGT CGA CAT GCA TT
<b>Id1_25</b>	ATG TCT GCT TTT TCA ATA AAA CAG AAA CAC GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id1_26</b>	TTG TTT AAT AAC AAC AAA AAA CTC ACC CCC ATT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_01</b>	TTC TAC TTA GCA GTC TGG TCG ACA ACA CTT ATT ACA CTC GGA CCT CGT CGA CAT GCA TT
<b>Id4_02</b>	AGG ATG TAG TCG ATA ACG TGC TGC AGG ATC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<b>Id4_03</b>	GTC GTT CAT ATC GCA CTG CAG GCA CAG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<b>Id4_04</b>	ACC GCG ACA AGC GGT AGA GCG AGC TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_05</b>	TGT AGC CTC TAA GGT TGG ATT CAC GAT TGC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<b>Id4_06</b>	TCT CTC TCT CTC TCT TGG AAT GAC AAG ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_07</b>	GAC GAC GTT TCT AGA TTT GCT GAA GAT TTC CCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_08</b>	CCA TCC ATC GCA GCT CAG CGG CAG AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_09</b>	GGC CGG GTC AGT GTT GAG CGC GGT GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_10</b>	CGG GCG GTG GCG GCT GTC TCA GCA AAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_11</b>	GTG AGT CTC CAG CGC CAG CTG CAG GTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_12</b>	CAC TTT GCT GAC TTT CTT GTT GGG CGG GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_13</b>	GCA CGA GCC TCC GCA GGC GAC TGT AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_14</b>	CTT CAT CGC GCG CTT CCT GCG CGA GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_15</b>	GAA TTC ACT CAG AAT CTA TTT TTG ACC TCA GTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_16</b>	TTA CCA TGT ACA CGT CAA ATA CAA AGG TTT CCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_17</b>	AAG AAA GCA CAT TTG AAA GGA CTC GTA ACT GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_18</b>	TAT TTT GTG ATT GAA CAC CTC ATG CAA TCA TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_19</b>	AGA CCA GTA CCA GAG AGC TGT TAC CTC TTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<b>Id4_20</b>	TAC AGT AGT CTA TTA AAG CGC ATA TAC TTT CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_21</b>	CAC TTA TAG AAC ATC TCT ATA TAT ACA GGG TAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_22</b>	AAT GTC ACT CAC TTA TCT ATA CAA TAT GTA ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>Id4_23</b>	TCT TGC AGA GAA AAA GTT CCC CGC CCT TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<b>Id4_24</b>	AAA CGA AAG AGA ATA CGT ACG GTG AAT GCT CGT TAC ACT CGG ACC TCG TCG ACA TGC ATT



<a href="#">Id4_25</a>	GAA TTT AAG TTT TAT TTT TCC CCT TCT CTC TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_26</a>	CCT TTC CTC CGG TGG CTT GTT TCT CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_27</a>	TGC TGT CAC CCT GCT TGT TCA CGG CGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_28</a>	TAT AAA ACC CTG TAC TTA AAA CCC GAG AAG GTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_29</a>	GCC TCA GAA ACT GGA TAC TGG GCA AAA CAA ATT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Id4_30</a>	AAA AAA ACC AAA TCA ACA GCC TGG CCA AAC AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_31</a>	AAC ATC CAG ATA GCA AAA GCT CTG CAA GGG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Id4_32</a>	ATA GAT GTA AAA CAA CAT ATC TAC AGT GCT CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_33</a>	AGC TTC AAA AAC AAA AAC AGT TCC CTT GTC ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_34</a>	CTT CGA AGT TTA ACT GCA CCT TCA AAA GGG TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_35</a>	AAA ATA ACC ACA GAA ACG TAC AAT TCA CAG CGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Id4_36</a>	AAA CCC CAC ACA CTT AAT TCT TTA TAT ATT ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_01</a>	GAT CTT ATC TGC CTT CAA GAC AGC CTG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_02</a>	TTT ATT GTG CAT ATT GTC AAG AAG CAG TTT CCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_03</a>	TTT TCT TCA TCT CGA TTT CAT ACA GCA AAT TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_04</a>	GGA CAT CAC GCT CTT TCA TAA CTC TGC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_05</a>	CTT TGT ATA TTT CTT CCT CCA GAT CAA GAA CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_06</a>	CAT CAG TTG CCT GAT AGC TTG GCC TTA TTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_07</a>	CAC CTC TGC CTA CAA AGG GTG GTC CAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_08</a>	CAT AGG GTA GGC GTA TTT CTT TGT TGA TTC AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_09</a>	ATT CTT TCT CCT TCT CAA CTG TAA GGG CGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_10</a>	TAT TTA TTT ATG GCT ATT TGC TGA ATG TGA GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_11</a>	CCT GAA TTT CCA GTT TCT CTC TGG TCA CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_12</a>	GAG GCT TTT CTC CTT TTC AAG CTC CTG GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_13</a>	ATT GTT CCT TAG CCT CTA TTT TCC TTT GTC TTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_14</a>	CTT CTT CCT CTT TAT TTT TCA TCA CAG AGG CTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_15</a>	TAT ATT CTG CAA TTG CTT TCA AGT CTG CTT GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_16</a>	TCA TGT TTT TCT TTT TCT CTT TTC TCC AGT TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_17</a>	AGG AAA TTA TTT ATT CTT TCT CTC CGT TCT TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_18</a>	TTA GCC TAT GTG TTT CAG CAT CTT TGT CCA TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_19</a>	TGG CTT TGA TAA ACT TTC TAA TCT TTT CGT CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_20</a>	TTT GTA TCT ATC TCT TCC TGT TTC TTG CTT AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_21</a>	CCG CTT CAT CTC CTC AGC ATC TTT TTC TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_22</a>	CAC TTT TCT TTC TTG CTT CTT CTT CCT CTT TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_23</a>	TGT TCC TCA ATT TGT TTC AGA TGA TCC TCG GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_24</a>	AGA GCT ACT CTT TCT CTG CGC CGT TTT TTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_25</a>	TGA GCT TCA CTT GCT CCT CCC ATT TTT TTA CAC TCG GAC CTC GTC GAC ATG CAT T

<a href="#">Ccdc173_26</a>	TCT GAT TTC ACT GCA TTC TTT TTG TAC TGA ATT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_27</a>	TCC GTC TGG TGA AAC TGG CAC TGC CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_28</a>	CAC TCT CAA TGG CCT TCT TTC TTT CTC CTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_29</a>	TCT TTC AGC CTC AAT CTC TTC ATC ACG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_30</a>	TTT GCT TGG CTT TAA GTT TCT GTT CTT TCA TTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_31</a>	TGC ATA TGT GTT AGT CCA GTG TTT TAC CAA CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_32</a>	TGG GAT TTC ACA TGC ATT TTC TTC TTT GCT GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">Ccdc173_33</a>	CTT TCT GCA CGA AGG ACT GCT GCT TCT TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_34</a>	TGT CAA CCT GTT GAG GCT ATC CCG GAT TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">Ccdc173_35</a>	ATC TGC TGG AGA TCC ACC TTG CTA GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">Ccdc173_36</a>	GGA GGG GTG GAT AGG GAA TCT CTT CTT TTT CAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_01</a>	TAC AAT GAT TAG TCA TTC CAT TTG TTC TCT ATT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_02</a>	TAC ACT CGG AAA TGA TGG GGT ACT GGA TGG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">mNog_03</a>	GCA CAG AGC AGG AGC GCT TGC TGA AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_04</a>	GCA CCG GGC AGA AGG TCT GTG ACC ACT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_05</a>	AGC CAT AAA GCC CGG GTC GTA GTG GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_06</a>	CAG ATC CTT CTC CTT AGG GTC AAA GAT AGG GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">mNog_07</a>	CCC AGG ACC ACC ACC AGG GCG TAG AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_08</a>	GGG CAT CCG AGA TTA CTC CAG CGC GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_09</a>	GGT TTT AGT GGT TTT CTT TAA TCC TGT TCT GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_10</a>	TTT CTC TGC TCT TTT TTC CTT TTG CTT GCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_11</a>	GTG AAA CTG GTT GGA GGT GGT GGG GGT AGG GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">mNog_12</a>	GGC GTT GGT GGG GAT CCA TCA AGT GTC TTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">mNog_13</a>	CCA CCG CAG CAC CGT GAG GTG CAC AGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_14</a>	TTG GAT GGC TTA CAC ACC ATG CCC TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_15</a>	CGG CTG CCT AGG TCA TTC CAC GCG TAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_16</a>	CCA CAT CTG TAA CTT CCT CCT CAG CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_17</a>	TTG CTC AGG CGC TGT TTC TTG CCT TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_18</a>	CAA GCC CTC GGA GAA CTC CAG CCC TTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_19</a>	TGG ATG TTC GAT GAG GTC CAC CAG GGG TTA CAC TCG GAC CTC GTC GAC ATG CAT T
<a href="#">mNog_20</a>	GGT TGT CGC TGG GTG CTG GGC GGA TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_21</a>	TAG ATA GTG CTG GCC GCC GGC TGG TGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_22</a>	GCG CAG GGC GCG TGG ACG AGC CTT TTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_23</a>	TCT GTG CGG AGC CTC CGG TAG AGC GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_24</a>	ATA ACA TTG AAC TCT ATA ACT TCT TCG AGG TCT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<a href="#">mNog_25</a>	CGC CAT GCG AAG GGT ACT GGG ATA TAA ATA GTT ACA CTC GGA CCT CGT CGA CAT GCA TT
<a href="#">mNog_26</a>	AAC ATT ATT ACC AAC AAC CAG AAT AAG TCT CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT

<b>mNog_27</b>	AAA TAC AGT AGA AGC CGG GTA ACT TTT GAC GTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_28</b>	TAC ATT CCT ACA CAG TTA AAC AGT GCA TTA CAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_29</b>	AAC CAG AAA GCC AGG TCT CTG TAG CCA ATT ACA CTC GGA CCT CGT CGA CAT GCA TT
<b>mNog_30</b>	GGC CCC CCC GAG TTC TAG CAG GAA CAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_31</b>	TGC CCA CCT TCA CGT AGC GTG GCC AAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_32</b>	CTC CTC CGG TCG CTC GGC GTC TTG TTT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_33</b>	AAA TTA AAA CTG GGA CCG TAT ATA CAC ACA CAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_34</b>	AAA AAA GTT CAT TGA AAA CCC TCG CTA GAG GGT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_35</b>	TCT TCG TCC CGC GTC CCC GGA GGA GAT TAC ACT CGG ACC TCG TCG ACA TGC ATT
<b>mNog_36</b>	AAA AAA AAA GTC CTT CTA CAA AAG TTC CCC CTT TAC ACT CGG ACC TCG TCG ACA TGC ATT

**Table 2:** List of probes designed for smFISH with their sequence.

## 7.0: List of buffers

- **Cell fractionation buffer:** 0.1% NP-40 in 1x PBS
- **IP150 buffer:** 10 mM Tris-HCL (pH 7.5), 150 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 1% NP-40
- **Laemmli buffer:** 63 mM Tris-HCl (pH 6.8), 0.1% 2-Mercaptoethanol, 2% SDS
- **NET2 buffer:** 50 mM Trish-HCL (pH 7.5), 200 mM NaCl, 0.1% NP-40
- **RIPA buffer:** 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mm Na<sub>2</sub>EDTA, 1mm Na<sub>2</sub>EGTA, 1% NP-40, 1% Sodium deoxycholate, Protease inhibitors (added fresh each time)
- **SDS sample loading buffer:** 50 mM Tris·HCl (pH 6.8), 2% SDS, 10% (wt/vol) glycerol, 1.4 M β-mercaptoethanol, 0.05% bromophenol blue
- **Splicing dilution buffer:** 20 mM Tris-HCl (pH 7.9), 100 mM KCl.
- **Western blot transfer buffer:** 25 mM Tris, 192 mM glycine, 20% Ethanol



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